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Jack bean (*Canavalia ensiformis*) urease induces eicosanoid-modulated hemocyte aggregation in the Chagas' disease vector *Rhodnius prolixus*

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ABSTRACT

Ureases are multifunctional proteins that display biological activities independently of their enzymatic function, such as induction of exocytosis and insecticidal effects. *Rhodnius prolixus*, a major vector of Chagas' disease, is a model for studies on the entomotoxicity of jack bean urease (JBU). We have previously shown that JBU induces the production of eicosanoids in isolated tissues of *R. prolixus*. In insects, the immune response comprises cellular and humoral reactions, and is centrally modulated by eicosanoids. Cyclooxygenase products signal immunity in insects, mainly cellular reactions, such as hemocyte aggregation. In searching for a link between JBU's toxic effects and immune reactions in insects, we have studied the effects of this toxin on *R. prolixus* hemocytes. JBU triggers aggregation of hemocytes after injection into the hemocoel and when applied to isolated cells. On *in vitro* assays, the eicosanoid synthesis inhibitors dexamethasone (phospholipase A_2 indirect inhibitor) and indomethacin (cyclooxygenase inhibitor) counteracted JBU's effect, indicating that eicosanoids, more specifically cyclooxygenase products, are likely to mediate the aggregation response. Contrarily, the inhibitors esculetin and baicalein were inactive, suggesting that lipoxygenase products are not involved in JBU's effect. Extracellular calcium was also necessary for JBU's effect, in agreement to other cell models responsive to ureases. A progressive darkening of the medium of JBU-treated hemocytes was observed, suggestive of a humoral response. JBU was immunolocalized in the cultured cells upon treatment along with cytoskeleton damage. The highest concentration of JBU tested on cultured cells also led to nuclei aggregation of adherent hemocytes. This is the first time urease has been shown to affect insect hemocytes, contributing to our understanding of the entomotoxic mechanisms of action of this protein.

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1. Introduction

Seeds of the legume *Canavalia ensiformis* (jack bean) are rich sources of proteins with biotechnological interest. These proteins include enzymes such as ureases (Carlini and Polacco, 2008), which catalyze the hydrolysis of urea into ammonia and carbon dioxide (Dixon et al., 1975). Ureases display biological activities such as entomotoxicity and exocytosis induction, which are independent of their catalytic function (Follmer et al., 2001; Follmer et al., 2004; Staniscuaski and Carlini, 2012). *C. ensiformis* urease isoforms display insecticidal effects mainly due to the digestive processing of ingested urease molecules and subsequent release of derived peptides within the lumen of the insect digestive tract (Carlini et al., 1997; Defferrari et al., 2011; Ferreira-Dasilva et al., 2000; Piovesan et al., 2008). Susceptible insects such as the hemipterans *Rhodnius prolixus*, *Oncopeltus fasciatus* and *Dysdercus peruvianus*, rely on cathepsin-like digestive enzymes, which cleave urease molecules on specific sites for the release of entomotoxic peptides (Carlini et al., 1997; Defferrari et al., 2011; Piovesan et al., 2008; Real-Guerra et al., 2013). Additionally, it has been found that hours after feeding on a solution containing *C. ensiformis* urease, intact protein molecules can be detected in the hemolymph of *R. prolixus* (Staniscuaski et al., 2010) and that urease is insecticidal even when injected directly into the insect's hemolymph, indicating that the entomotoxic activity is also due to actions of the whole molecule (Defferrari et al., 2013; Staniscuaski et al., 2010). When tested *ex vivo* on different *R. prolixus* tissues, urease interferes with cell signaling pathways modulated by eicosanoids, specifically cyclooxygenase products (Staniscuaski et al., 2009; Staniscuaski et al., 2010). Also modulated by eicosanoids, plant and bacterial ureases are capable of inducing inflammation and activating several mammalian cell types such as platelets and neutrophils. However, in contrast to what has been seen for *R. prolixus*, ureases act through lipoxygenase products in mammalian models (Barja-Fidalgo et al., 1991b; Carlini et al., 1985; Olivera-Severo et al., 2006; Uberti et al., 2013; Wassermann et al., 2010). Eicosanoids are synthesized from fatty acids, mainly arachidonic acid (AA), released from membrane phospholipids upon cell stimulation, via activation of a phospholipase A_2 (Harizi et al., 2008). Arachidonic acid then follows different enzymatic oxygenation pathways involving cyclooxygenases (COX) to yield prostaglandins (PGs) and thromboxanes, and lipoxygenases (LOX) producing, among other compounds, lipoxins and leukotrienes (Lone and Tasken, 2013). Most of the work done with insects focused on the PGs, which serve, among other functions, as central mediators of the immune response (Stanley, 2006; Stanley et al., 2009). The insect immune system encompasses broadly two types of defense reactions, the immediate cellular responses and, subsequently, the humoral responses (Marmaras and Lampropoulou, 2009). It has been shown in *R. prolixus* that hemocyte functions, including aggregation and phagocytosis, are modulated by eicosanoids (Figueiredo et al., 2008; Garcia et al., 2004a; Garcia et al., 2004b). Insect's humoral responses tend to appear a few hours after challenge and infection, and result in the activation of the prophenoloxidase (PPO) cascade (Kanost et al., 2004). The active form of PPO, phenoloxidase (PO), is required for melanization of nodules and capsules formed upon the cellular response. Melanin is also necessary for protection against toxic compounds after an invasion or damage to the cuticle (Cerenius and Soderhall, 2011). Here, based on previously reported data on eicosanoid-modulated effects of ureases on insects and on diverse mammalian cell models, we studied the effects of the *C. ensiformis* major urease (JBU) on hemocytes and immune responses of the Chagas' disease vector *R. prolixus*.

