Article

CALCIUM-INDUCED LIPID PEROXIDATION IS MEDIATED BY *RHODNIUS* HEME-BINDING PROTEIN (RHBP) AND PREVENTED BY VITELLIN

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Lipid peroxidation is promoted by the quasi-lipoxygenase (QL) activity of heme proteins and enhanced by the presence of free calcium. Unlike mammalian plasma, the hemolymph of Rhodnius prolixus*, a vector of Chagas disease, contains both a free heme-binding protein (RHBP) and circulating lipoproteins. RHBP binds and prevents the heme groups of the proteins from participating in lipid peroxidation reactions. Herein, we show that despite being bound to RHBP, heme groups promote lipid peroxidation through a calcium-dependent QL reaction. This reaction is readily inhibited by the presence of ethylene glycol tetraacetic acid (EGTA), the antioxidant butylated hydroxytoluene or micromolar levels of the main yolk phosphoprotein vitellin (Vt). The inhibition of lipid peroxidation is eliminated by the in vitro dephosphorylation of Vt, indicating that this reaction depends on the interaction of free calcium ions with negatively charged phosphoamino acids. Our results demonstrate that calcium chelation mediated by phosphoproteins occurs via an antioxidant mechanism that protects living organisms from lipid peroxidation.*

Keywords: *Rhodnius* heme-binding protein; quasi-lipoxygenase; vitellin; antioxidant

INTRODUCTION

Hematophagous organisms continuously face metabolic challenges. During blood digestion, a massive amount of the known pro-oxidant molecule heme is released into the guts

of these organisms, thereby triggering a potential set of harmful free radical reactions. Heme participates in the reactions of oxygen radicals that lead to the degradation of proteins, lipids, carbohydrates, and DNA (Aft and Mueller, 1984). A series of antioxidant mechanisms have been developed during evolution and they confer protection to blood-sucking organisms (Graça-Souza et al., 2006). These mechanisms include the induction of heme aggregation into larger molecules, such as hemozoin in the Triatominae *Rhodnius prolixus*, or its association with the peritrophic matrix in the mosquito *Aedes aegypti*. In the case of *Rhodnius*, antioxidant enzymes such as extracellular glutathione peroxidase (GPX) and catalase operate in a concerted manner, and their inhibition largely increases H_2O_2 production in the midgut (Paes and Oliveira, 1999; Paes et al., 2001). Both bugs and mosquitoes express heme oxygenase, which catalyzes the oxidative degradation of heme to less-toxic molecules, such as biliverdin and carbon monoxide (Paiva-Silva et al., 2006; Pereira et al., 2007). Despite all of these antioxidant mechanisms, a large amount of free heme is still able to reach the insect hemolymph. Several mammalian circulating proteins have been shown to bind heme, including albumin and hemopexin (Morgan et al., 1976; Gutteridge and Smith, 1988). *Rhodnius* heme-binding protein (RHBP) is a 15-kDa polypeptide (Oliveira et al., 1995) isolated from the hemolymph and oocytes of *Rhodnius* that binds a single molecule of heme. RHBP is synthesized in the *Rhodnius* fat body, and the levels of both RHBP mRNA and secreted protein increase (Paiva-Silva et al., 2002) in response to a blood meal. Following a meal, bugs engage in blood digestion and the synthesis of proteins such as lipophorin (Lp) and the yolk protein vitellogenin (Vg). These proteins are highly enriched in meal-derived lipids and synthesized in the fat body of *Rhodnius*. After being secreted into the insect hemolymph, Lp delivers lipids to the ovary while Vg is removed by receptor-mediated endocytosis. Once inside oocytes, Vg is called vitellin (Vt) because Vg can be altered during uptake into the ovary. Both Lp and Vg are essential for oocyte growth because they provide lipids, carbohydrates, and protein backbones. Upon oocyte fertilization, Lp-transported lipids and Vt are required to provide nourishment for the growing embryo. Lp, Vg, and RHBP are in close contact in the hemolymph. The binding of free heme by RHBP inhibits the heme-dependent peroxidation of both linolenic acid liposomes and Lp in the bug hemolymph (Dansa-Petretski et al., 1995). Oxidized Lp is functionally impaired because it cannot be loaded with phospholipids from the fat body and lacks the ability to deliver phospholipids to growing oocytes. Thus, RHBP prevents heme-induced oxidative damage to yolk proteins and ensures proper vitellogenesis and egg formation.

