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Evaluation of the anti-inflammatory activity of riparin II (O-methil-N-2-hidroxi-benzoyl tyramine) in animal models

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ABSTRACT

Riparin II (RipII), an alkamide isolated from the green fruit of *Aniba riparia*, was tested in the various animal models of inflammation to investigate its anti-inflammatory activity. Male Wistar rats (180–240 g) were treated with RipII by gavage at doses 25 or 50 mg/kg, before initiating the inflammatory responses. The tests used were paw edema induced by carrageenan, dextran, histamine or serotonin; peritonitis induced by carrageenan and fMLP, as well as the measurement of MPO activity, TNF-α and Il-1β amount in the peritoneal fluid. In the animal models of carrageenan and dextran-induced paw edema, the animals treated with RipII showed lower edema than those of the control group. Treatment with RipII also reduced the paw edema induced by histamine but not serotonin. In the carrageenan-induced peritonitis model, treatment with RipII reduced leukocyte migration, the MPO activity and the amount of TNF-α and IL-1β in the peritoneal fluid. In summary, these results indicate that RipII has an anti-inflammatory activity in chemical models of acute inflammation. RipII might be directly or indirectly inhibiting the activity, production or release of pro-inflammatory mediators involved in the generation of the pain associated with inflammation.

1. Introduction

Over the years, natural products have contributed significantly to the discovery and development of important modern therapeutic drugs [1–3].

Aniba riparia (Ness) Mez is a species belonging to the Lauraceae family, and the genus *Aniba* is typical of the Amazon (Brazil), where it is popularly known as "louro" [4]. Its fruits contain various chemical constituents such as flavonoids, neolignans, stirilpironas and alkamides [5].

In earlier studies, the substances N-methyl benzoiltyramine (riparin I), N-(2-hydroxybenzoyl)-tyramine (riparin II) and N-(2,6-dihidroxibenzoil)-tyramine (riparin III) (Fig. 1), isolated from the unripe fruit of *A. riparia* (Ness) Mez, have demonstrated several biological activities, including antimicrobial and antimalarial efficacy [6–8].

These three alkamides have been shown to induce non-specific and reversible relaxation of contractions produced by acetylcholine and histamine in guinea pig ileum and by oxytocin and bradykinin in the uterus of virgin rats [9]. This relaxing effect was previously shown to be associated with the inhibition of the influx of calcium ions into the intracellular compartment and inhibition of the release of intracellular calcium stores, without affecting cyclic adenosine monophosphate (cAMP) generation [10].

Riparins contain tyramine, a sympathomimetic amine with an indirect mode of action, in their chemical structure. For this reason, studies conducted by our group have evaluated the effects from these substances on the central nervous system. Data from these studies showed that riparin I [11], riparin II [12] and riparin III [13,14] exhibited anxiolytic-like effects in mice, while riparin II [15] and riparin III [16] produced antidepressant effects. Riparin I also demonstrated antinociceptive activity, which could involve

Abbreviations: TNF-α, tumor necrosis factor; IL1-β, interleukin; MPO, myeloperoxidase; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine.

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peripheral mechanisms (for example, the nitric oxide pathway) and central mechanisms [17].

2. Materials and methods

2.1. Riparin II isolation, purification and identification

Barbosa-Filho et al., described the process of extraction of the Riparin II (RipII) from *A. riparia*. A sample of RipII were deposited in the Bank of Standards of Natural and Synthetic Products of the Laboratório de Tecnologia Farmacêutica of the Universidade Federal da Paraíba, and to be used in this work, it was repurified by Preparative Thin Layer Chromatography (PTLC) silica gel Merck using the same system as described in the original paper. Spectroscopically pure RipII was confirmed by Analytical Thin Layer Chromatography (TLC) and High-performance Liquid Chromatography (HPLC). The identification of the RipII was performed by analyzing ^{1}H and ^{13}C NMR spectral data compared with those published in the original literature [18].

