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Edema and Nociception Induced by *Philodryas patagoniensis* Venom in Mice: A Pharmacological Evaluation with Implications for the Accident Treatment

Priscila Hess Lopes, Marisa M.T. Rocha, Alexandre Kazuo Kuniyoshi, Fernanda Calheta Vieira Portaro, and Luís Roberto C. Gonçalves

Laboratório de Imunoquímica (P.H.L., A.K.K., F.C.V.P.), Laboratório de Herpetologia/Venenos (M.M.T.R.), and Laboratório de Fisiopatologia (L.R.C.G.) Instituto Butantan, São Paulo-SP, Brazil

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ABSTRACT

We have investigated the mechanisms involved in the genesis of edema and nociception induced by *Philodryas patagoniensis* venom (PpV) injected into the footpad of mice. PpV induced dose-related edema and nociceptive effects. Pretreatment of mice with cyclooxygenase inhibitor (indomethacin), but not with cyclooxygenase 2 inhibitor (celecoxib) markedly inhibited both effects. Pretreatments with H_1 receptor antagonist (promethazine) or with dual histamine-serotonin inhibitor (cyproheptadine) failed in inhibiting both effects. In groups pretreated with captopril (angiotensin-converting enzyme inhibitor) the edema was unaltered, but nociception was clearly increased, suggesting the participation of kinins in the pathophysiology of the nociception but not of the edema-forming effect of PpV. When PpV was treated with EDTA, the nociception was similar to the one induced by untreated venom, but edema was markedly reduced. We concluded that PpV-induced edema and nociception have cyclooxygenase eicosanoids as the main mediators and no participation of vasoactive amines. Kinins seem to participate in nociception but not in edema induced by PpV. The results also suggest that metalloproteinases are the main compounds responsible for the edema, but not for the nociception induced by this venom.

Introduction

In Brazil, accidents induced by venomous snakes of the Viperidae and Elapidae families have significant human and veterinary importance. They are responsible for a high number of accidents in humans; however, from 20% to 40% of the snakebite cases are caused by the so-called nonvenomous snakes, which belong to the families Boidae and Colubridae (Salomão et al., 2003). The latter is the largest group of snakes with approximately 300 genera and 1850 species (Vidal, 2002). This group was recently divided into five new families, among which is the Dipsadidae family (Zaher et al., 2009).

Among the colubrid snakes belonging to the Dipsadidae family, the genus *Philodryas* is widespread in South America and is considered to be of medical interest. These snakes produce venom (Duvernoy's gland secretion) with sufficient toxicity to elicit serious lesions at the site of the bite. Although most accidents caused by these snakes do not result in serious consequences, some reports emphasize the importance of their toxins in causing local manifestations such as pain, edema, ecchymosis, hemorrhage, and in some cases necrosis, but no coagulation disturbances (Ribeiro et al., 1999; Prado-Franceschi and Hyslop, 2002; de Medeiros et al., 2010). Similarly, *Philodryas patagoniensis* venom (PpV) causes local tissue damage as hemorrhage, edema, myonecrosis, and dermonecrosis, as well as pain (Acosta et al., 2003; Peichoto et al., 2004; Rocha and Furtado, 2007; de Medeiros et al., 2010).

The complexity of rear-fanged snake venom composition is reflected in the clinical symptoms of envenomation (Kuch and Mebs, 2002; Peichoto et al., 2012; Weinstein et al., 2013). Metalloproteinases play a critical role in the pathophysiology of colubrid envenomation since they are responsible for the hemorrhagic activity exhibited by many rear-fanged snake venoms (Peichoto et al., 2007; Weldon and Mackessy, 2012). Recent studies have described the purification and characterization of various components of colubrid venoms. Peichoto et al. (2007, 2009, 2010) have isolated and characterized a metalloproteinase from PpV. This toxin, named patagonfibrase, presents α-fibrinolytic and hemorrhagic activities, and can induce hemorrhage and inflammation when injected in mice (Peichoto et al., 2011). These observations and proteomic studies (Weldon and Mackessy, 2010, 2012; Peichoto et al., 2012) have demonstrated that colubrid snake venoms have many proteins in common with the Viperidae venoms, and that severity of envenomation depends on the nature of the venom components (Peichoto et al., 2012).