Lipoxygenases (LOXs) constitute a heterogeneous family of lipid peroxidizing enzymes. They are nonheme iron-containing dioxygenases that catalyze the addition of O_2 to unsaturated fatty acids that contain a *cis,cis*-1,4-pentadiene moiety on the corresponding hydroperoxy derivatives. LOXs are characterized by the carbon atom of the fatty acid that is predominantly oxygenated during the peroxidation reaction (Prescott and John, 1996; Schneider and Brash, 2002). However, the details of their kinetic mechanism are still not well understood (Segraves and Holman, 2003). Lipoxygenase-like or *quasi*-lipoxygenase (QL) activity is observed when polyunsaturated fatty acids are oxidized to hydroperoxy, epoxy, keto, and hydroxy-epoxy derivatives by free heme or hemeproteins (hemoglobin, myoglobin, myeloperoxidase, catalase, and cytochrome c) in the presence of calcium (Iwase et al., 1998). These proteins oxidize linoleic acid to hydroperoxy, epoxy, hydroxy-epoxy, and keto derivatives at a physiological pH value. QL activity has been described for classic hemeproteins, such as hemoglobin, myoglobin, myeloperoxidase, and cytochrome c. In vertebrate blood, the QL reaction is inhibited by the following two mechanisms: the binding of plasma calcium albumin and the location of hemoglobin

(the main blood hemeprotein) inside erythrocytes, in which calcium concentrations are usually kept at submicromolar levels. In the blood-sucking bug *R. prolixus*, RHBP is found both in the hemolymph and eggs in close contact with Lp, and phosphoproteins (e.g., Vg or Vt). Additionally, the bug hemolymph, growing oocytes and developing eggs are in compartments in which the calcium concentrations are usually maintained at millimolar levels (Ramos et al., 2011). Thus, these compartments are suitable for the generation of lipid peroxidation products. In the present study, we show that RHBP-mediated lipid peroxidation is prevented by calcium sequestration mediated by yolk proteins, a novel antioxidant mechanism found in hematophagous organisms.

MATERIALS AND METHODS

Materials

Hemin also known as protoporphyrin IX, hemoglobin (bovine), butylated hydroxytoluene (BHT), and thiobarbituric acid (TBA) were supplied by the Sigma Chemical Co. (St. Louis, MO). Linoleic acid and 9-octadecanoic acid were supplied by the Cayman Chemical Co. (Ann Arbor, MI). All of the other chemicals used were of high purity.

Insects. Insects were obtained from a colony of *R. prolixus* maintained at 28°C and 70% relative humidity. Normal mated females were fed rabbit blood at 2-week intervals. Four days after a blood meal, the hemolymph was collected and diluted 10 times with PBS. The samples were then immediately used in experiments.

Animal Experimental Procedures

Bug feeding was conducted in rabbit ears in accordance with the standards of the local committee for animal experimentation at the Health Sciences Center (UFRJ, approval # IBQM 011).

Purification of RHBP and Vt

Vt and RHBP were purified as previously described (Oliveira et al., 1995; Silva-Neto et al., 1996). Both molecules were readily isolated from *R. prolixus* oocytes. A total of 100,000 g supernatants obtained from 4,000 oocytes were combined and used as the starting material. Supernatants were dialyzed overnight at 4°C against buffer A (20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.02% NaN_3,10 mM NaF, 1 mM benzamidine). The dialysate was then applied to a Sephadex G-75 gel filtration column (2.0 × 128 cm) equilibrated in buffer A. The column was eluted with a flow rate of 15 ml/h, and 2.5-ml fractions were collected. Fractions from the Sephadex G-75 containing Vt and RHBP, as evaluated by absorbance at 280 nm (Vt) and 413 nm (RHBP), were separately pooled and applied to a DEAE Toyopearl 650 M column (1.4 × 24 cm) equilibrated in buffer A. The column was washed with buffer A, and a linear gradient of 100 ml containing 0.05–0.4 M NaCl in buffer A was developed. Protein content of fractions (0.1 ml) was measured by absorbance as described above. Fractions containing the proteins of interest were pooled and dialyzed extensively at 4°C against 10 mM Tris-HCl pH 7.5, NaCl 0.15 M (TBS).