2.2. Animals

Male rats (180–240 g) and Male Swiss mice (27–32 g) were used in this study. The animals were maintained on a 12/12 h light/dark cycle, with access to water and food *ad libitum*, and the experiments were performed at an ambient temperature of 26 ± 2 °C. All experiments were performed in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals [19]. The study was also performed under the consent and surveillance of the Ethics Committee from the Department of Physiology and Pharmacology of Federal University of Ceará (Protocol number 40/10).

2.3. Drugs

RipII was emulsified with 3% Tween 80 (VETEC, USA) and administered intragastric in male rats at single doses of 25 or 50 mg/kg. Control groups (vehicle) received the same volume of 3% Tween 80 as the treated groups dissolved in distilled water. The following drugs were used: indomethacin, cyproheptadine, dexamethasone, carrageenan, dextran, histamine, serotonin, and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). All drugs were purchased from Sigma Chem. Co® (St. Louis, MO, USA). All drugs were dissolved in saline solution immediately before use, with the exception of indomethacin, which was dissolved in 8.4% $NaHCO_3$. The vehicles used alone had no effects per se on the inflammatory responses in rats.

2.4. Experimental procedures

2.4.1. Paw edema induced by carrageenan

Rats were divided in four groups that received vehicle (3% of Tween 80 with distilled water, by gavage), RipII (25 or 50 mg/kg, by gavage) or indomethacin (10 mg/kg, by gavage). Sixty minutes later, the animals received an intraplantar injection of 1% carrageenan (100 μl) to induce edema in the right hind paw. The volume of the paws was measured before and 1, 2, 3, 4 and 24 h after the administration of carrageenan [20].

The volume of the edema in milliliters was measured using a Pletismometer (Ugo Basile, Italy), where the right hind paw was submerged up to the tibio-tarsal joint, in the measuring chamber of the device. The volume of fluid displaced was recorded and considered the volume of the paw. The results were expressed as the difference between the volume of the paw at the specified time intervals and the volume before the carrageenan injection ($t = 0$).

2.4.2. Paw edema induced by dextran

Animals were divided in four groups and pre-treated with vehicle (3% of Tween 80 with distilled water, by gavage), RipII (25 or 50 mg/kg, by gavage) or cyproheptadine (10 mg/kg, by gavage). Sixty minutes later, the animals received an intraplantar injection of dextran 1.5% (100 μl) to induce the edema in the right hind paw. The volume of the paws was measured before and 1, 2, 3 and 4 h after dextran administration [21].

The volume of the edema in milliliters was registered using a Pletismometer (Ugo Basile, Italy), where the right hind paw was submerged until the tibio-tarsal joint in the measuring chamber of the device. The volume of fluid displaced was recorded and considered the volume of the paw. The results were expressed as the difference between the volume of the paw at the referred time intervals and the volume before of the dextran injection.

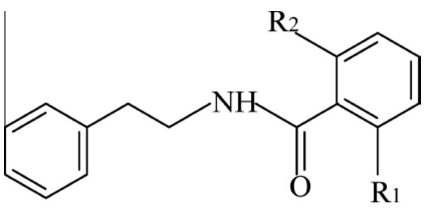
2.4.3. Paw edema induced by histamine and serotonin in rats

The experiments were performed in accordance with the methodologies of earlier studies [22]. Rats were divided in three groups and treated with vehicle (3% of Tween 80 with distilled water, by gavage) or RipII (25 or 50 mg/kg, by gavage). Sixty minutes later, the animals received an intraplantar injection of histamine (200 μg/paw) or serotonins (200 μg/paw) induce edema in the right hind paw. The volume of the paws was registered using a pletismometer at several times after the injection of the inflammatory stimulus. In the histamine-induced paw edema, the measurements were taken before and after 15, 30, 60 and 90 min; in the serotonin-induced paw edema, the measurements were taken before and after 15, 30 and 60 min; and in the bradykinin-induced paw edema, the measurements were taken before and after 15 and 30 min.