Similarities between the pathophysiological manifestations of *Bothrops* spp. and *P. patagoniensis* may result in a misidentification

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ABBREVIATIONS: MED, minimum edematogenic dose; PpV, *Philodryas patagoniensis* venom.

of accidents by *Philodryas* as *Bothrops* envenomation (Cardoso and Fan, 1995; França and Málaque, 2003). In many instances, *Bothrops* antivenom was administered (Nishioka and Silveira, 1994; de Araújo and dos Santos, 1997; Ribeiro et al., 1999), potentially causing adverse effects (Nishioka and Silveira, 1994). Since edema and pain are the main local signs in accidents caused by *Philodryas* snakes, in some cases conservative treatments with antihistamine and anti-inflammatory drugs are employed (de Medeiros et al., 2010), but there is no information on the inflammatory mediators involved in these symptoms. Here, we studied the mechanisms involved in the genesis of the edema and the nociceptive effect induced by the venom of *P. paragoniensis* in mice.

Material and Methods

***Philodryas Patagoniensis* Venom.** Venom from adult specimens of *P. patagoniensis* (90–120 cm body length) was obtained after a previous injection of pilocarpine (10 mg/kg) to induce secretion in Duvernoy's glands. The venom was collected with the aid of capillary tubes, lyophilized, and stored at −20°C (Ferlan et al., 1983). The venom solutions were prepared with sterile physiologic saline (0.15 M NaCl) at the moment of use, and the protein content was evaluated by the method employed in Markwell et al. (1978) using bovine serum albumin as the standard.

Animals. Male Swiss mice weighing 18–22 g, supplied by the Central Animal House of the Butantan Institute were used. Animals were maintained and used under strict ethical conditions according to the Brazilian Society of Laboratory Animal Science (SBCAL, http://www.cobea.org.br). This study was submitted to the Institutional Animal Care Committee at the Butantan Institute and was approved by protocol number 295/06.

Evaluation of Edema. The footpad of one of the hind paws of mice (n = 6/group) was injected with 50 μl of sterile saline solution (0.15 M NaCl) containing different doses of PpV, and the contralateral paw received the same volume of saline as the control. The paw volumes were evaluated with the aid of a plethysmometer (Panlab, Barcelona, Spain) at 15 and 30 minutes and 1, 2, 3, 4, 6, and 24 hours after the paw injections. The edema was expressed as the difference (%) between the volumes of venom and saline-injected paws. The minimum edematogenic dose (MED) was defined as the venom dose able to induce 30% of paw increase (Yamakawa et al., 1976). In experiments of pharmacological modulation, the edema was evaluated only at 30 minutes after the injection of 2 MED of PpV.

Evaluation of Nociception. Nociception was assessed after subcutaneous injection of 50 μl of solutions containing saline or 0.5, 1.0, 3.0, or 5.0 μg of PpV into the footpad of the right hind paw of mice (n = 9/ group). The animals were placed in a glass chamber on a reflector surface for observation of nociceptive behavior. During 30 minutes of observation, we recorded the time (in seconds) that animals spent in nociceptive behavior (licking, shaking, or lifting the injected paw), as described in Hunskaar et al. (1985). In experiments of pharmacological modulation, the nociception was induced with 2.0 μg of PpV.