2. Materials and methods

2.1. Insects

Fifth instars *R. prolixus* Stål (1859) were used throughout this study. The colony was reared at 25 °C under 60% humidity, and maintained by feeding once each instar on defibrinated rabbit blood (Cedarlane Laboratories, Burlington, ON, Canada).

2.2. Rhodnius prolixus *saline and jack bean urease*

Rhodnius prolixus saline was prepared as follows: NaCl 150 mM, KCl 8.6 mM, $CaCl_2$ 2.0 mM, $MgCl_2$ 8.5 mM, NaHCO3 4.0 mM, glucose 34.0 mM, HEPES 5.0 mM, pH 7.0 (Lane et al., 1975). Crystalline Jack bean urease (JBU) was obtained from Sigma–Aldrich (Mississauga, ON, Canada) and diluted in *R. prolixus* saline to the appropriate concentrations.

2.3. In vivo *hemocyte aggregation assays*

Unfed insects (mean weight 50 mg) were injected into the hemocoel with solutions of JBU diluted in *R. prolixus* saline to a final dose of 6 μg per insect and control insects were injected solely with saline. Six hours after injections, the insects had their surface sterilized by immersion in 70% ethanol and hemolymph was collected from a cut in one of the legs. Hemolymph samples were immediately diluted in cold anticoagulant solution (EDTA 10 mM, glucose 100 mM, NaCl 62 mM, sodium citrate 30 mM, citric acid 26 mM, pH 4.6) at a ratio of 1:5 (anticoagulant: hemolymph), as described by Azambuja et al. (Azambuja et al., 1991). hemocyte viability was assessed by the Trypan Blue dye (Sigma–Aldrich, Mississauga, ON, Canada) exclusion method (Boyes et al., 1964). Briefly, 0.1% Trypan Blue diluted in *R. prolixus* saline was added directly to the tubes containing the suspension of hemolymph for 10 min at room temperature. The number of cells and aggregates (defined as a cluster of nine or more cells) was then determined for each sample by counting in a hemocytometer with phase-contrast optical microcopy, according to Miller and Stanley (Miller and Stanley, 2001).

2.4. In vitro *hemocyte aggregation assays*

Unfed insects had their surface sterilized by immersion in 70% ethanol for 5 min and the hemolymph was collected

from a cut in one of the legs. Hemolymph samples were immediately added to cold, sterile, *R. prolixus* saline at a ratio of 1:1 in sterile tubes. In other sterile tubes 100 μM EGTA (ethylene glycol tetraacetic acid) and eicosanoid synthesis inhibitors at different final concentrations were diluted in saline. The final concentrations were 50 and 100 μM for dexamethasone and indomethacin, 100 and 300 μM for esculetin and baicalein The inhibitors of the eicosanoid pathway synthesis, namely dexamethasone, indomethacin, baicalein and esculetin were obtained from Sigma–Aldrich (Mississauga, ON, Canada) and the stock solutions were prepared in 95% ethanol. The diluted hemolymph was mixed with solutions of EGTA plus inhibitors, or equivalent volumes of saline, and the tubes were incubated at room temperature for 30 min under gentle shaking. Subsequently, JBU was added in different concentrations to the tubes containing hemolymph and EGTA plus inhibitors or saline, followed by incubation at room temperature with gentle shaking, for 1 h. The final ethanol concentration in the experiments was 0.5%–1% v/v (ethanol:hemocytes suspension). As controls in each experiment, hemolymph samples were incubated in the same conditions without JBU. Hemocyte viability was again assessed by the Trypan Blue dye exclusion method as described above.