2.4.4. Carrageenan-induced leukocyte migration in the rat peritoneal cavity (peritonitis)

The carrageenan solution (500 μg/ml) or sterile saline (0.9%, w/v) was injected in the peritoneal cavities of rats (1 mL). Four hours later, the rats were sacrificed, and the peritoneal cavity was washed with 10 ml of saline containing heparin 5 IU/ml. The peritoneal fluid was recovered for the analysis of leukocyte numbers with a Neubauer chamber, IL-1β and TNF-α concentrations, and total protein [23] and for the quantification of myeloperoxidase (MPO) activity. The rats were treated orally with RipII (25 or 50 mg/kg), dexamethasone (5 mg/kg) or vehicle 1 h before receiving the i.p. injection of the carrageenan solution [24].

MPO activity, a marker for neutrophil infiltration, was quantified in the peritoneal fluid using an assay adapted from the method



Riparin I (R1=R2=H)
Riparin II (R1=OH, R2=H)
Riparin III (R1=R2=OH)

Fig. 1. Structure of riparins.

of Bradley et al. [25]. Briefly, samples of peritoneal fluid (0.1 ml) were vigorously mixed with 0.9 ml of 0.5% hexadecyltrimethylammonium bromide potassium phosphate buffer solution. Aliquots of 30 μl were transferred to 96-well plates. Hydrogen peroxide and θ-dianisidine were added to the samples. The reaction between H_2O_2 and θ-dianisidine, catalyzed primarily by MPO in the samples, generates a color solution that was measured spectrophotometrically (absorbance at 460 nm) to provide an index of MPO activity. The results are shown as MPO units per milliliter. IL-1β and TNF-α were quantified in the peritoneal fluid by enzyme-linked immunosorbent assays (ELISA); Amersham© TNF-α Rat Biotrak ELISA System (assay range: 31–2500 pg/ml and sensitivity: <15 pg/ml) and Amersham© IL-1β Rat Biotrak ELISA (assay range: 25.6–2500 pg/ml and sensitivity: 12 pg/ml), according to the instructions of the manufacturer.

2.4.5. Leukocyte migration induced by bacterial chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) in the rat peritoneal cavity (peritonitis)

A procedure similar to that used to produce carrageenan-induced peritonitis was performed to evaluate the capacity of RipII (25 or 50 mg/kg) to block the neutrophil migration in response to fMLP, a direct stimulator of leukocytes. A solution of fMLP (100 nmol) or sterile saline (0.9%, w/v) was injected (1 mL) in the peritoneal cavities of the rats. Four hours later, the rats were sacrificed and the peritoneal cavity was washed with 8 ml of saline containing 5 IU/ml heparin. The peritoneal fluid was recovered for the analysis of leukocyte numbers with a Neubauer chamber [26].

2.4.6. Measurement of membrane lipids peroxidation

The rate of lipoperoxidation in the paw was estimated by determination of malondialdehyde (MDA) using the thiobarbituric acid reactive substances (TBARS) test. The paws were washed with saline to eliminate the interference of hemoglobin with free radicals. The paws were homogenized to 10% of tissue with potassium phosphate buffer. Then 63 μL was removed and add 100 μL of 35% perchloric acid, and the mixture was centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant (approximatetly 150 μL) was removed, mixed with 50 μL of 1.2% thiobarbituric acid and incubated at 100 °C for 30 min. After cooling, the absorbance at 532 nm was measured. The results were expressed as nmol MDA/g tissue.

2.4.7. Measurement of nitrite amount

The nitrite amount was determined by a colorimetric assay similar to describe by Green et al. [27]. Briefly, 50 μL of each paw homogenate was mixed with the same volume of Griess reagent. This reagent consist of equals parts of 5% phosphoric acid, 1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride (NEED) and distilled water. The absorbance was read at 540 nm on microplate reads (UVM-340, Asys Hitech, Netherlands). The amount of nitrite was calculated from a $NaNO_2$ standard curve. NO^{2-} is a major unstable product of NO and molecular oxygen reaction, and gives an indication of free radicals generation.

3. Statistical analyses

Graph Pad Prism 5.0 software was used for the statistical analyses. The results are shown as the mean ± SEM. The statistical significance of difference between the groups was assessed by one-way ANOVA, followed by the Student–Newman–Keuls *post hoc* test. Values of p less than 0.05 were considered significant.