Treatment of Animals. To evaluate the pharmacological modulation of edema and nociception, PpV was injected into a hind paw of nonblinded groups of 6–9 mice after the following pretreatments: (1) dexamethasone (corticosteroid, phospholipase A_2 inhibitor; Hypofarma, Ribeirão das Neves, Brazil), 1 mg/kg i.p., 1 hour before; (2) indomethacin (cyclooxygenase inhibitor; Fluka Chemie AG, Buchs, Switzerland), 2 mg/kg s.c., 30 minutes before; (3) celecoxib (cyclooxygenase 2 inhibitor; Pfizer, Itapevi, Brazil), 5 mg/kg i.p., 30 minutes before; (4) promethazine (H_1 receptor antagonist; EMS Laboratories, Hortolândia, Brazil), 5 mg/kg i.p., 15 minutes before; (5) cyproheptadine (histamine and serotonin receptors antagonist; Sigma, São Paulo, Brazil), 2 mg /kg s.c., 30 minutes before; or (6) captopril (angiotensin-converting enzyme inhibitor; Medley, São Paulo, Brazil), 10 mg/kg i.p., 2 hours before. Control groups received only PpV into the paw.

Effect of EDTA on PpV-Induced Edema and Nociception. The effectiveness of EDTA treatment as a metalloprotease inhibitor was evaluated using a free resonance energy transfer peptide

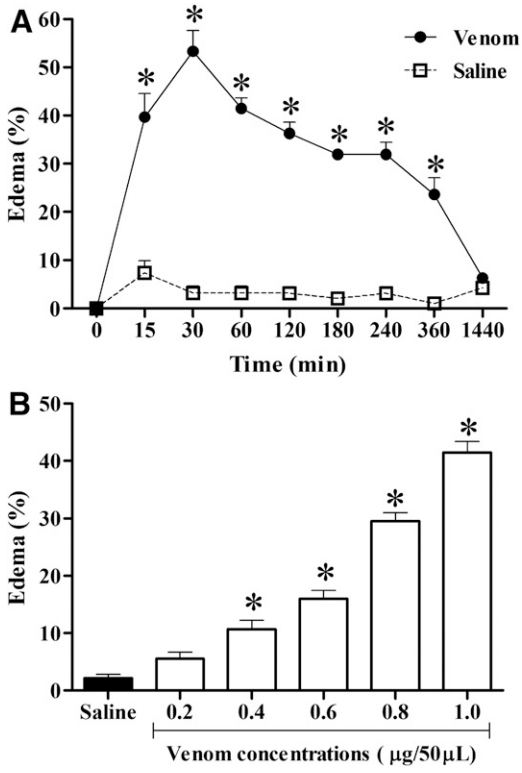

Fig. 1. Edema-forming activity of PpV. Time course of edema induced by 1.0 μg/50 μl of PpV in the footpad of mice (A) and effect of different doses of PpV on edema evaluated 30 minutes after injection (B). Edema (%) is expressed as mean ± S.E.M. of six animals, analyzed by one-way analysis of variance and Bonferroni post-test. (*) Statistically different from saline-induced edema.

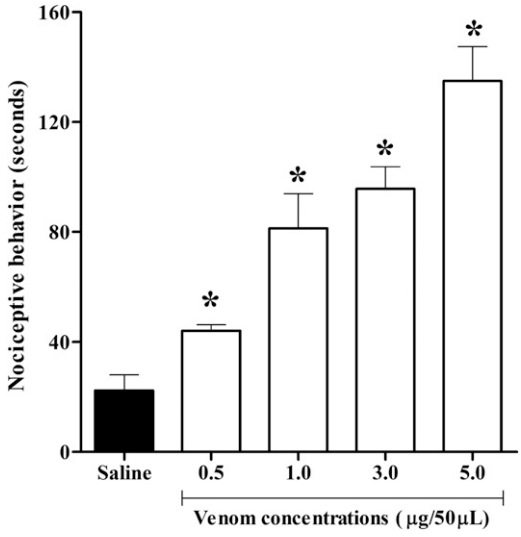

Fig. 2. Nociceptive effect of PpV. Effect of different doses of PpV in 50 μl injected in the footpad of mice. Nociceptive behavior, represented as licking, shaking, or lifting time in seconds, is expressed as mean ± S.E.M. of nine animals for each dose, analyzed by one-way analysis of variance and Bonferroni post-test. (*) Statistically different from saline-induced nociception.