2.5. Hemocytes culture

To establish hemocyte cultures, 5th instars (5 days after feeding) had their surface sterilized by immersion in 70% ethanol for 5 min and then hemolymph was collected from a cut in one of the legs under aseptic conditions using a laminar flow cabinet. Hemolymph samples were immediately added to Schneider's insect medium (Sigma–Aldrich, Mississauga, ON, Canada) with supplements at a ratio of 1:1 in sterile tubes. The hemolymph/medium suspension was placed in 9-well plates containing glass cover slips inside each well, and allowed to settle for 30 min. Subsequently, 1 mL of the supplemented culture medium was slowly added to the wells such that the glass cover slips were fully immersed, and the plates were kept inside an incubator at 23 °C. The supplemented Schneider's insect medium was prepared as follows: 2 mg/mL tryptose phosphate, 10% inactivated fetal bovine serum (FBS), 0.5 mg/mL glucose, 30 mg/mL L-glutamine, 1× insect medium supplement (low protein), 0.04 mg/mL tetracycline, 0.05 μg/mL amphotericin B and 0.05 mg/mL gentamicin, pH 7.0 (all reagents from Sigma–Aldrich, Mississauga, ON, Canada).

2.6. Immunocytochemistry

One day after being collected, cultured insect cells were exposed to JBU at different concentrations for 24 h. Controls cells were treated solely with saline. The glass cover slips with the attached cells were removed from the culture plates and the monolayers were fixed with 4% paraformaldehyde for 20 min, room temperature (RT), followed by 3 washes, 5 min each, with phosphate buffered saline – PBS (Na_2HPO_4 10 mM, NaCl 150 mM, pH 7.2). After the washes, the cells were permeabilized with 0.1% Triton and 100 mM glycine in PBS for 20 min, RT, and washed with PBS as described. Subsequently, cells were blocked with 5% FBS in PBS for 1 h, RT, and, without washing, the blocking solution was replaced by 1% FBS plus the primary antibodies, anti-α-tubulin and anti-JBU (1:10,000 dilution each), in PBS. The incubation with primary antibodies was carried out for 1 h, RT, and cells were again washed 3 times with PBS. As secondary antibodies, anti-mouse IgG-Cy5 and anti-rabbit IgG-Cy3 conjugates (Jackson Immuno, West Grove, PA, USA, and Invitrogen-Life Technologies, Burlington, ON, Canada, respectively), were diluted (1:1000) in 1% FBS in PBS and incubated for 1 h, RT. DAPI (4′,6-diamidino-2-phenylindole, Invitrogen-Life Technologies, Burlington, ON, Canada) was added during the last 10 min of secondary antibody incubation for nuclear staining. Finally, cells were washed 3 times with PBS, 5 min each wash, and twice with double distilled water, 2 min each wash. The cells were mounted with glycerol/*n*-propyl gallate mounting medium (glycerol 90% and 0.5% *n*-propyl gallate in 1 M TRIS-HCl, pH 8.0) for observation under a laser scanning confocal microscope with LSM image browser software (Zeiss, Jena, Germany).

2.7. Statistical analysis

Results are expressed as mean ± standard error. Significance of differences between means was determined using one-way ANOVA followed by Holm–Sidack method (SigmaPlot software), and results were considered statistically different when $P < 0.05$.

3. Results

3.1. *JBU induces hemocyte aggregation* in vivo

Rhodnius prolixus 5th instars show nearly 100% mortality rate 24 h after injections of approximately 12 μg of JBU per insect (Staniscuaski et al., 2010). To be able to observe JBU's effects upon hemolymph cells, we used lower doses and shorter periods in the following experiments. The insects were injected with 6 μg of JBU each, or saline in the case of controls. Formation of cell aggregates in the hemolymph was analyzed 6 h after injection. This time interval allowed sealing of the wound caused by the needle on injection thereby facilitating hemolymph extraction. The number of free non-aggregated cells was very similar in both JBU and saline injected insects (Fig. 1A). In contrast JBU injections triggered hemocyte aggregation (Figs. 1B and 2) while saline injections had not such effect (Fig. 1B).