4. Results

4.1. Evaluation of the anti-inflammatory properties of RipII on rat paw edema

The administration of RipII at both doses (25 and 50 mg/kg, p.o.) significantly reduced the carrageenan-induced paw edema two hours after the administration of the stimulus compared to the animals pre-treated with vehicle. Indomethacin (10 mg/kg, p.o.), a non-steroidal anti-inflammatory used as a drug of reference, significantly reduced the volume of paws at all periods observed compared to the vehicle (Fig. 2A). RipII (25 or 50 mg/kg, p.o.) also reduced the dextran-induced edema compared to the vehicle, except in the third hour following dextran application. As expected, cyproheptadine (10 mg/kg, p.o.) was effective at all times (Fig. 2B). Both doses of RipII significantly reduced the histamine-induced paw edema (Fig. 2C) but were not able to reduce the volume of the edema induced by serotonin compared to the vehicle group (Fig. 2D) at any time.

4.2. Evaluation of the anti-inflammatory properties of RipII in carrageenan or fMLP-induced peritonitis

Administration of carrageenan increased the total number of leukocytes in the peritoneal fluid. Pretreatment with RipII at doses of 25 or 50 mg/kg (p.o.) significantly reduced the numbers of leukocytes, corresponding to an inhibition of 23.58% and 39.92%, respectively. Dexamethasone (5 mg/kg, p.o.), used as reference drug, diminished the cellular infiltrate by 81.80% relative to the vehicle-treated group (Fig. 3A). Intraperitoneal administration of fMLP resulted in a strong migration of leukocytes into the peritoneal cavity compared to the group treated only with saline. The pretreatment with RipII (25 and 50 mg/kg, p.o.) significantly reduced the cell migration, corresponding to inhibitions of 26.21% and 22.52%, respectively. As expected, dexamethasone (5 mg/kg, p.o.) was able to suppress leukocyte recruitment by 49.18% (Fig. 3B). RipII, at both doses, significantly reduced the protein concentration in the peritoneal fluid compared to the vehicle, corresponding to an inhibition of 41.6% and 40.36%, respectively, while dexamethasone reduced this parameter by 61.92% (Fig. 3C). The intraperitoneal injection of carrageenan solution (500 μg/cavity) induced an increase in MPO activity, an indirect evaluation of the accumulation of neutrophils, compared to the values obtained from the animals treated with saline. RipII (25 or 50 mg/kg, p.o.) and dexamethasone significantly reduced the activity of MPO when compared to the vehicle (by 49.95%, 26.44% and 53.05%, respectively) (Fig. 3D). RipII at both doses tested, as well as dexamethasone, also decreased the concentration of TNF-α to 38.54%, 66.7% and 48.46%, respectively, of the values obtained for the vehicle-treated animals (Fig. 4B). RipII at 25 mg/kg and dexamethasone reduced the amount of IL-1β to 41.8% and 62.3%, respectively, of that of the vehicle-treated group (Fig. 4A).

4.3. Effect of RipII on the membrane lipid peroxidation marker – MDA

Administration of carrageenan increased the MDA amount in the paws. The treatment with RipII in the both doses decreased the MDA amount, a reduction of 59.02% and 59.94%, respectively. Indomethacin, used as reference drug, diminished the parameter by 52.36% compared to the vehicle-treated group (Fig. 5A).

4.4. Effect of RipII on the nitrite amount

The quantity of nitrite was increased in the paws of animals treated only with vehicle. The pretreatment with RipII in the both

