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Inflammatory oedema induced by *Lachesis muta muta* (Surucucu) venom and LmTX-I in the rat paw and dorsal skin

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ABSTRACT

The ability of crude venom and a basic phospholipase A_2 (LmTX-I) from *Lachesis muta muta* venom to increase the microvascular permeability in rat paw and skin was investigated. Crude venom or LmTX-I were injected subplantarly or intradermally and rat paw oedema and dorsal skin plasma extravasation were measured. Histamine release from rat peritoneal mast cell was also assessed. Crude venom or LmTX-I induced dose-dependent rat paw oedema and dorsal skin plasma extravasation. Venom-induced plasma extravasation was inhibited by the histamine H_1 antagonist mepyramine (6 mg/kg), histamine/5-hydroxytriptamine antagonist cyproheptadine (2 mg/kg), cyclooxygenase inhibitor indomethacin (5 mg/kg), nitric oxide synthesis inhibitor L-NAME (100 nmol/site), tachykinin NK_1 antagonist SR140333 (1 nmol/site) and bradykinin B_2 receptor antagonist Icatibant (0.6 mg/kg). Platelet-activating factor (PAF) antagonist PCA4248 (5 mg/kg) had no effect. LmTX-I-induced skin extravasation was inhibited by cyproheptadine, mepyramine, indomethacin and PCA4248, while L-NAME and SR140333 had no effect. Additionally, both *Lachesis muta muta* venom and LmTX-I concentration-dependently induced histamine release from rat mast cells. In conclusion, *Lachesis muta muta* venom and LmTX-I increase microvascular permeability by mechanisms involving *in vivo* mast cell activation and arachidonic acid metabolites. Additionally, crude venom-induced responses also involve substance P, nitric oxide and bradykinin release, whether LmTX-I-induced responses involve PAF.

1. Introduction

Lachesis, Crotalus and *Bothrops* are the genera belonging to the *Crotalinae* subfamily of the *Viperidae* snakes found in South America. The bushmaster *Lachesis muta muta* (Surucucu) is the largest *Crotalinae* snake species in the world, and lives in forested regions of hard access. Accidents induced by *Lachesis* present many symptoms like local pain, oedema, haemorrhage and necrosis at the site of the bite. Moreover, systemic complications such as nausea, vomiting, diarrhoea, hypotension and bradycardia, coagulation disturbances and renal failure are observed during *Lachesis* envenomation (Jorge et al., 1997; Rucavado et al., 1999). However, because of the difficult capture and maintenance in captivity of this snake, few studies have attempted to investigate the physiopathology of *Lachesis* envenomation in animal models (Damico et al., 2005b). Recent works have shown that *Lachesis muta muta* venom possesses active components that act on neuromuscular junction and muscle fibres (Damico et al., 2005a) and exerts toxic effects on cultured Madin–Darby canine kidney cells (Damico et al., 2007). Some components of this venom have been

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isolated, such as the haemorrhagic metalloproteases, mutalysin I and II (Estevão-Costa et al., 2000), thrombin-like enzyme (Magalhães et al., 2006), and phospholipases A_2 (PLA_2) isoforms (Fuly et al., 2002; Damico et al., 2005b, 2006). Generally, *Lachesis muta muta* crude venom possesses higher levels of procoagulant, proteolytic and PLA_2 activities when compared with several *Bothrops* snake venoms (Fuly et al., 1993).

Bothrops venoms are well known for their ability to cause oedema, protein leakage, leukocyte recruitment and pain (Chacur et al., 2001; Farsky et al., 2005). The inflammatory effects induced by these venoms generally involve the degranulation of tissue mast cells and generation of lipid-derived metabolites, bradykinin, neuropeptides, cytokines and nitric oxide (NO), as demonstrated by studies using venoms from *Bothrops* genus such as *Bothrops asper* (Chaves et al., 1995, 2006), *Bothrops insularis* (Barbosa et al., 2003), *Bothrops jararaca* (Trebien and Calixto, 1989; Guzzo et al., 2000) and *Bothrops lanceolatus* (de Araújo et al., 2000; de Faria et al., 2001; Guimarães et al., 2004). In addition, it has been shown that PLA_2s can account for part of the oedematogenic or hyperalgesic activities induced by bothropic venoms (Chacur et al., 2003; Teixeira et al., 2003).

Phospholipases A_2 can be divided into 11 groups based on their structure similarity and localization. Groups I, II and III consist of low molecular weight secretory PLA_2s found in snake and bee venoms, mammalian pancreas and synovial fluid (Six and Dennis, 2000). Venom PLA_2s cause local inflammatory effects characterized mainly by increase in vascular permeability (Cirino et al., 1989; Chaves et al., 1995; Landucci et al., 2000; Câmara et al., 2003) or leukocyte infiltration into tissues (de Castro et al., 2000) which take place by mechanisms dependent and independent of their catalytic activity (see Teixeira et al., 2003, for review). These secretory enzymes are also capable of activating human inflammatory cells (Gambero et al., 2002; Triggiani et al., 2005) and inducing acute pancreatitis in rats (Camargo et al., 2005). LmTX-I is a basic PLA_2 isolated from the *Lachesis muta muta* venom with p*I* 8.8 and a single polypeptide chain of 14.2 kDa (Damico et al., 2005b). The primary structure of LmTX-I showed a high level of homology with Asp-49-containing PLA_2s such as those from the Mojave rattlesnake *Crotalus scutulatus scutulatus*, *Crotalus durissus terrificus,* and *Agkistrodon halys pallas* (Agkistrodotoxin) venoms (Damico et al., 2005b). Other PLA_2 isoforms from *Lachesis muta muta* venom were also isolated, such as LmTX-II (Damico et al., 2005b) and the acid isoforms LM-PLA_2-I and II (Fuly et al., 2002). Since no studies have been carried out to explore the inflammatory actions of *Lachesis muta muta* crude venom and LmTX-I, this work was designed to investigate its ability to induce inflammatory oedema and mechanisms involved using the rat paw oedema and skin model of inflammation.