3.2. *JBU induces hemocyte aggregation* in vitro

After 1 h of *in vitro* incubation, JBU (200 nM or 500 nM) induced a decrease in the number of freely suspended cells along with induction of hemocyte aggregation (Fig. 3A and B). Control hemocytes, which were collected and kept in the same conditions but in the absence of JBU, did not show any significant formation of aggregates (Fig. 3B). In addition to aggregate formation, a darkening of the hemolymph in response to JBU treatment was observed (Fig. 3C).

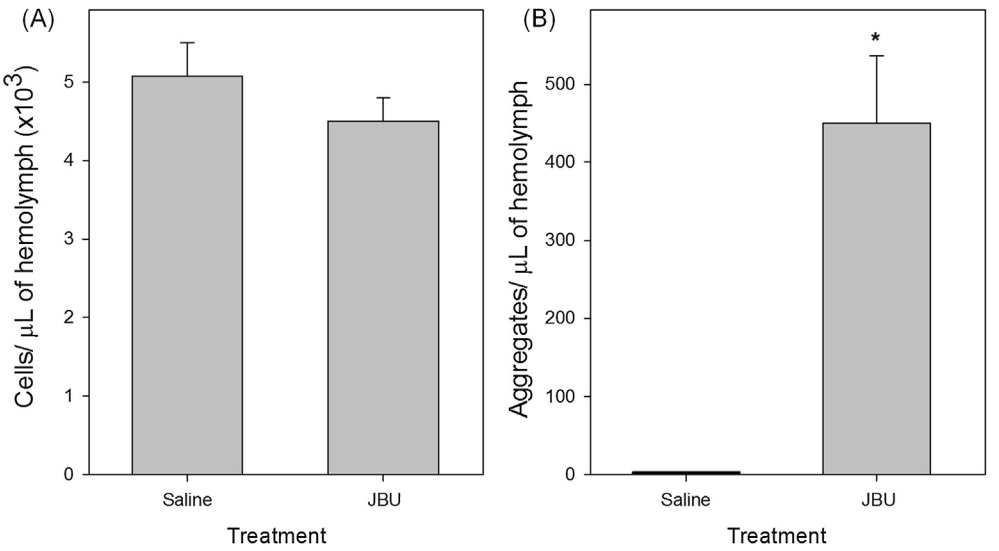

Fig. 1. *In vivo* hemocyte aggregation induced by JBU. Unfed 5th instars (mean weight 50 mg) were injected with 6 µg of JBU per insect or solely with saline for the control insects and 6 h after injections hemolymph was collected in anticoagulant solution. In order to verify cell viability, 0.1% Trypan Blue was added to the solution 10 min prior to counting. Hemocytes and aggregates were counted under phase-contrast optical microscopy with a hemocytometer. (A) Number of cells per µL of hemolymph, (B) Number of aggregates per µL of hemolymph. Results are shown as means of 3 biological replicates with standard error. "*" indicates statistically significant difference from saline controls.

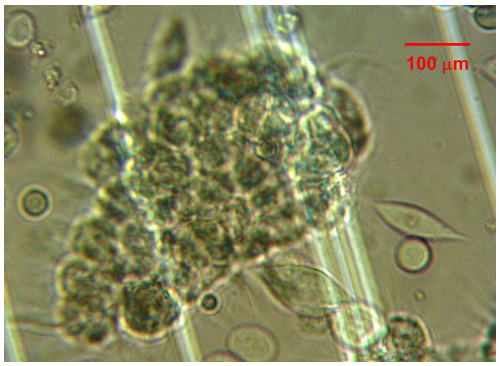

Fig. 2. Hemocyte aggregation induced by JBU *in vivo*. Unfed 5th instars (mean weight 50 mg) were injected with 6 µg of JBU and hemolymph was collected in anticoagulant solution 6 h after injections. The aggregates were observed under phase-contrast optical microscopy with a hemocytometer. Typical result. 20×/0.5 magnification.