Abz-RPPGFSPFRQ-EDDnp as previously described in Kuniyoshi et al. (2012). Briefly, PpV activity assay was conducted in 7.4 pH phosphate-buffered saline (final volume 100 μl) containing 50 mM phosphate and 20 mM NaCl, using Corning 96-well plates (Oakville, Canada) and a peptide substrate in a final concentration of 5 μM. The reactions occurred at 37°C and were initiated by the addition of 0.5 μg of PpV. There was an incubation period of 30 minutes at room temperature when phenylmethanesulfonylfluoride (1 mM), 1,10-phenantroline (5 mM), and EDTA (5 mM) where tested. When necessary, control samples were made in the presence of the same volume of ethanol used in the preparation of inhibitor stock solutions (phenylmethanesulfonylfluoride and 1,10-phenantroline). The experiments were made in triplicate.

Next, PpV venom was treated with 5 mM of EDTA for 30 minutes at room temperature for the in vivo experimental procedure. EDTA-treated venom was injected into the hind paw of mice at doses used to evaluate edema or nociception as described previously. In the control groups crude venom or with the same concentration of EDTA was injected into the hind paws.

Statistical Analysis. The results are presented as mean ± S.E. They were analyzed by one-way analysis of variance followed by Bonferroni test, or when appropriate by Student's t test using the GraphPad Prism 5.01 software (GraphPad, San Diego, CA). Results were considered significant when $P < 0.05$.

Results

Evaluation of Edema and Nociception Induced by PpV. PpV induced edema of rapid onset, which peaked 30 minutes after venom injection, decreased after that, and disappeared 24 hours after the experimental envenomation (Fig. 1A). The edematogenic activity was intense and dose dependent (Fig. 1B) and the MED was 0.82 μg. A dose of 1.64 μg (2 MED) was used for the study of pharmacological modulation. This venom also induced dose-dependent nociception (Fig. 2). A dose of 2.0 μg was used to study the pharmacological modulation.

Pharmacological Evaluation of PpV-Induced Edema and Nociception. The edema induced by PpV was significantly inhibited in groups pretreated with dexamethasone and indomethacin (Fig. 3). In contrast, nociception was significantly inhibited in the group pretreated with indomethacin. In the group treated with captopril the nociception was significantly increased (Fig. 4). In groups pretreated with celecoxib, promethazine, or cyproheptadine, edema and nociception were not affected (Figs. 3 and 4).

Effect of EDTA on PpV-Induced Edema and Nociception. Figure 5 shows that Abz-RPPGFSPFRQ-EDDnp hydrolysis was totally inhibited by both EDTA and 1,10-phenantroline, thus indicating complete inhibition of metalloproteinases present in the venom. This was unlike the results obtained with phenylmethanesulfonylfluoride, a serine protease inhibitor. Treatment of PpV with EDTA significantly inhibited its edema-forming activity (Fig. 6A) but did not affect the venom-induced nociception (Fig. 6B). Groups injected only with EDTA did not present significant edema or nociception (data not shown).

Discussion

In accidents caused by colubrid snakes, prominent edema and hyperalgesia is frequently present (Prado-Franceschi and Hyslop, 2002), as well as characteristic signs of acute inflammation. The venom of *P. patagoniensis* causes a rapid inflammatory response with marked edema and hyperalgesia. These local effects are intense, and are similar to the effects observed in *Bothrops*-induced envenomation, which can cause misinterpretation in the clinical diagnosis, despite the lack of blood coagulation disturbances (Puorto and França, 2003; de Medeiros et al., 2010).