2. Materials and methods

2.1. Venom and drugs

Lachesis muta muta venom was purchased from a private serpentarium CETA (Centro de Extração de Toxinas Animais, Morungaba, SP, Brazil). The crude venom and PLA_2 from *Lachehis muta muta* venom (LmTX-I) used in our study were isolated in house according to previous studies (Damico 2005a,b). Crude venom, PLA_2 from *Lachesis muta muta* venom (LmTX-I) and other test agents were diluted with modified Tyrode solution prior to use. The Tyrode composition was (in mM): NaCl, 137; KCl, 2.7; $MgCl_2$, 0.5; NaH_2PO_4, 0.4; $NaHCO_3$, 11.9 and glucose, 5.6.

Bradykinin, cyproheptadine, compound 48/80, histamine, indomethacin, N^{ω}-nitro-L-arginine methyl ester (L-NAME), mepyramine and substance P were purchased from Sigma Chemical Co. (St Louis, MO, USA). ^{125}I-Human serum albumin was radio-labelled in IPEN/CENEN-USP (São Paulo, Brazil). SR140333 ((*S*)1-{2-(3(3-4-dichlorophenyl)-1-(3-iso-propoxyphenylacetyl) piperidine-3-yl)ethyl}-4-phenyl-1-azoniabicyclol(2.2.2) octone, chloride) was provided by Sanofi Recherche (Montpellier, France). PCA4248 (2-phenyltio)ethyl-5-methoxycarbonyl-2,4,5,-trimethyl-1, 4-dihydropyridine-3-carboxylate from Tocris (Bristol, UK). The selective tachykinin NK_1 receptor agonist GR73632 was a gift from Glaxo Group Research (Ware, UK). Icatibant (JE049) was provided by Aventis Pharma Deutschland GmbH (Frankfurt, Germany).

2.2. Animals

Male Wistar rats were used for all the experiments and were provided by UNICAMP Central animal House Services (CEMIB). The animals were housed in temperature-controlled rooms and received water and food *ad libitum* until used. All experiments were carried out in accordance with the State University of Campinas (UNICAMP) guidelines for animal care.

2.3. Rat paw oedema

Wistar rats (120–150 g) were anaesthetized with inhaled halothane and received a subplantar injection of crude *Lachesis muta muta* venom or LmTX-I (0.3–3 and 0.1–1 μg/paw, respectively) in one paw in a final volume of 0.1 ml. Control groups received sterile saline (0.1 ml). The paw volume was assessed immediately before venom or LmTX-I injection for the basal measurement, and thereafter at 0.25, 0.5, 1 and 2 h , using a hydroplethysmometer (model 7150, Ugo Basile, Italy). The results are expressed as the increase in paw volume (ml) calculated by subtracting the basal volume (Landucci et al., 2000). The area under the time–course curve (AUC_{0-2h}) was also calculated using trapezoidal rule, and the results expressed as total oedema volume (ml/h) by comparison with the control rats.

2.4. Measurement of rat dorsal skin plasma protein extravasation

Male Wistar rats (200–300 g) were anaesthetized with sodium thiopental (50 mg/kg, given i.p.) and maintenance doses were administered when required. Local plasma protein extravasation was measured in the shaved dorsal rat skin, in response to intradermally injected crude *Lacheshis muta muta* venom (0.03–10 μg/site), PLA_2 (0.003–0.3 μg/site) or other agents (100 μl/site in Tyrode solution),

according to Brain and Williams (1985). Agents were injected in a random order, according to a balanced site pattern. Plasma protein extravasation was measured by the accumulation of intravenously injected (i.v.) ^{125}I-human serum albumin (2.5 μCi/rat) with Evan's blue dye (20 mg/kg) to act as a visual marker. Antagonists and other test agents were injected as required by specific protocols (see Table 1). At the end of the accumulation period (30 min), a cardiac blood sample (5 ml) was taken and the rats killed by anaesthetic overdose. The blood samples were centrifuged at 8,000 × *g* for 10 min to obtain a plasma sample. The injected sites were punched out and counted for radioactivity, with the plasma samples in a γ-counter. Plasma extravasation was expressed as the volume (μl) of plasma accumulated at each skin site compared to total counts in 1 ml of plasma.