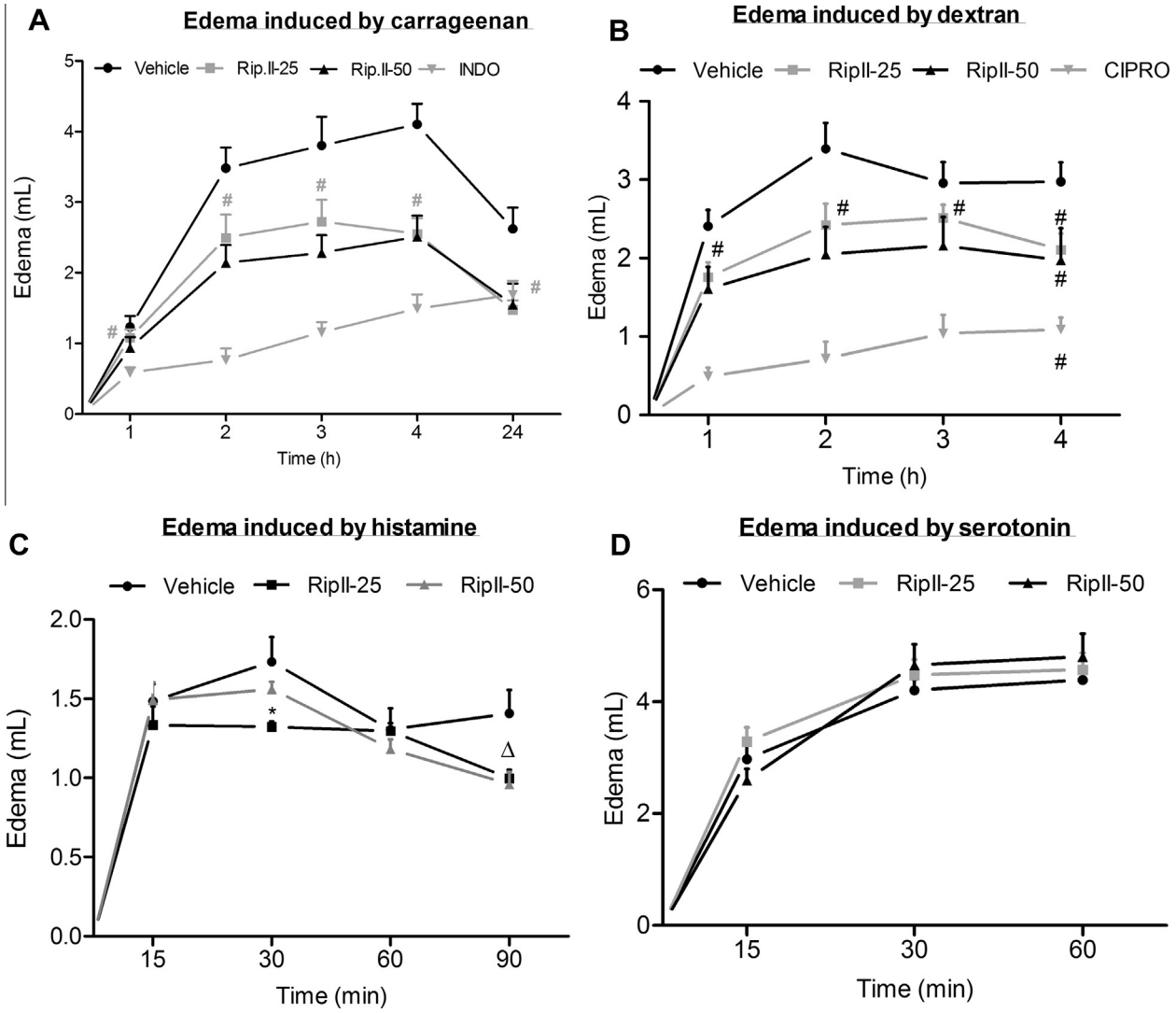


Fig. 2. Time course of the effect of RipII treatment on paw oedemas induced by carrageenan (1%) (A), dextran (0.15%) (B), serotonin (200 μg/paw) (C) or histamine (200 μg/paw) (D) in rat. Animals were pre-treated with RipII (25 or 50 mg/kg, intragastrically), indomethacin (10 mg/kg, i.p.), ciproheptadine (5 mg/kg, i.p.) or vehicle, before receiving intraplantar injections of the inflammatory agents. The oedema was measured with a plethysmometer at each time point marked. Each point represents the mean ± SEM. $^{\#}p < 0.05$ RipII-25, RipII-50, Indomethacin and ciproheptadine *vs.* vehicle, $^{*}p < 0.05$ *vs.* vehicle; $\Delta p < 0.05$ RipII-25, RipII-50 *vs.* vehicle (ANOVA and Student–Newman–Keuls *post hoc* test).

doses was able to reduce the nitrite amount, corresponding a reduction of 45.99% and 47.44%, respectively (Fig. 5B).