3.3. Effect of EGTA and inhibitors of eicosanoid synthesis on JBU-induced hemocytes aggregation

JBU-induced aggregation was tested in the presence of the eicosanoid synthesis inhibitors, dexamethasone (inhibits PLA_2 activity) (Herbert et al., 2007), indomethacin (inhibits COX-1 and COX-2) (Rainsford, 2004), baicalein (inhibits 12-LOX) (Donald et al., 2012) and esculetin (inhibits 5-LOX and 12-LOX) (Buyukguzel et al., 2010), as well as EGTA, a calcium ion chelating agent. EGTA (Fig. 3B), as well as dexamethasone and indomethacin (Fig. 4A and B), inhibited JBU-induced hemocyte aggregation besides increasing the number of cells counted in the suspension. These results suggest that JBU's effect on hemocytes are mediated by COX-derived eicosanoids products and are dependent on extracellular calcium. The LOX inhibitors, baicalein and esculetin, did not alter JBU-induced effects (Fig. 5B), indicating that products of LOX have no roles in hemocyte aggregation. From the controls of each treatment, it was found that hemocytes did not aggregate when tested only with the inhibitors, with EGTA or with ethanol in the absence of JBU (data not shown). The number of non-aggregated cells in the presence of JBU preceded by dexamethasone or indomethacin (Fig. 4A), or EGTA (Fig. 3A) pre-treatments reverted to values similar to those in the control groups. On the other hand the decreased number of non-aggregated cells in presence of JBU was not prevented by baicalein or esculetin pre-treatments (Fig. 5A). When the aggregation reactions were inhibited by dexamethasone and indomethacin the darkening of the hemolymph caused by JBU also diminished (data not shown), in contrast to the effect of EGTA which inhibited aggregation but not the darkening of the medium (Fig. 3C).

3.4. Immunolocalization of JBU in cultured cells

Cell cultures treated with 50 nM JBU appeared to internalize the protein into vesicles, accompanied by slight damage to the cytoskeleton (as shown by some α-tubulin immunoreactivity spreading onto the cover slip around the cells, Fig. 6); however in cells incubated with 100 nM JBU severe structural damage could be seen, with intense α-tubulin immunoreactive sites around aggregated nuclei (Fig. 6). The pattern of JBU immunoreactivity was different between the two tested concentrations and nuclei aggregation was observed only at the higher JBU dose. While at the lower concentration JBU appears to be present inside cell vesicles, at 100 nM JBU appears also associated to α-tubulin found outside cells (Fig. 6).

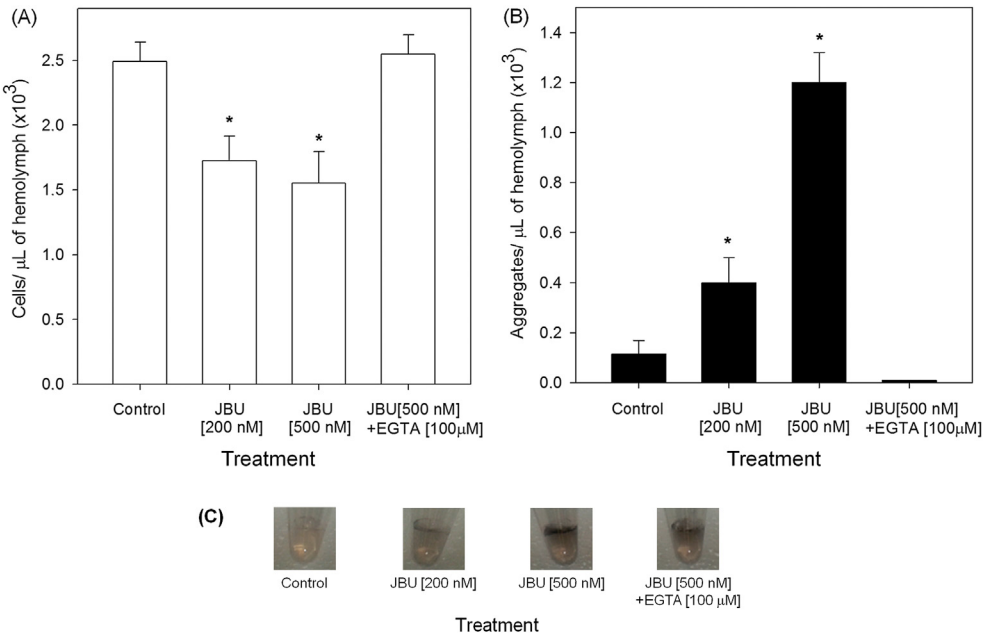

Fig. 3. *In vitro* hemocyte aggregation induced by JBU. Hemolymph was collected from unfed 5th instars in *R. prolixus* saline and incubated at room temperature for one hour in the presence of JBU at 200 and 500 nM. Controls were incubated in saline alone. EGTA (100 μM) was added to the cells 30 min prior to JBU addition. In order to verify cell viability, 0.1% Trypan Blue was added to the solution 10 min prior to counting. Hemocytes and aggregates were counted under phase-contrast optical microscopy with a hemocytometer. (A) Number of cells per μL of hemolymph, (B) Number of aggregates per μL of hemolymph, (C) Reaction tubes containing hemolymph and saline after the incubation period. Results are shown as means of 3 biological replicates with standard error. "*" indicates statistically significant difference from saline controls.