2.5. Pharmacological investigation with different drugs

Several drugs were used in order to investigate the mechanisms involved in the *Lachesis muta muta* venom and LmTX-I-induced rat skin oedema. Cyproheptadine, a dual histamine H_1 and serotonin 5-HT_2 receptor antagonist, was used at 2 mg/kg, given intraperitoneally (i.p.), at 30 min before injection in the dorsal skin sites (Câmara et al., 2003). Mepyramine, a non-selective histamine H_1 receptor antagonist, was given at 6 mg/kg, intravenously (i.v.), 15 min before injection (Barbosa et al., 2003). Indomethacin, a cyclooxygenase (COX) inhibitor, was given at 5 mg/kg, i.p., 1 h before injection (Barbosa et al., 2003). The B_2 receptor antagonist Icatibant was given at 6 mg/kg, i.p., 30 min before injection (Costa et al., 2001). The platelet-activating factor (PAF) receptor antagonist PCA 4248 was given at 5 mg/kg, i.v., 1 h before injection (Filliatre et al., 2001). The non-selective nitric oxide (NO) synthase inhibitor L-NAME or the NK_1 receptor antagonist SR140333 were co-injected with *Lachesis muta muta* venom or LmTX-I at 100 and 1 nmol/site, respectively (Ridger et al., 1997; Câmara et al., 2003).

2.6. Mast cell isolation and degranulation

Mast cells from the peritoneal cavity of four to six rats were purified on a Percoll gradient as described previously (Enerback and Svenson, 1980). Briefly, the rats were exsanguinated under halothane anaesthesia and 10 ml of Krebs–Ringer phosphate solution (pH 7.3) were injected into the intraperitoneal cavity. The Krebs–Ringer solution had the following composition (mM): NaCl 150, KCl 6.1, Na_2HPO_4 10, $MgSO_4$.1.5, $CaCl_2$.42.9 and glucose 5.6. The abdomen was carefully massaged and the fluid was withdrawn, placed in polypropylene centrifuge tubes and centrifuged at 300 *g* for 5 min at 4 °C. The resulting cell pellet (of which mast cells comprise 10%) was gently resuspended in a small volume of Krebs–Ringer phosphate solution, layered over the isotonic Percoll gradient and left at room temperature for 10 min prior to centrifugation (150 × *g*, 25 min at 4 °C). The gradient zone containing the mast cells was removed and washed twice in Krebs–Ringer phosphate solution. The purity of the cells in the final preparation was 90–95% and their viability (assessed by 0.1% Trypan blue dye exclusion) was approximately 95%. Aliquots of mast cell suspension (0.5 ml containing 4×10^5 cells/ml) were warmed to 37 °C for 10 min. The stimulus was added to the suspension (final volume of 1.0 ml) followed by incubation for a further 15 min, after which the reaction was stopped by placing the test tubes in ice-cold water. The cells were then centrifuged (300 × *g*, 10 min at 4 °C), and the supernatant was removed for histamine determination. Krebs–Ringer phosphate solution (1.0 ml) was added to the cell pellet which was boiled at 100 °C for 10 min to release the residual histamine. Histamine concentrations were determined spectrofluorometrically, and the histamine release was expressed as a percentage of the total cellular content of the amine.

Table 1
Area under curve (AUC_{0-2h}) of rat paw oedema induced by *Lachesis muta muta* venom (0.3–3 μg/ml), LmTX-I (0.1–1 μg/ml).

	Dose (μg/paw)	Area under curve (ml/h)
Saline	–	0.3 ± 0.05
Lachesis muta muta venom	0.3	0.5 ± 0.02
	1	0.9 ± 0.15*
	3	1.5 ± 0.12*
LmTX-I	0.1	0.4 ± 0.04
	0.3	0.6 ± 0.1*
	1	1.3 ± 0.1*

The values represent the mean ± SEM of five rats. *$P < 0.05$ compared to saline group.

2.7. Statistical analysis

Results are presented as mean values ± SEM for *n* experiments. The values were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's modified test or Student's unpaired *t*- test, when appropriate. $P < 0.05$ was taken as significant.

3. Results

3.1. Effects of Lachesis muta muta venom and LmTX-I in the rat paw

The subplantar injection of either *Lachesis muta muta* crude venom (0.3–3 μg/paw; $n = 5$) or LmTX-I (0.1–1 μg/paw; $n = 5$) induced dose-dependent rat paw oedema, as shown in Fig. 1 and Table 1. The paw oedema induced by crude venom and LmTX-I had a similar time–course profile as characterized by a rapid onset (maximal responses obtained at 15 min post-injection) which was relatively sustained during the 2-h observation period, particularly with the highest doses of crude venom (3 μg/paw) and LmTX-I (1 μg/paw).

3.2. Effects of Lachesis muta muta venom and LmTX-I in the rat dorsal skin

The intradermal injection of *Lachesis muta muta* venom (0.03–10 μg/site; $n = 5$) induced a dose-dependent plasma extravasation in the rat skin (Fig. 2). Doses higher than 10 μg/site were not tested because the plasma