TBA (TBARs) Assay

Hemin was dissolved in 0.1 ml of 0.1 M NaOH and suspended in 1.5 ml of 10 mM PBS (10 mM phosphate, 0.15 M NaCl, pH 7.4) to yield a 1 mM stock solution. Either linolenic or linoleic acid micelles (0.2 mM) were prepared by vortexing in 0.1 M sodium phosphate buffer (pH 7.2). Fatty acid micelles were incubated at 37°C in 1.5 ml of 1 mM TBS (pH 7.4), 0.1 mM deferoxamine, and in the presence or in the absence of 2 mM $CaCl_2$. The lipid peroxidation reactions were initiated by the addition of hemin or hemeproteins to the reaction mixture, and then, the mixture was incubated at 37°C for 1 h. The reactions were stopped by the addition of 0.2 ml of TBA (1%, w/v) and 0.1 ml of TCA (12%). In order to measure the TBA reactive substances (TBARS), particularly malondialdehyde (MDA) formation, these samples were heated for 30 min at 95°C, placed on ice, and centrifuged at room temperature for 5 min at 13,000 × *g*. Then, the pellets were discarded. The products of the reactions specially MDA were determined by the absorbance at 532 nm. Such values are plotted in figures as MDA (U.A.) as arbitrary units in recognition of the fact that MDA is the major but not the only product of such reaction. Further conditions as described (Gutteridge and Quinlan, 1983).

Gas Chromatography (GC) and GC-Mass Spectrometry (MS)

Linolenic and 9-octadecanoic acid micelles (0.2 mM) prepared as above in the presence or in the absence of RHBP (2 μM), Vt (5 μM), $CaCl_2$ (2 mM), dephosphorylated Vt (dVt; 5 μM; defined just below) incubated for 1 h at 37°C. Fatty acids were further methylated with diazomethane at 4°C for 1 h and fatty acid methyl ester were extracted with heptane, dried under N_2, and trimethylsilylated with bis(trimethylsilyl)trifluoracetamide/pyridine (1:1, v/v) for 1 h at room temperature. GC-MS analyses were performed on a Shimadzu GC 17 gas chromatograph equipped with a DB-1 capillary column interfaced with a GC-MS-QP5050 quadruple mass spectrometer (Shimadzu, Nakagyo-ku, Kyoto, Japan). The column temperature was maintained at 50°C for the first 3 min, increased to 250°C at a rate of 3°C/min, and then maintained at 250°C for 10 min. Electron ionization was performed using an ionization potential of 70 eV and an ionization current of 0.2 mA. Linoleic acid was quantitated relative to the standard, 9-octadecanoic acid based on peak area calculations.

Vt Dephosphorylation

The protein concentration was determined as previously described (Lowry et al., 1951). Purified Vt was incubated overnight at 37°C in the presence or absence of 200 U of recombinant lambda phosphatase obtained from Upstate Biotechnology (Waltham, MA) according to the manufacturer's instructions. The extent of dephosphorylation was evaluated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by Western blot analyses using an antiphosphoserine antibody (EMD Millipore Corporation, Billerica, MA). In the experiments in which dVt was used, the controls contained Vt naturally phosphorylated by insect tissue obtained as described above.

Isothermal Titration Calorimetry (ITC)

Binding assays of Ca^{2+} to Vt and dVt were performed at 25°C in a VP-ITC microcalorimeter from MicroCal/Malvern (Northampton, MA) described by Wiseman et al. (1989).