4. Discussion

Here we demonstrated that *C. ensiformis* urease (JBU) triggers the immune system of the hemipteran model *R. prolixus*, activating the insect's hemocytes both *in vivo* and *in vitro*. Based on previous evidences we aimed to evaluate if eicosanoid metabolites could be involved in the response triggered by JBU. Pretreatment with inhibitors of eicosanoid synthesis, dexamethasone and indomethacin, blocked the *in vitro* JBU-induced hemocyte aggregation also reducing the concomitant darkening of the medium, suggestive of activation of the PPO cascade. We have already shown the involvement of a PLA_2 gene in JBU's lethal effect on *R. prolixus*, with the toxicity being significantly reduced

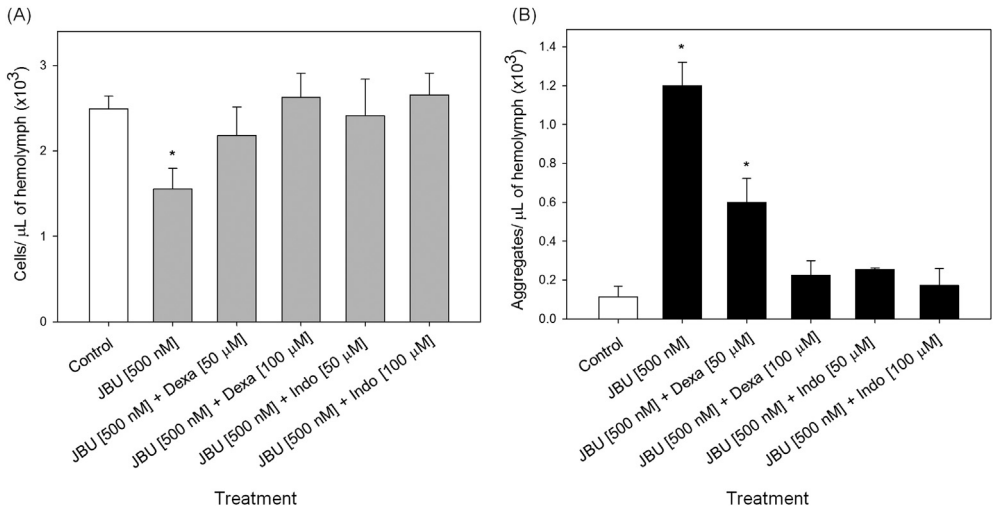

Fig. 4. Inhibition of JBU-induced *in vitro* hemocyte aggregation. Hemolymph was collected from unfed 5th instars in *R. prolixus* saline and incubated at room temperature for one hour in the presence of JBU at 500 nM. Controls were incubated with saline only. Dexamethasone and indomethacin were added to the cells 30 min prior to JBU addition. In order to verify cell viability, 0.1% Trypan Blue was added to the solution 10 min prior to counting. Hemocytes and aggregates were counted under phase-contrast optical microscopy with a hemocytometer. (A) Number of cells per μL of hemolymph, (B) Number of aggregates per μL of hemolymph. Results are shown as means of 3 biological replicates with standard error. "*" indicates statistically significant difference from saline controls.