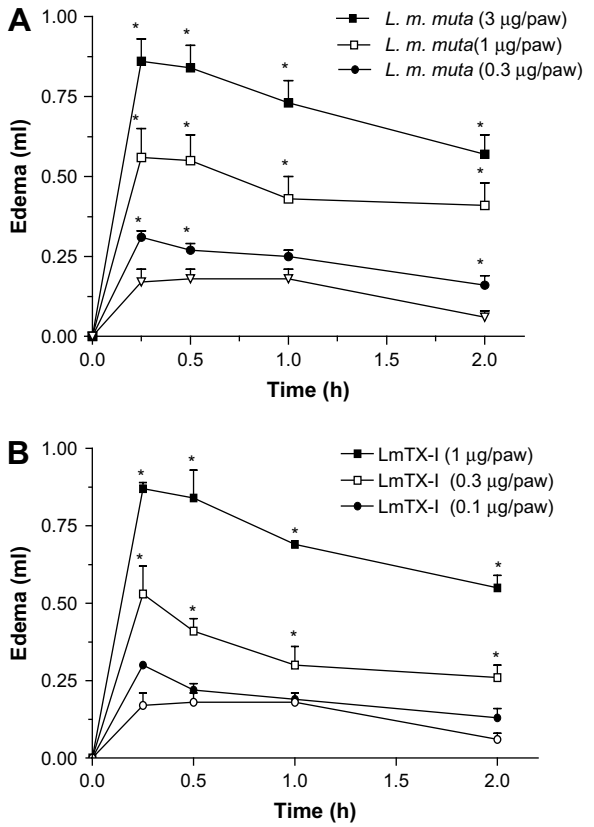

Fig. 1. *Lachesis muta muta*- and LmTX-I-induced rat paw edema. Venom (Panel A) was subplantarly injected at doses of 0.3, 1 and 3 μg/paw, whereas LmTX-I (Panel B) was injected at doses of 0.1, 0.3 and 1 μg/paw. Rat paw volume was measured at the indicated times. The columns and lines represent the mean ± SEM of five rats. Control animals received sterile saline instead of venom. *$P < 0.05$ compared with saline.

extravasation exceeded the area of the site. The intradermal injection of LmTX-I (0.003–0.3 μg/site; $n = 5$) in the rat skin also induced a dose-dependent plasma extravasation with a similar profile of crude venom, except that much lower doses were used to produce maximum extravasation (Fig. 2). For further experiments, the rat skin model was chosen, and doses of 1 and 0.1 μg/site for *Lachesis muta muta* crude venom and LmTX-I, respectively, were routinely used.

3.3. Mast cell involvement

In order to investigate the role of amines derived from mast cells, rats were pretreated with either cyproheptadine (2 mg/kg) or mepyramine (6 mg/kg). Both cyproheptadine and mepyramine markedly reduced by the rat skin plasma extravasation induced by either *Lachesis muta muta* venom ($n = 5$) or LmTX-I ($n = 5$, Fig. 3). In rats pretreated with cyproheptadine or mepyramine, the plasma extravasation caused by the mast cell degranulator compound 48/80 (1 μg/site; $n = 5$) and histamine (30 nmol/site, $n = 5$) was nearly abolished, confirming the efficacy of the treatments used. These treatments did not affect the basal values observed in Tyrode-injected sites.

We next tested the hypothesis that mast cell degranulation by *Lachesis muta muta* venom or LmTX-I was a consequence of a direct action on the mast cell membranes causing the amine release. To achieve this, rat isolated peritoneal mast cells incubated with either *Lachesis muta muta* venom (0.3–10 μg/ml) or LmTX-I (0.1–3 μg/ml) were used. Our results showed that both *Lachesis muta muta* crude venom and LmTX-I markedly and concentration-dependently induced histamine release (Fig. 4). At the higher concentrations, the histamine release extent by crude venom and LmTX-I was similar to that produced by compound 48/80 (1 μg/ml; 89.1 ± 4.6%), used as a positive control.

3.4. Participation of COX metabolites and PAF

Pre-treatment of rats with indomethacin (5 mg/kg), a non-selective COX inhibitor, significantly inhibited the rat skin plasma extravasation induced by either *Lachesis muta muta* venom ($n = 5$) or LmTX-I ($n = 5$; Fig. 5A).

A similar profile of inhibition of crude venom and LmTX-I-induced plasma extravasation was observed when rats were pretreated with PCA4248 (5 mg/kg), a PAF receptor antagonist (Fig. 5B). Both treatments did not affect significantly the basal values observed in Tyrode-injected sites.

3.5. Role of NO and tachykinin NK_1 receptors

The participation of NO in the plasma protein extravasation induced by *Lachesis muta muta* venom and LmTX-I was investigated through the co-injection of the non-selective NO synthase inhibitor L-NAME (100 nmol/site). This substance reduced by 67% ($P < 0.05$) the *Lachesis muta muta* venom-induced plasma extravasation, but failed to significantly affect the LmTX-I- induced protein extravasation (Fig. 6A; $n = 5$). The basal extravasation was not modified by L-NAME.

The contribution of neuropeptide substance P in the *Lachesis muta muta* venom- and LmTX-I-induced inflammatory activities in the rat dorsal skin was also investigated. Co-injection of SR140333 (1 nmol/site, $n = 5$), a selective tachykinin NK_1 receptor antagonist, decreased significantly ($P < 0.05$) the rat skin plasma extravasation induced by *Lachesis muta muta* venom (1 μg/site), without affecting that produced by LmTX-I ($n = 5$, Fig. 6B). The tachykinin NK_1 receptor agonist GR73632 (100 pmol/site, $n = 4$), caused a significant rat skin plasma accumulation that was completely nearly abolished by SR140333 (35.9 ± 4.3 and 8.5 ± 3.5 μl, for control and treated sites respectively, $n = 5$).