Aliquots of 2.8 mM $CaCl_2$ were injected into the sample cell ($V = 1.422$ ml), which was then filled with a 5 μM solution of either Vt or dVt in 10 mM TBS (pH 7.4). A total of 27 injections (4×5 μl; 23×10 μl) were performed at 5-min intervals under continuous stirring at 300 rpm. The heat of $CaCl_2$ dilution was determined in the same condition by using 10 mM TBS (pH 7.4) in the sample cell and used to subtract from the thermograms obtained in the presence of the proteins. The enthalpy of Ca^{2+} binding to Vt or dVt (ΔH) was calculated by dividing the area under each peak (heat absorbed) by the concentration (in moles) of $CaCl_2$ injected. ΔH is given in kilocalories per mole of injectant as it represents the enthalpy change for each injection. The thermograms were analyzed with the ORIGIN 5.0 software provided by MicroCal.

Statistical Analysis

Statistical analysis was conducted with GraphPad Prism 3 software (GraphPad Software, Inc., San Diego, CA). Data are presented as the mean ± standard deviation, and all of the experiments were repeated at least three times. Data were analyzed by one-way analysis of variance, and differences between groups were assessed with Tukey's post-test. The level of significance was set at $P < 0.05$.

RESULTS

The peroxidation of linolenic and linoleic acids was determined by the accumulation MDA. As shown in Figure 1A, the incubation of hemin with linolenic acid triggered lipid peroxidation to some degree, which is enhanced by calcium. MDA production in the presence of Ca^{2+} largely decreased in the presence of EGTA or the antioxidant BHT. Similar results were observed when the source of heme was either hemoglobin or RHBP (Fig. 1B). Vt did not alter the overall production of MDA compared with the control, which contained only lipid micelles (Fig. 1C). However, MDA production by incubating hemin with Ca^{2+} was largely reduced by Vt. The addition of the phosphoprotein casein to this reaction also inhibited MDA production (data not shown). Similar results were verified in the presence of RHBP instead of hemin (Fig. 1D).

For the TBARS assay, we used linolenic acid because which is the more sensitive substrate for this assay. Nevertheless, linoleic acid was also used to monitor lipid peroxidation by using the QL reaction shown in Figure 2.

To test the role of phosphate groups on the inhibition of lipid peroxidation catalyzed by heme, RHBP, and hemoglobin, we used dVt. Dephosphorylation was verified by Western blot analysis using phosphoserine antibodies. Typically, 60–75% of the phosphate groups in the protein were removed by this procedure (data not shown). The binding of calcium to both Vt and dVt was studied by ITC, which provides a direct measurement of the enthalpy involved in a reaction (Wiseman et al., 1989). The enthalpy of Ca^{2+} binding was calculated after subtracting the heat of $CaCl_2$ dilution in buffer. Figure 3 shows that calcium binding to Vt occurs by an endothermic reaction that clearly reaches a saturation point. However, the injection of $CaCl_2$ to dVt did not generate heat, suggesting that dVt lacks the ability to bind calcium ions since no heat is developed by the injection of $CaCl_2$ into the protein solution (Fig. 3).

Vt and dVt were incubated in vitro with linoleic acid micelles in the presence or in the absence of RHBP and calcium (Fig. 4A–C). The oxidation of linoleic acid micelles catalyzed by QL was monitored by the amount linoleic acid consumed. Figure 4