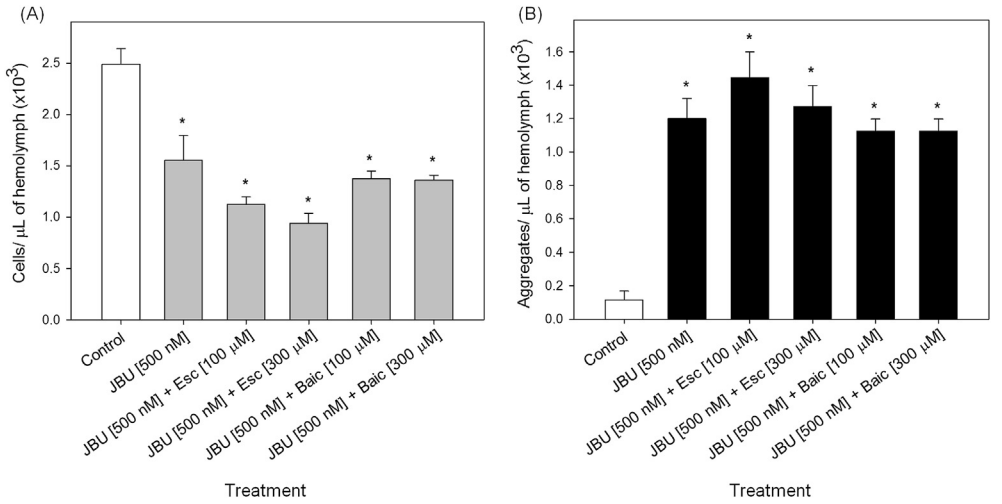

Fig. 5. Effect of lipoxygenase inhibitors on *in vitro* hemocyte aggregation induced by JBU. Hemolymph was collected from unfed 5th instars in *R. prolixus* saline and incubated at room temperature for one hour in the presence of JBU at 500 nM. Controls were incubated in saline only. Esculetin and baicalein were added to the cells 30 min prior to JBU addition. In order to verify cell viability, 0.1% Trypan Blue was added to the solution 10 min prior to counting. Hemocytes and aggregates were counted under phase-contrast optical microscopy with a hemocytometer. (A) Number of cells per µL of hemolymph, (B) Number of aggregates per µL of hemolymph. Results are shown as means of 3 biological replicates with standard error. "*" indicates statistically significant difference from saline controls.

after knocking down the expression of this PLA_2 gene (Defferrari et al., 2013). The glucocorticoid dexamethasone acts by increasing the expression of annexin A1, a protein that binds to PLA_2 and blocks its activity, therefore inhibiting eicosanoids production at the beginning of the cascade (Herbert et al., 2007). Indomethacin directly inhibits cyclooxygenase (COX) activity and subsequent release of prostaglandins (PGs) (Rainsford, 2004). Although

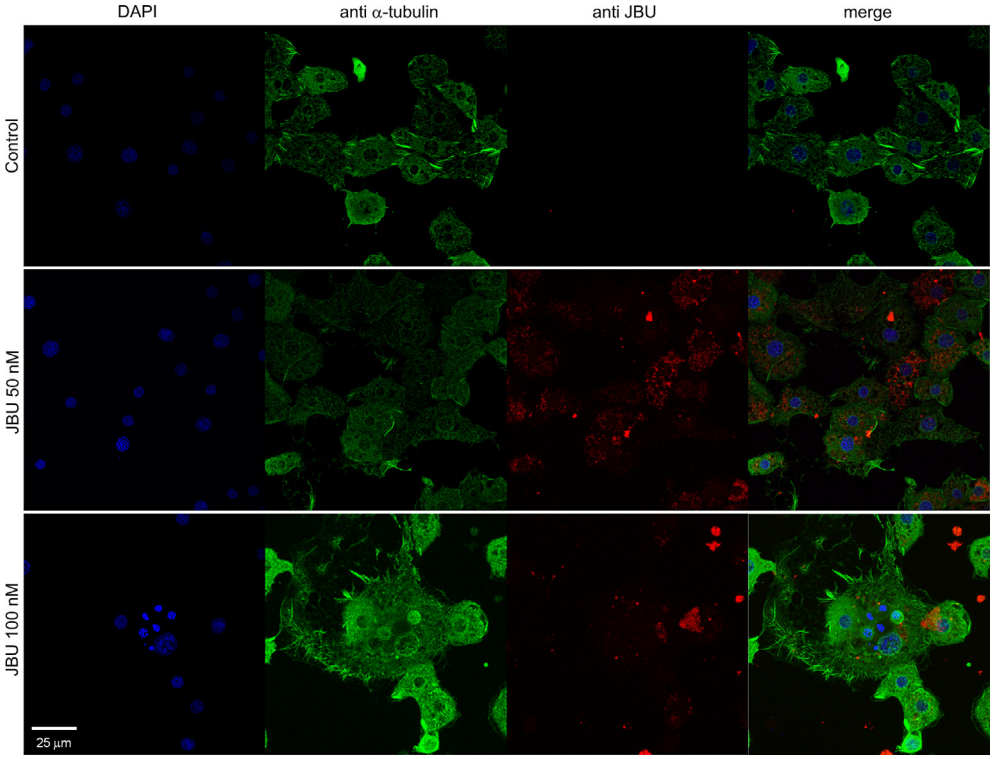

Fig. 6. JBU and α-tubulin immunoreactivity in cultured hemocytes. Hemolymph was collected from 5th instars 5 days after feeding and placed into supplemented Schneider's insect medium. Cells were kept inside incubators at 23 °C for 24 h and then JBU was added (50 and 100 nM) to the medium. After JBU addition, the cells were incubated in the same conditions for another 24 h. Confocal microscopy images of JBU immunoreactivity (red) and α-tubulin immunoreactivity (green). The nuclei of the cells were stained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