Regarding the time course and intensity of the edematogenic response, our results are in agreement with studies that have shown a peak of activity 30 minutes after venom injection, with a dose-effect response (Rocha and Furtado, 2007). In fact, the edema induced by PpV reached the maximal intensity faster than in edema induced by some viperid

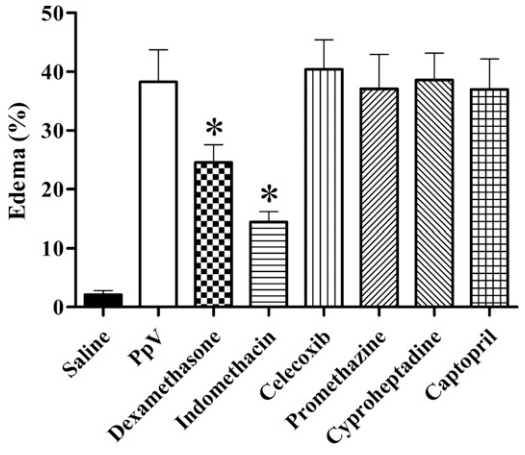

Fig. 3. Effect of the pretreatment with different drugs on paw edema induced by PpV. The edema was evaluated 30 minutes after injection of 1.64 μg/50 μl of PpV in the footpad of mice. Edema (%) is expressed as mean ± S.E.M. of six animals, analyzed by one-way analysis of variance and Bonferroni post-test. (*) Results statistically lower ($P < 0.05$) than the control untreated PpV-injected group.

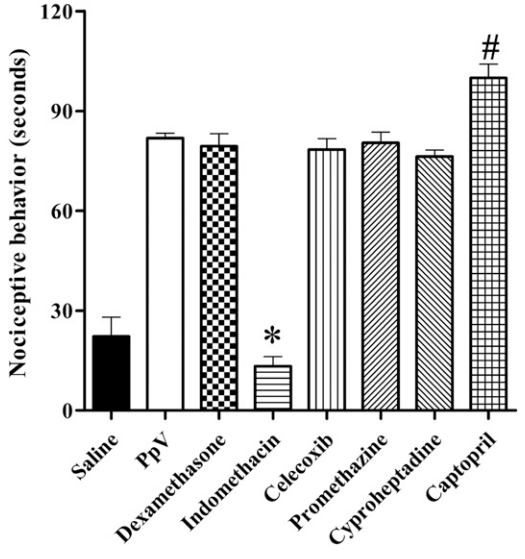

Fig. 4. Effect of the pretreatment with different drugs on the nociceptive behavior induced by PpV. The nociceptive behavior induced by the injection of 2.0 μg/50 μl of PpV is expressed as mean ± S.E.M. of the licking time (seconds) of nine animals, analyzed by one-way analysis of variance and Bonferroni post-test. (*) Results statistically lower or (#) results statistically higher ($P < 0.05$) than the control untreated PpV-injected group.

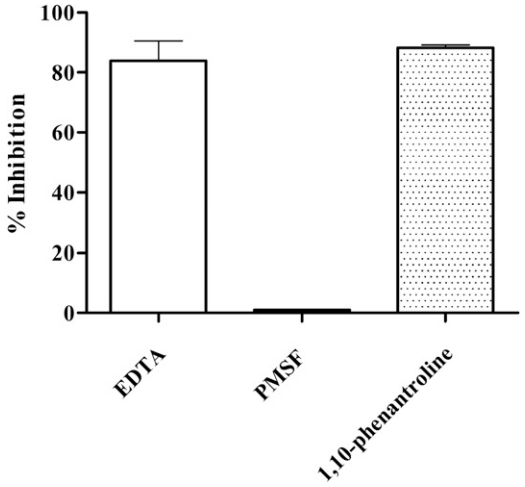

Fig. 5. Hydrolysis of the free resonance energy transfer substrate by PpV and the effect of classic inhibitors of metalloproteases and serine proteases. Inhibition effect of EDTA (5 mM), 1,10-phenantroline (5 mM), and phenylmethanesulfonylfluoride (PMSF) (1 mM) upon the hydrolysis of Abz-RPPGFSPFRQ-EDDnp by Ppv (0.5 μg). The results are expressed as mean ± S.E.M. of triplicates.