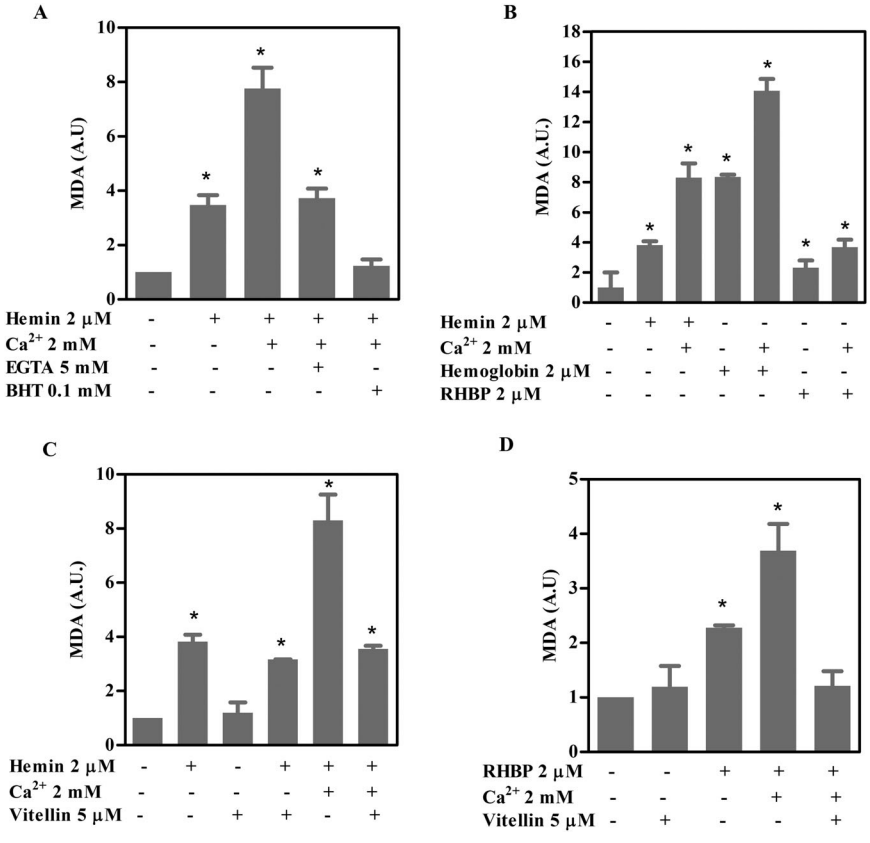

Figure 1. Linolenic acid (0.2 mM) was added to 1.2 ml of 1 mM TBS (pH 7.4) and 0.1 mM deferoxamine in the presence or absence of the following at 37°C for 60 min: (A) 2 μM hemin, 2 mM $CaCl_2$, 5 mM EGTA, and 0.1 mM BHT; (B) 2 μM hemin, 2 μM hemeproteins (hemoglobin or RHBP), and 2 mM $CaCl_2$; (C) 2 μM hemin, 5 μM vitellin, and 2 mM $CaCl_2$; or (D) 2 μM RHBP, 5 μM vitellin, and 2 mM $CaCl_2$. Lipid peroxidation was measured by TBARS as described in Materials and Methods and plotted as MDA arbitrary units (MDA (U.A.)). The data shown represent the mean ± SE ($n = 3$), *$P < 0.05$ compared with the control group.

shows GC chromatograms of the separation of linoleic acid (black arrow on the left) and 9-octadecenoic acid (gray arrow on the right; used as internal standard). The area of linoleic acid peaks obtained in the four chromatograms was transformed in percentage of linoleic acid consumption and is shown in Figure 4E. We observed that RHBP, calcium, and Vt cause a slight decrease in the area of the first peak, which corresponds to linoleic acid, indicating its consumption via oxidation (Fig. 4B). However, by using dVt instead of Vt, there is a dramatic increase in the consumption of linoleic acid (Fig. 4C) and almost a 50% decrease in the peak area (Fig. 4E). This effect was absent in the absence of calcium, indicating the inhibition of lipid peroxidation by the phosphate groups of Vt, most probably due to Ca^{+2} chelation (Fig. 4D).

DISCUSSION

The oxidation of lipoprotein particles has been widely studied in mammalian systems due to its role in atherosclerosis. In mammals, oxidative events targeting LDL particles may