3.6. Role of bradykinin B_2 receptors

The participation of bradykinin B_2 receptors in the plasma protein extravasation induced by *Lachesis muta muta* venom and LmTX-I was investigated using the bradykinin B_2 receptor antagonist Icatibant (0.6 mg/kg). This substance significantly reduced the *Lachesis muta muta* venom-induced plasma extravasation, but failed to affect the LmTX-I- induced protein extravasation (Fig. 7; $n = 5$). In rats pretreated with Icatibant, the plasma extravasation

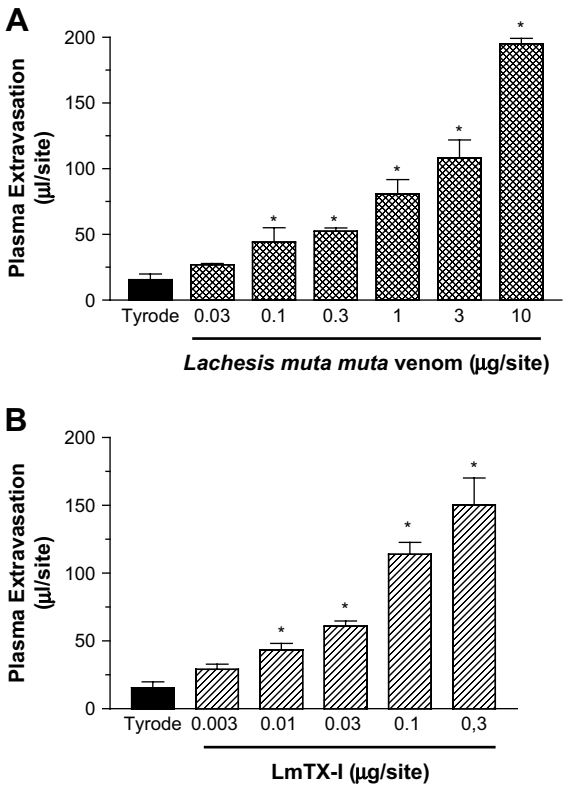

Fig. 2. Rat skin plasma extravasation induced by *Lachesis muta muta* venom (0.03–10 µg/site, Panel A) and LmTX-I (0.003–0.3 µg/site; Panel B). Results are expressed as µl of plasma extravasated per site, and each bar represents the mean ± SEM of four to six animals. $^*P < 0.05$ compared with Tyrode-injected sites.

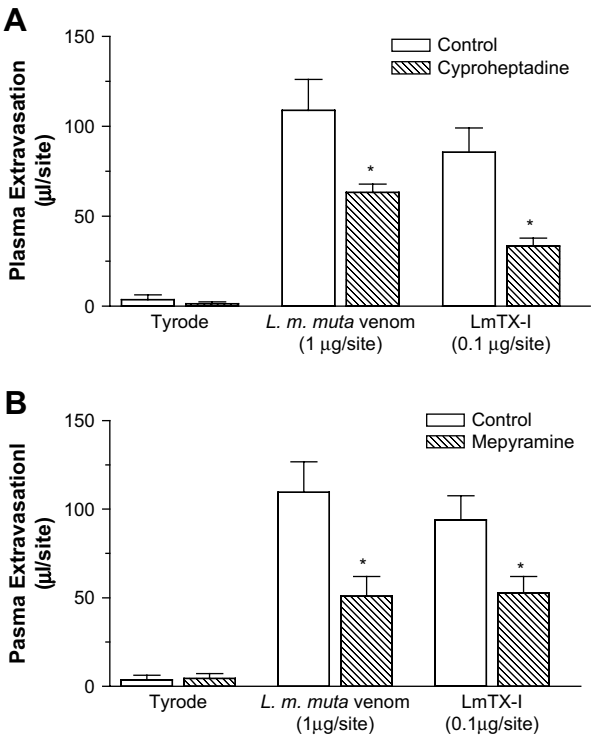

Fig. 3. Inhibitory effect of cyproheptadine or mepyramine (Panel B) on plasma protein extravasation induced by *Lachesis muta muta* venom (1 µg/site) or LmTX-I (0.1 µg/site). Cyproheptadine (Panel A) was given at 2 mg/kg, i.p., 30 min prior venom (or toxin) injection, whereas mepyramine was given at 6 mg/kg, i.v., 15 min prior venom (or toxin) injection. Results are expressed as µl of plasma extravasated per site, and each bar represents the mean ± SEM of five animals. $^*P < 0.05$ compared with the respective control.

caused by bradykinin (30 nmol/site; $n = 5$) was significantly inhibited, confirming the efficacy of the treatment used (160.3 ± 6.8 and 85.0 ± 3.7 µl, for control and treated sites, respectively, $n = 5$). These treatments did not affect the basal values observed in Tyrode-injected sites.