it has been shown that LOX products can modulate some cellular responses in *Spodoptera exigua* (Shrestha and Kim, 2009), COX products, particularly PGs, are known to mediate cellular reactions in insects infected with fungi, bacteria and other pathogens (Dean et al., 2002; Downer et al., 1997; Jurenka et al., 1999; Lord et al., 2002; Stanley, 1997; Stanley-Samuelson et al., 1997; Stanley-Samuelson et al., 1991). COX-derived eicosanoids have also been shown to signal PPO release and activation in *S. exigua* (Shrestha and Kim, 2008), *Galleria mellonella* (Downer et al., 1997) and *R. prolixus* (Garcia et al., 2004a), leading to PO, the active form of PPO, which initiates the melanization process (Cerenius and Soderhall, 2004). Further studies are needed to evaluate the production of melanin by JBU-treated hemocytes, and to determine if the toxin could also be triggering a humoral response.

The involvement of COX-derived eicosanoids in JBU-induced hemocyte aggregation contrasts to what has been observed in mammalian cells activated by different ureases, which require LOX-derived products, as reported in platelets (Carlini et al., 1985; Olivera-Severo et al., 2006; Wassermann et al., 2010), pancreatic β-cells (Barja-Fidalgo et al., 1991a), rat brain synaptosomes (Barja-Fidalgo et al., 1991b) and neutrophils (Uberti et al., 2013). On the other hand, activation of murine macrophages by canatoxin, an isoform of JBU, does not seem to require LOX products (Ghazaleh, 1992). It has been shown that insect hemocytes are very similar to mammalian phagocytic cells such as neutrophils, despite the facts that the insect cells are larger, show a higher density of granules and do not have a multilobed nucleus (Browne et al., 2013). EGTA also inhibited the *in vitro* aggregation response, suggesting that extracellular calcium is necessary for the reaction. The dependence of calcium ions in most biological activities of JBU has been reported in previous studies, such as JBU-induced inhibition of diuresis in *R. prolixus* Malpighian tubules (Staniscuaski et al., 2009) and platelet activation (Ghazaleh et al., 1997; Wassermann et al., 2010).

To examine the fate of JBU and its cytotoxic effect in a more controlled manner, we used cultured cells and developed 24 h long experiments. Cultured cells were considerably more sensitive and vulnerable than fresh isolated hemocytes, thus lower doses of JBU had to be used. JBU appeared to be internalized in vesicles into the hemocytes, causing mild to severe damage to the cytoskeleton and nuclei aggregation. Programmed cell death of hemocytes is one of the mechanisms to control infections. For example, *Drosophila melanogaster* cells fight bacterial infections by triggering cytoplasmatic autophagy (Yano et al., 2008). Apoptosis mechanisms are especially important in insects during viral infections, reducing viral replication and the consequent spreading within the host (Clem, 2005). The damage we observed in insect cells treated with JBU could possibly be part of programmed cell death. It has been shown that canatoxin, a JBU isoform, induces damage and cytolytic effect in cultures of various types of mammalian cell in doses ranging from 50 to 500 nM (Campos et al., 1991). Interestingly, *Helicobacter pylori* urease has been reported to increase survival of human neutrophils by affecting their apoptosis pathways (Uberti et al., 2013).

Altogether we conclude that in *R. prolixus* JBU activates cellular responses through modulation by COX metabolites, similar to what has been observed in previous studies for the activation of the insect immune system by pathogens. These reactions might contribute to the overall entomotoxicity of JBU, acting synergistically with the effects of urease-derived peptides released in the insect's digestive tract. We have shown for the first time that a urease directly affects hemocytes and hemolymph in an insect, contributing to the overall understanding of the mechanism of action of these toxins.

Authors contributions

M.S.D. conceived the study, conducted all the experiments and wrote the manuscript, R.S. helped with cell cultures and imaging, C.R.C. and I.O. conceived the study, revised the manuscript and supervised all the work.

Ethical statement

The authors declare that: a) the material has not been published in whole or in part elsewhere, except in the form of an abstract or part of academic thesis; b) the work is not currently being considered for publication elsewhere; c) all authors have agreed upon the content and form of the manuscript; d) all relevant ethical safeguards have been met regarding animal experimentation.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

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