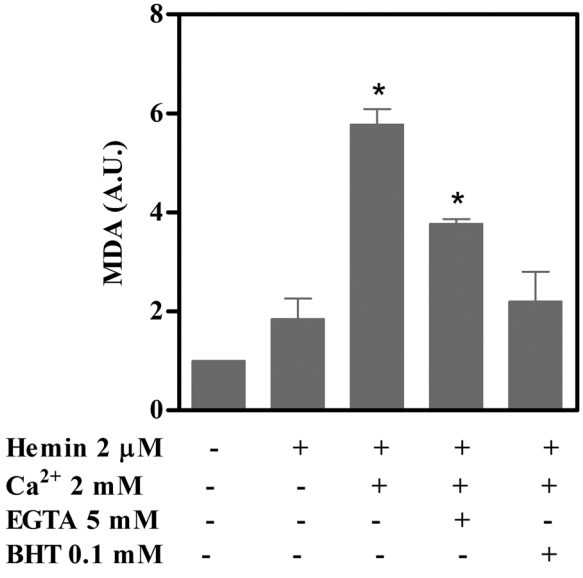

Figure 2. Linoleic acid (0.2 mM) was added to 1.2 mL of 1 mM TBS (pH 7.4) and 0.1 mM deferoxamine in the presence or absence of the following at 37°C for 60 min: 2 μM hemin, 2 mM $CaCl_2$; 5 mM EGTA or 0.1 mM BHT. Lipid peroxidation was measured by TBARS as described in Material and Methods and plotted as MDA arbitrary units (MDA (U.A.)). The data shown represent the mean ± S.E. ($n = 3$).

have several sources, such as reactive oxygen species (ROS) derived from cells involved in inflammatory reactions, the production of nitric oxide and derivatives from endothelial dysfunction and innate immunity. Heme is also involved in LDL oxidation, but its interaction with unsaturated fatty acids on LDL only occurs following a hemolysis event. Heme-mediated peroxidation is also largely dependent on an H_2O_2 source (Klouche et al., 2004) and may result from the generation of ferryl and perferryl radicals derived from hemic iron and H_2O_2 interactions. Blood-sucking arthropods constitute a unique system to study lipid peroxidation catalyzed by heme and its derivatives because they are in seasonal contact with blood, a major source of heme. In the case of *R. prolixus*, heme derived from a blood meal reaches the hemolymph despite several antioxidant and detoxification mechanisms. In the hemolymph, the presence of RHBP and the generation of biliverdin in the dorsal vessel constitute major mechanisms to avoid the harmful effects of free heme. Once RHBP binds to a heme moiety, Ca^{2+} ions are another source of pro-oxidant reactions, such as the heme-mediated QL reaction. In mammals, Ca^{2+} is chelated by albumin, while heme contact to LDL rarely occurs. Thus, in the mammalian system, the QL reaction is considered to be a minor source of LDL oxidation. As mentioned above, heme-mediated peroxidation in mammalian systems requires an H_2O_2 source. In the hemolymph of *Rhodnius*, this peroxidation is largely prevented due to the activity of GPX, which was found in several tissues of the blood-sucking bug *R. prolixus*. In vertebrates, GPX is predominantly intracellular. However, in *Rhodnius,* the highest levels of this enzyme are found in the hemolymph (Paes and Oliveira, 1999). In *Rhodnius*, the hemolymphatic GPX keeps H_2O_2 levels in this insect compartment very low, acting as an antioxidant enzyme and preventing the generation of free radical molecules to react with free heme.