4. Discussion

The acute inflammatory reaction is characterised by exudation of fluid and plasma proteins leading to a local oedema formation consisting of leukocyte-dependent and leukocyte-independent components. These vascular changes are produced by different mediators which act mainly by increasing the microvascular permeability to macromolecules in the postcapillary venules thus enhancing plasma protein efflux. The local inflammatory responses of *Lachesis muta muta* venom have been poorly studied. This is of clinical importance because *Lachesis* envenomation is still a health problem in the Amazon region, given the efficacy of commercial bothropic antivenoms to neutralize the lethal effects of the bushmaster venom is rather uncertain (Jorge el al., 1997). Some recent findings improved the production of specific *Lachesis* antivenom by the removal of a component, present in the venom, that possesses a suppressive effect on the immune system (Stephano et al., 2005). In the present study, we have demonstrated that *Lachesis muta muta* crude venom dose-dependently induced rat paw oedema and plasma protein extravasation in the rat dorsal skin, leading to local inflammatory oedema formation. The oedema formation induced by *Lachesis muta muta* venom was similar to that of other *Lachesis* species (Soares de Moura et al., 1998) and of other bothropic venoms, including *Bothrops asper* (Chaves et al., 1995, 2006), *Bothrops insularis* (Barbosa et al., 2003), *Bothrops jararaca* (Trebien and Calixto, 1989) and *Bothrops lanceolatus* (de Araújo et al., 2000; de Faria et al., 2001; Guimarães et al., 2004; see Farsky et al., 2005, for review).

It is well established that PLA_2s play an important role in inflammatory processes since they provide precursors for pro-inflammatory lipid substances such as arachidonic acid-derived mediators and PAF (Teixeira et al., 2003). PLA_2 obtained from snake venoms are largely used as pharmacological tools because they present structural homology with human endogenous PLA_2 isoforms and hence production of inflammatory mediators. Little is known about the pathophysiology of LmTX-I, an Asp-49 basic PLA_2 isolated from *Lachesis muta muta* venom (Damico et al., 2005b); therefore we decided to carry out parallel experiments with this $sPLA_2$ in the rat paw and dorsal skin. Our data showed that LmTX-I dose-dependently induced increased plasma protein extravasation in the rat paw and

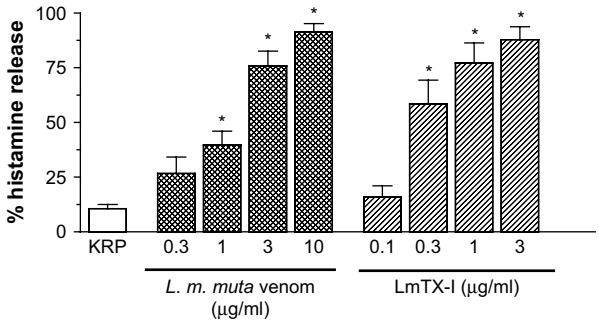

Fig. 4. Histamine release induced by *Lachesis muta muta* venom (0.3–10 μg/ml) and LmTX-I (0.1–3 μg/ml) in rat isolated peritoneal mast cells. Results (mean ± SEM of three to five experiments) are expressed as % of histamine release. $^{*}P < 0.05$ compared to Krebs–Ringer phosphate.

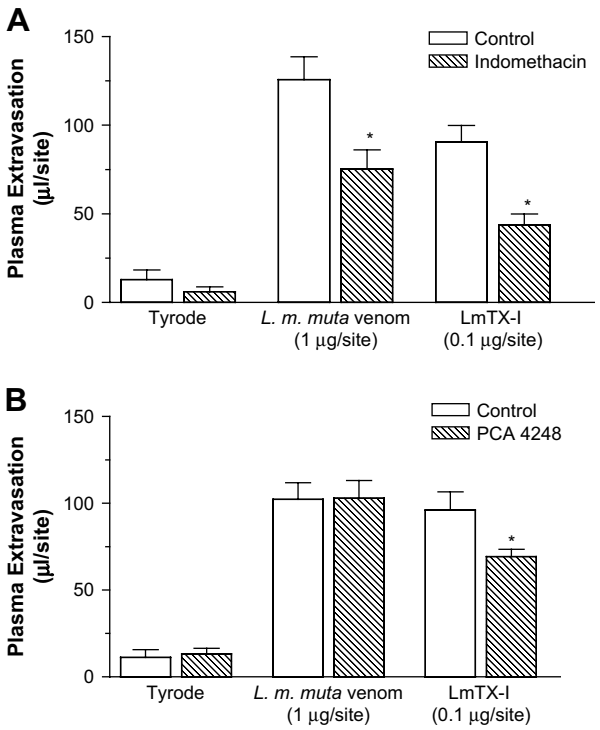

Fig. 5. Effects of the cyclooxygenase inhibitor indomethacin and the PAF receptor antagonist PCA4248 on the skin plasma protein extravasation induced by *Lachesis muta muta* venom (1 μg/site) and LmTX-I (0.1 μg/site). Indomethacin (Panel A) was given at 5 mg/kg, i.p., 1 h prior venom (or toxin) injection, whereas PCA4248 (Panel B) was given at 5 mg/kg, i.v., 1 h prior venom (or toxin) injection. Results are expressed as μl of plasma extravasated per site, and each bar represents the mean ± SEM of five animals. $^{*}P < 0.05$ compared with respective control.

dorsal skin in the same magnitude as crude venom. LmTX-I comprises approximately 2.5% of crude venom (Damico et al., 2005b), which corresponds to approximately 25 ng contained in 1 μg of crude venom. In the rat skin model, significant plasma extravasation by LmTX-I was obtained with doses higher than 3 ng/site (see Fig. 2), thus suggesting that at least part of the inflammatory actions of crude venom is due to LmTX-I. However, other venom components, such as proteases, metalloproteinases or lectins may also contribute to the oedematogenic properties of crude venom.