5. Discussion

Injection of carrageenan into the hind paw of an animal produces the cardinal signs of inflammation: edema, erythema (rubor or redness) and hyperalgesia (increased sensitivity to painful stimuli). These signs develop rapidly due to the activity of many pro-inflammatory mediators derived from plasma or cells involved in the inflammatory response [28].

The present results demonstrate that RipII reduced the carrageenan-induced paw edema. This effect was observed 2 and 24 h after carrageenan administration, suggesting that the mechanism of action of RipII might involve suppression of the inflammatory process induced by this agent. After the carrageenan injections, there is a characteristic sequence of release of inflammatory mediators. The initial edematous phase primarily involves histamine, serotonin and bradykinin. This phase is followed by an increase of prostaglandins in the damaged tissue. The prostaglandin increase coincides with the migration of leukocytes, which can amplify the inflammatory response through the production of other inflammatory mediators, reactive oxygen species (ROS), and increases myeloperoxidase activity and production of NO [29–32].

Edema induced by intraplantar injection of dextran proceeds through a different mechanism from that evoked by carrageenan. Dextran induces edema without the involvement of polymorphonuclear leukocytes in the inflamed tissue. Instead, mast cell activation and degranulation result in the release of high concentrations of biologically active amines, such as histamine and serotonin [33]. In the present work, RipII decreased the dextran-induced edema formation in the mouse paws, suggesting that the RipII effects could involve blocking the histamine and/or serotonin receptors or inhibition of their release from mast cells. Effects on histamine are more likely than effects on serotonin, as the direct effect of serotonin was not prevented by RipII treatment.

Thus, RipII appears to have the capacity to suppress the edemas induced by direct injection of histamine, a vasoactive amine that plays a fundamental role at inflammatory process by increasing of cell permeability, expansion of venules, increased fluid secretion