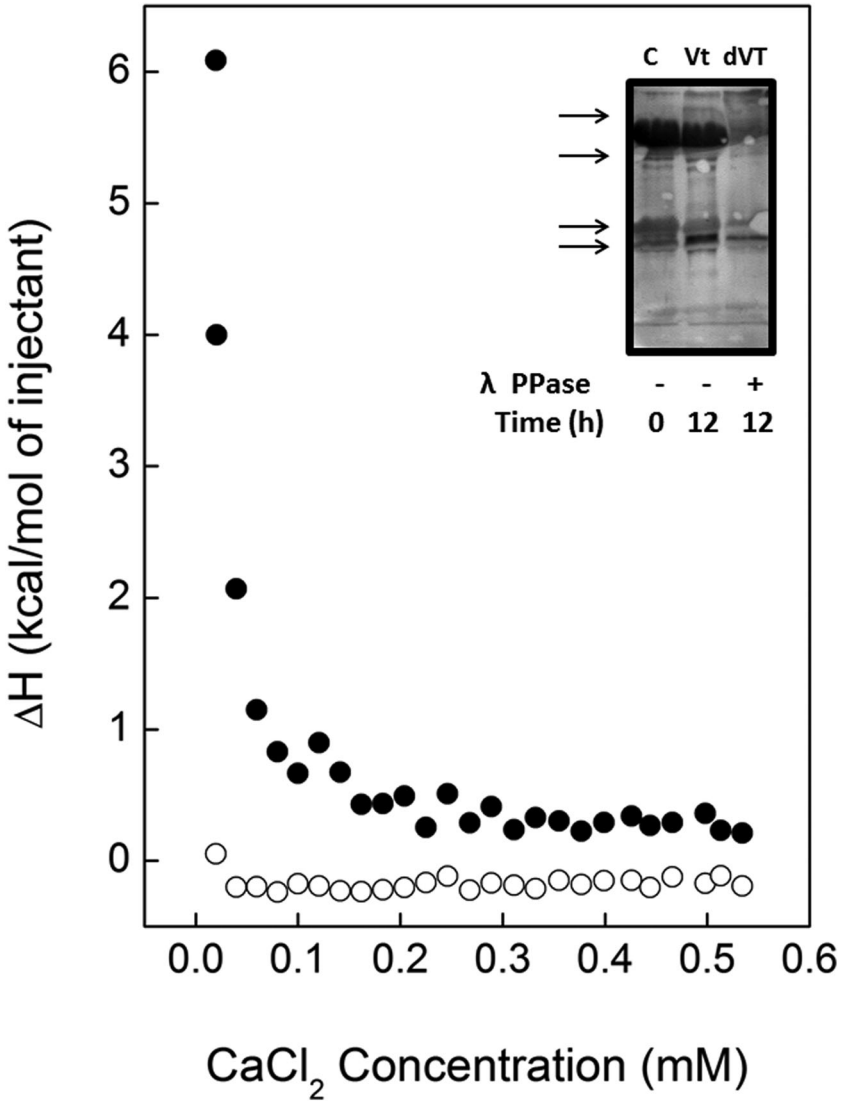

Figure 3. Calcium binding to Vt and dVt as analyzed by ITC. The enthalpy of binding (ΔH) was calculated after subtracting the heat of $CaCl_2$ dilution by dividing the heat absorbed (Q, in kilocalories) by the amount of $CaCl_2$ added in each injection (in moles). Positive peaks indicating an endothermic reaction were found for calcium binding to Vt (closed circles). The injection of $CaCl_2$ to dVt did not generate heat and after the subtraction of the heat of dilution, the enthalpy change was negligible (open circles). Inset shows the dephosphorylation of Vt upon incubation with lambda phosphatase. Following the incubation in the presence and in the absence of the enzyme, samples were applied on an SDS-polyacrylamide gel electrophoresis gel, transferred to blot membranes that were developed with antiphosphoserine antibodies.

The dependence of the QL reaction on Ca^{2+} availability poses a new question in blood-sucking arthropods. The regulation of the release of these ions in the hemolymph and their interactions with circulating proteins, such as Lp, Vt, and RHBP, regulate the extension of the QL reaction and lipid peroxidation by protein-bound heme. The Ca^{2+} ingested from a rabbit-blood meal is largely deposited in the upper cells of the Malpighian