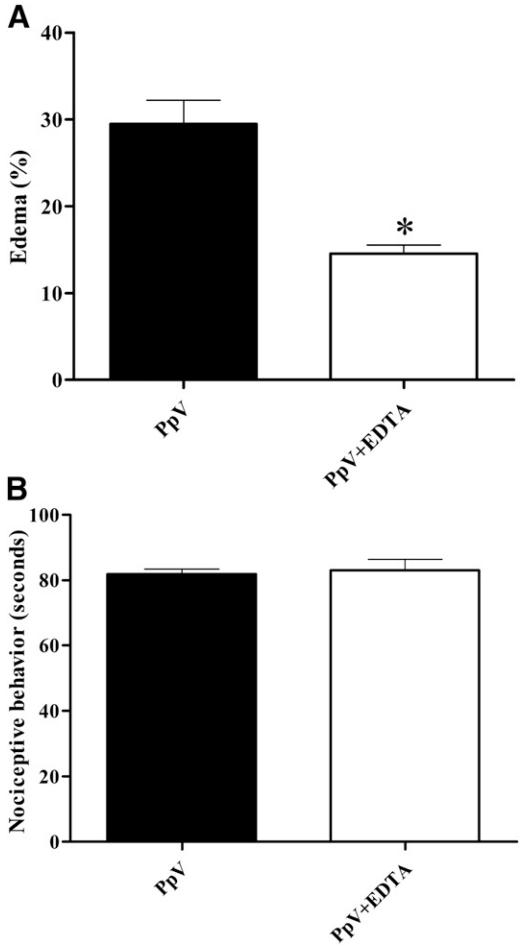

Fig. 6. Effect of treatment of PpV with EDTA on its edematogenic and nociceptive effect. Venom (1.64 and 2.0 μg/ 50 μl) treated with 5 mM of EDTA was injected into the footpad of mice for evaluation of edema (A) and nociception (B). Results are expressed as mean ± S.E.M., analyzed by Student's t test. (*) Statistically different ($P < 0.05$).

venoms (Chaves et al., 1995; Gonçalves and Mariano, 2000; Barbosa et al., 2003; Al-Asmari and Abdo, 2006). We also found that PpV elicits a marked dose-dependent nociceptive response, which is more intense than that described in relation to *P. olfersii* venom (Rocha and Furtado, 2007).

Apart from case reports, epidemiologic studies, and studies on the characterization of the biologic activities of PpV, to the best of our knowledge, this is the first experimental study on the pharmacological evaluation of inflammatory effects induced by this venom. Our results show that derivatives of arachidonic acid are the main mediators of edema induced by PpV since treatment with dexamethasone significantly inhibited this effect. The results of treatment with indomethacin and celecoxib indicate that eicosanoids from the cyclooxygenase pathway, but not from cyclooxygenase 2, participate in this mediation. Arachidonic acid derivatives are the major players in the modulation of edema induced by some viperid snake venoms (Perales et al., 1992; Chaves et al., 1995; Gonçalves and Mariano, 2000; Barbosa et al., 2003)

In contrast, results obtained in groups treated with promethazine (H_1 receptor antagonist), cyproheptadine (histamine and serotonin inhibitor), or captopril (angiotensin-converting enzyme inhibitor) suggest that vasoactive amines and kinins do not play a role in the edema induced by PpV. Other studies have shown that histamine does not participate in the edema induced by *Bothrops jararaca* or *B. asper* venom in mice (Perales et al., 1992; Chaves et al., 1995). However, this mediator can participate in the edema induced by other animal venoms, such as viperid venoms (Al-Asmari and Abdo, 2006; Galvão Nascimento et al., 2010; Sebia-Amrane and Laraba-Djebari, 2013), *Lonomia* caterpillar venom (de Castro Bastos et al., 2004), *Potamotrygon motoro* stingray venom (Kimura et al., 2015), and *Echinometra lucunter* sea urchin coelomic fluid (Sciani et al., 2014).