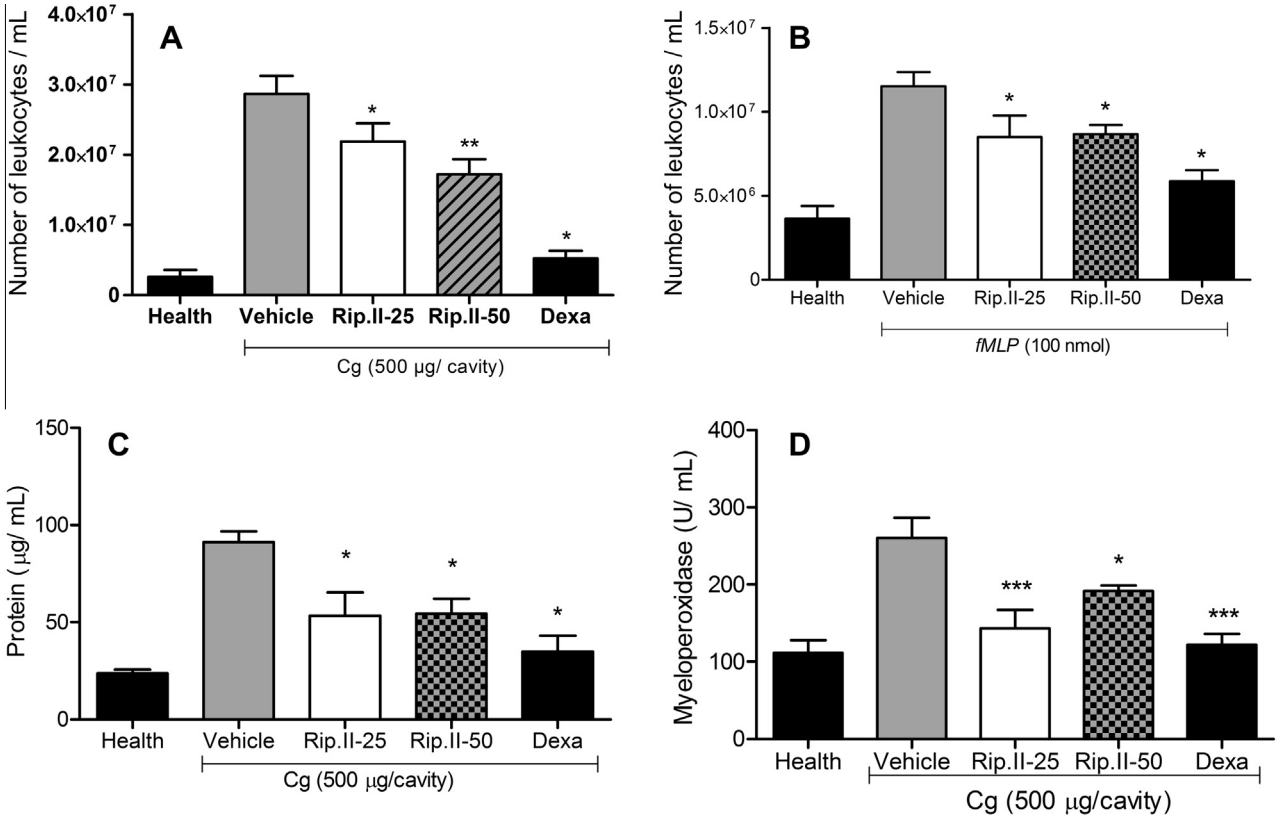


Fig. 3. Effect of RipII at 25 and 50 mg/kg doses and dexamethasone at 5 mg/kg p.o. on total leukocyte count in animals with peritonitis induced by carrageenan (A), or fMLP (B); protein concentration (C) and myeloperoxidase activity (D) were measured in the peritoneal fluid of animals with carrageenan-induced peritonitis. Results are presented as mean ± SEM. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ *vs.* vehicle (ANOVA and Student–Newman–Keuls *post hoc* test). Peritoneal fluids of animal without peritonitis are shown as healthy.

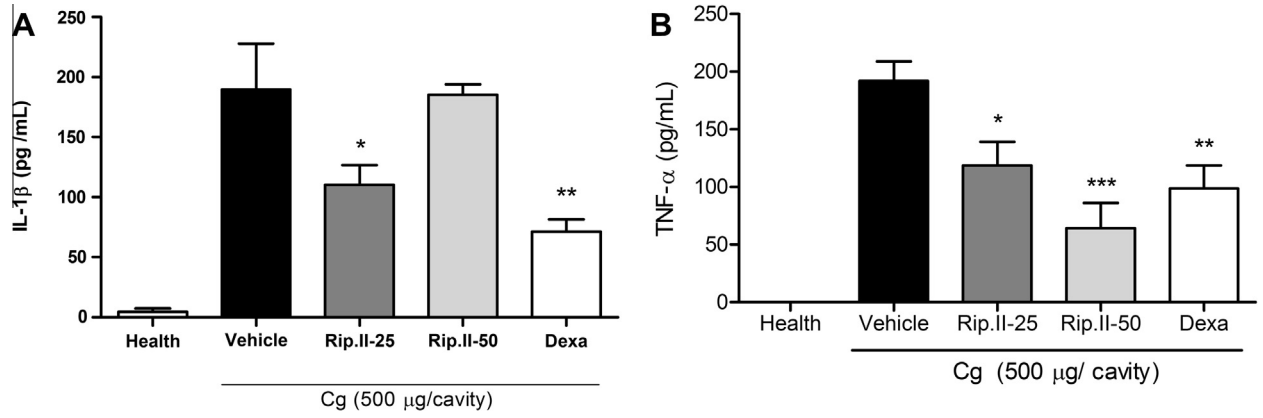


Fig. 4. Effect of RipII at 25 and 50 mg/kg doses and dexamethasone at 5 mg/kg p.o. on TNF-α and IL-1β in the peritoneal fluid of animals with carrageenan-induced peritonitis. Results are presented as mean ± SEM. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ *vs.* vehicle (ANOVA and Student–Newman–Keuls *post hoc* test). Peritoneal fluids of animal without peritonitis are shown as healthy.

and generation of pain and hyperalgesia. Suppression of leukocyte migration may explain the anti-inflammatory profile of RipII in the carrageenan-induced paw edema [34–37].