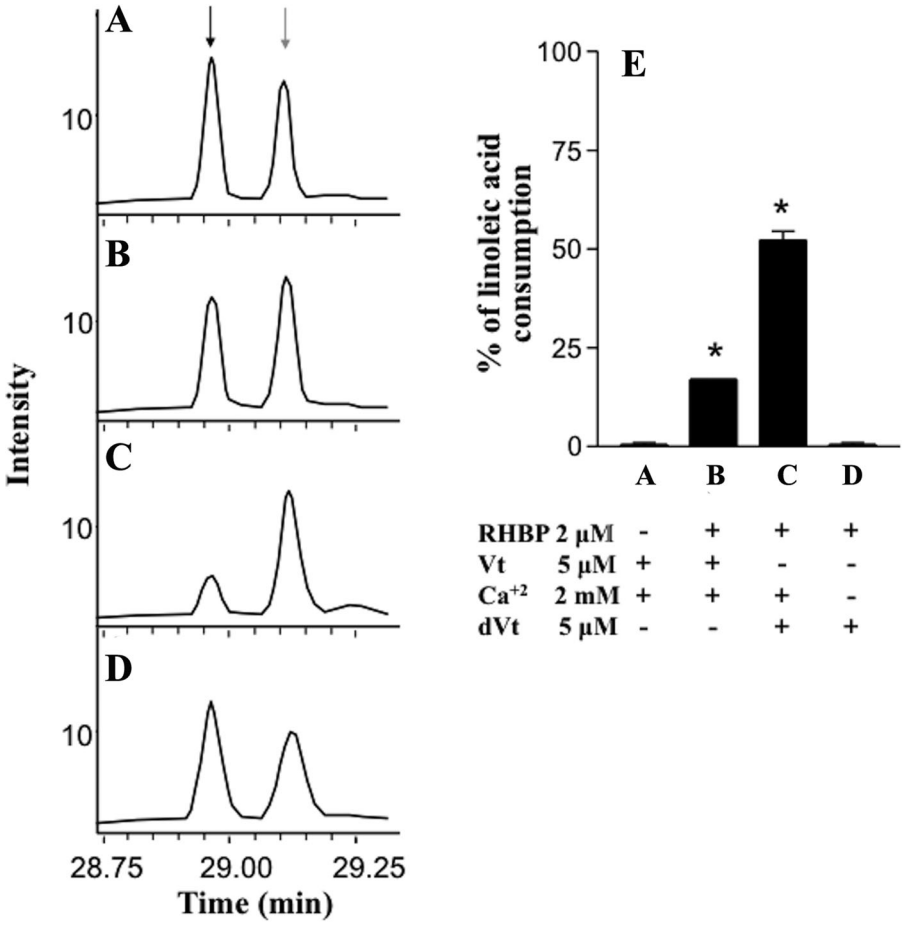

Figure 4. Consumption of linoleic acid by RHBP. GC-MS chromatograms of 0.2 mM of linoleic acid (black arrow on the left) and 0.2 mM of the internal standard 9-octadecanoic acid (gray arrow on the right) were incubated as following: (A) in presence of Vt and Ca^{+2}; (B) in the presence of RHBP, Vt, and Ca^{+2}; (C) in the presence of RHBP, Ca^{+2}, and dVt; and (D) in presence of RHBP and dVt. Left panels: GC-MS chromatograms (A–D). (E) Linoleic acid consumption. Linoleic acid was quantitated relative to the standard, 9-octadecanoic acid based on peak area calculations, and plotted as the mean ± SE ($n = 3$). (A–D) Representative of three independent experiments. $*P < 0.05$.

tubules (Maddrell et al., 1991). Ca^{2+} accumulation occurs 3–4 days after a blood meal, and a very small number of these ions are immediately excreted from the body of the insect. The concentration of total Ca^{2+} in the hemolymph is approximately 8 mM with a 2–3 mM calcium activity. Thus, enough Ca^{2+} is available to participate in the QL reaction. Additionally, hemin-mediated lipid peroxidation may represent an important mechanism of endothelial regulation in vertebrate plasma in vitro (Higdon et al., 2012). Thus, we speculate whether free heme-induced activation of intracellular signaling may contribute to calcium removal from the hemolymph to avoid lipid peroxidation catalyzed by the QL reaction (Graça-Souza et al., 1999). Such results suggest that QL reaction may be catalyzed by other *Rhodnius* hemeproteins such as salivary nitrophorins (Mesquita et al., 2005). In this case, QL products may modulate the hemostatic and immune response of the host

during bug feeding (Mesquita et al., 2008). Such possibility should be investigated in the future. Altogether, the results shown in the present study provide evidence for the role of circulating phosphoproteins as inhibitors of oxidative reactions mediated by heme-binding proteins.

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