Several previous works have confirmed that snake venom PLA_2s are able to activate mast cells in vitro leading to release of amines (Lau and Roche, 1997; Landucci et al., 2000) and attraction of neutrophils (Gambero et al., 2002). They also cause rat paw oedema and skin plasma extravasation (Cirino et al., 1989; Landucci et al., 2000; Câmara et al., 2003), as well as induce leukocyte influx in vivo (de Castro el al., 2000), partly due to release of histamine and serotonin. In our study, treatment of rats with the histamine H_1 receptor antagonist mepyramine or the dual histamine/serotonin receptor antagonist cyproheptadine markedly decreased the skin plasma extravasation induced by crude venom and LmTX-I. Moreover, both venom and LmTX-I dose-dependently stimulated the mast cells in vitro leading to histamine release, reinforcing that local mast cell activation play an important role for the oedematogenic properties of both substances.

Cyclooxygenase-derived mediators and PAF have been recognized as important mediators of diverse types of inflammatory conditions, including those induced by venoms and venom proteins (Mitchell et al., 1995). In our study, prior treatment of the animals with the non-selective COX inhibitor indomethacin significantly reduced the venom- and LmTX-I-induced plasma skin extravasation, indicating an important role for arachidonic acid metabolites, which is consistent with previous studies showing reduction by indomethacon of oedematogenic responses caused by different bothropic venoms including *B. jararaca* (Trebien and Calixto, 1989), *B. asper* (Chaves et al., 1995), *L. muta rhombeata* (Soares de Moura et al., 1998), *B. lanceolatus* (de Araújo et al., 2000) and *B. insularis* (Barbosa et al., 2003), as well as PLA_2s isolated from venoms (Fuly et al., 2002). Amongst the COX-derived metabolites, PGE_2 produced by resident cells such as macrophages has been shown to exert critical roles in diverse types of inflammatory conditions as a consequence of its ability to cause prominent local vasodilatation, and hence oedema formation and cell extravasation (Brock and Peters-Golden, 2007). It is likely, therefore, that increased microvascular permeability in response to venom and LmTX-I is secondary to large production of PGE_2 at the level of precapillary arterioles leading to an increase in the microvascular blood flow and protein extravasation. Accordingly, previous studies in skin show that oedema is due to synergistic actions of mediators of increased microvascular permeability and vasodilators (Brain and Williams, 1985).

Additionally, in our study, prior treatment of the animals with the PAF receptor antagonist PCA4248 failed to affect the crude venom-induced plasma skin extravasation, which is agreement with a previous study showing that the PAF receptor antagonist BN5221 failed to inhibit the oedema elicited by *B. asper* venom (Chacur et al., 2001). Interestingly, PCA4248 partly (but significantly) reduced the LmTX-I-induced skin extravasation that is likely to reflect the phospholipasic activity of LmTX-1. The reasons for the discrepancy between total venom and LmTX-I in animals pretreated with PCA4248 are unclear but may be

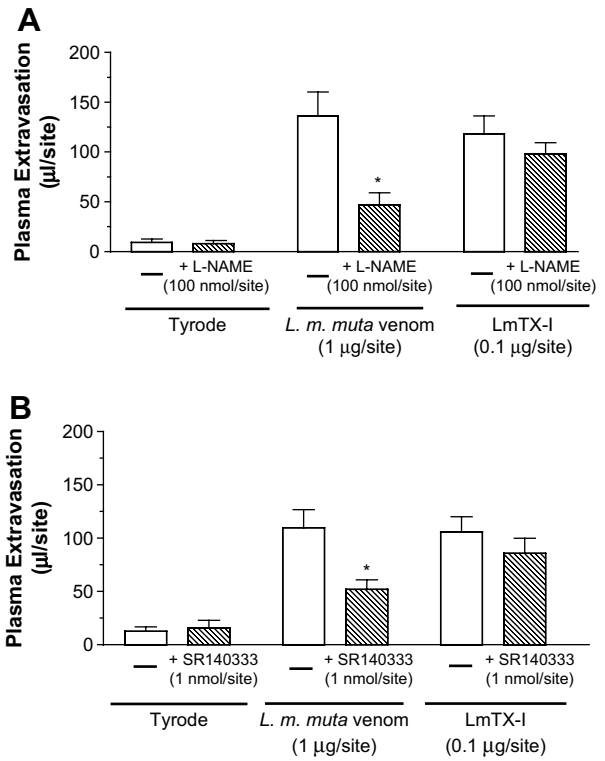

Fig. 6. Effects of the nitric oxide synthesis inhibitor L-NAME and the tachykinin NK_1 receptor antagonist SR140333 on the skin plasma protein extravasation induced by *Lachesis muta muta* venom (1 µg/site) and LmTX-I (0.1 µg/site). L-NAME and SR140333 were co-injected with venom (or toxin) at doses of 100 and 1 nmol/site, respectively. Results are expressed as µl of plasma extravasated per site, and each bar represents the mean ± SEM of four animals. $^*P < 0.05$ compared with the respective control.