The nociceptive effect of PpV has a pharmacologic modulation distinct from the edema induced by this venom. Besides eicosanoids, the increase in nociceptive behavior after inhibition of the angiotensin-converting enzyme by captopril is indicative of kinins participation in the modulation of nociception induced by PpV. Bradykinin participates in nociception induced by some *Bothrops* venoms (Chacur et al., 2001, 2002), but not in the edema induced by these venoms (Trebien and Calixto, 1989; Chacur et al., 2001). Nevertheless, this mediator participates in edema and nociception induced by *Thalassophryne nattereri* fish venom (Lopes-Ferreira et al., 2004).

The inhibition of free resonance energy transfer substrate hydrolysis by EDTA corroborates that metalloproteinases have a central role in the edematogenic effect since a significant reduction in this effect in EDTA-treated PpV was found. This fact agrees with previous studies with an isolated toxin and with proteomic studies showing metalloproteinases as the main class of toxins present in PpV (Peichoto et al., 2011, 2012). In contrast, treatment with EDTA did not affect the nociceptive action of PpV, suggesting the participation of other classes of toxins. It is well known that bradykinin is a major mediator of the pain induced by *B. jararaca* venom (Chacur et al., 2002) and that serine proteinases in this venom are the class of toxins responsible for the liberation of this nonapeptide (Serrano and Maroun, 2005; Serrano, 2013). Serine proteinases are present in colubrid venoms (Ching et al., 2006;

Peichoto et al., 2012); however, until now there has been no information on kinin-releasing enzymes from colubrid venoms. This issue is under investigation by our group. In *B. jararaca* venom, metalloproteinases are the main compounds responsible for the inflammatory effect, and serine proteinases do not have consequences in its local inflammatory effect, despite having kininogenase activity (Zychar et al., 2010).

As cited previously, some accidents caused by *Philodryas* snakes have been wrongly diagnosed as *Bothrops* envenomation, resulting in the use of antivenom (de Medeiros et al., 2010). The use of *Bothrops* antivenom to treat cases of *Philodryas* envenomation do not have a clinical basis, despite the fact that some of the toxins of *Philodryas* venom are immunologically recognized by the *Bothrops* antivenom (Rocha et al., 2006). Since specific antivenoms are not available for treatment of colubrid-induced envenomation in Brazil, knowledge of the pharmacological mediation of the edema and pain induced by PpV can allow a therapeutic strategy. Clinical studies describe the use of antihistamine drugs as supportive treatment for this envenomation (de Medeiros et al., 2010); however, the present results indicate that histamine does not participate in the mediation of edema and nociception induced by PpV. In contrast, our results suggest that treatment with cyclooxygenase inhibitors, such as indomethacin, may be beneficial in the treatment of edema and pain induced by PpV, but further experimental and clinical studies need to be carried out to confirm this observation. Experimental studies have shown that in envenomation-induced by *Bothrops* spp., the use of anti-inflammatory drugs, such as dexamethasone, associated with serum therapy has a beneficial effect by reducing inflammatory edema more quickly and avoiding muscle damage (Araújo et al., 2007; Patrão-Neto et al., 2013). In conclusion, our results show that eicosanoids are the primary mediators of edema and nociception induced by PpV, and suggest that nociception may also be mediated of kinins.

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Authorship Contributions

Participated in research design: Lopes, Rocha, Portaro, Gonçalves.

Conducted experiments: Lopes, Rocha, Kuniyoshi.

Performed data analysis: Lopes, Portaro, Gonçalves.

Wrote or contributed to the writing of the manuscript: Lopes, Rocha, Kuniyoshi, Portaro, Gonçalves.

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Address correspondence to: Dr. Priscila Hess Lopes, Laboratório de Imunoquímica, Instituto Butantan, Av. Vital Brazil, 1500, 05503-900, São Paulo-SP, Brazil. E-mail: priscila.lopes@butantan.gov.br