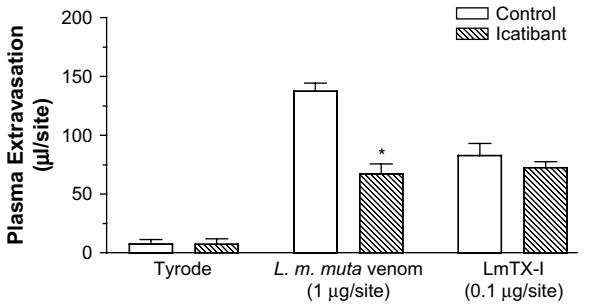

Fig. 7. Effect of the bradykinin B_2 receptor antagonist Icatibant on the skin plasma protein extravasation induced by *Lachesis muta muta* (1 µg/site) and LmTX-I (0.1 µg/site). Icantibant was given at 0.6 mg/kg, i.v., 15 min prior venom (or toxin) injection. Results are expressed as µl of plasma extravasated per site, and each bar represents the mean ± SEM of four animals. $^*P < 0.05$ compared with the respective control.

related to the low amounts of LmTX-I found in crude venom.

Substance P is released from afferent sensory fibres and causes plasma extravasation acting mainly through tachykinin NK_1 receptors in post-capillary venules (Holzer, 1998). A previous study showed that PLA_2s isolated from *Crotalus durissus* venoms (*C. d. terrificus, C. d. collilineatus* and *C. d. cascavella*) cause rat skin plasma extravasation in the rat dorsal skin induced by a mechanism partly involving C-fibre activation (Câmara et al., 2003). Our data showed that co-injection of the selective NK_1 receptor antagonist SR140333 significantly decreased the extravasation induced by *Lachesis muta muta* venom, but did not affect the LmTX-I responses. This suggests that components in crude venom that activate sensory fibres leading to substance P release and oedema formation are unrelated to LmTX-I.

Nitric oxide (NO) is a potent vasodilator agent produced by the endothelium and modulates the microvascular tone in several beds (Furchgott and Vanhoutte, 1989; Moncada and Higgs, 2006). Nitric oxide synthesis inhibitors such as L-NAME have been shown to affect the interaction of inflammatory cells with the endothelium and vascular protein leakage (Kubes, 1993; Ialenti et al., 2000; Ahluwalia et al, 2004). In our study, the involvement of NO in the plasma extravasation induced by *Lachesis muta muta* crude venom and LmTX-I was investigated by co-injection of L-NAME. It is well established that different vasodilators such as PGE_1, PGE_2, prostacyclin, tachykinins and CGRP potentiates the increased vascular permeability induced by various agents (Severini et al, 2002; Brain and Grant, 2004). Accordingly, acute administration of L-NAME reduces skin plasma extravasation in response to a decrease in local microvascular blood flow in different animal species (Ialenti et al., 1992; Teixeira et al., 1993; Mariani-Pedroso et al., 1995). Previous studies have reported that NO contributes to the local mice footpad oedema induced by *Bothrops insularis* (Barbosa et al., 2003) or *Bothrops asper* venom (Chaves el al., 2006) and to the rat paw oedema caused by *Lachesis muta rhombeata* venom (Soares de Moura et al., 1998). In accordance with these studies, co-injection of L-NAME significantly decreased the plasma extravasation induced by *Lachesis muta muta* venom. However, the LmTX-I-induced plasma extravasation was unaffected by L-NAME co-injection. Of interest, the arteriolar vasodilation is maintained by prostaglandins and/or endothelium-derived hyperpolarizing factor in eNOS knockout mice (Sun et al., 1999; Huang et al, 2000; Bucci et al., 2005). Thus, it is plausible to speculate that direct formation of prostaglandins (especially PGI_2) from LmTX-I PLA_2 activity can overcome the local absence of NO maintaining the vascular tone and consequently the plasma extravasation in rat skin microcirculation.

Bradykinin is released from high-molecular weight kininogen mainly by the enzymatic action of serine proteases, the plasma kallikreins, and exerts its effects through the activation of B_2 receptors (Bhoola et al., 1992). The B_2 receptor antagonism by HOE 140 has been shown to diminish the protein leakage, leukocyte accumulation and nitrite/nitrate levels in the synovial fluid of rabbits during arthritis induced by *B. jararaca* venom (Guzzo et al., 2000). In our study, the bradykinin B_2 receptor Icatibant significantly decreased the *Lachesis muta muta* venom-induced plasma protein extravasation, indicating that the local formation of kinins, at least in part, contributes to venom-induced oedema. In contrast, LmTX-I-induced plasma extravasation was not inhibited by pre-treatment with

Icatibant, indicating that bradykinin is not a pivotal mediator of LmTX-I-induced inflammatory response. Similarly, a previous study reported that Icatibant failed to affect the rabbit skin oedema induced by piratoxin-I, a Lys-49 PLA_2 isolated from *B. pirajai* venom (Landucci et al., 2000).

In conclusion, we have shown here that *Lachesis muta muta* venom and its constituent LmTX-I increase the microvascular permeability in the rat paw and skin by mechanisms partly involving in vivo mast cell activation and arachidonic acid metabolites. However, responses by crude venom and toxin may be triggered by different mechanisms. Whilst substance P, bradykinin and NO mediate skin plasma extravasation induced by crude venom (but not LmTX-I), PAF mediates the responses to LmTX-I (but not crude venom).

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Conflict of interest

The authors declare that there are no conflicts of interest.

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