In addition to histamine and serotonin, bradykinin is also present in the initial phase of carrageenan-induced edema. Some previous studies have demonstrated that administration of bradykinin causes a long-lasting inflammatory reaction, mainly mediated by B2 receptors. This inflammatory action is modulated, at least in part, by the activation of PKC (Protein kinase C) and later by sensitization of the TRPV1 (transient receptor potential vanilloid 1), which facilitates the release of other pro-inflammatory mediators by promoting the entry of calcium into cells [38,39]. Thus, we can hypothesize that RipII may have the capacity to decrease the nociceptive effect of TRPV1 agonists (data not published). This would suppress the mobilization of arachidonic acid, the precursor to prostaglandins and leukotrienes.

Taken together, the ability of RipII to reduce the edema formation induced by carrageenan, dextran and histamine suggests a high potential of this substance to intervene in the initial establishment of acute inflammatory process.

In the acute inflammatory process, there are two main changes: cellular and vascular events. The infiltration of leukocytes is one of

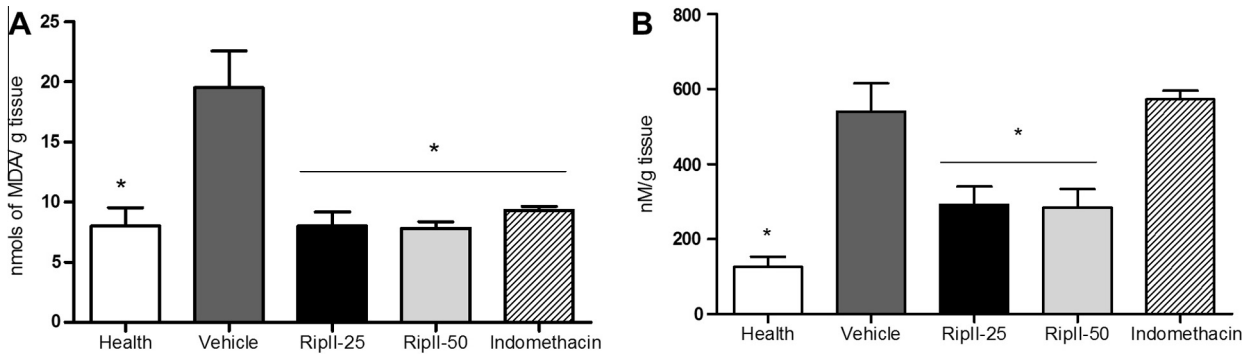


Fig. 5. Effect of RipII 25 and 50 mg/kg on lipoperoxidation level-content of MDA (A) and nitrite content (B) in the paws. One additional group received vehicle and was not exposed to carrageenan (health). Results are presented as mean ± SEM. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ *vs.* vehicle (ANOVA and Student–Newman–Keuls *post hoc* test).

the histological features of inflammation and represents a cellular event, while the increased vascular permeability with exudation or transudation constitutes a vascular event [40–43].

In this work, we evaluated the effect of RipII at doses of 25 and 50 mg/kg in the inflammatory model of carrageenan-induced peritonitis, which causes leukocyte migration to the peritoneal cavity of rats. Pre-treatment with RipII at both doses decreased leukocyte migration, protein concentration and MPO activity in the peritoneal fluid of the rats with peritonitis. The lower protein content in these rats demonstrates the ability of RipII to decrease vascular permeability and also supports the results obtained in the edema models.

Cytokines serve as messengers between cells in many physiological and pathological processes [44]. IL-1β causes both dose- and time-dependent neutrophil migration in various models and animal species [45]. This cytokine might act directly on the target cells or through the induction and secretion of eicosanoids and chemokines [46]. TNF-α, another pro-inflammatory cytokine, is released primarily by activated macrophages and has a fundamental role in many conditions, including inflammation, immune-modulation, cytotoxicity and apoptosis [47,48].

In this study, we showed the ability of RipII to decrease TNFα and IL-1β in the peritoneal fluid of rats with carrageenan-induced peritonitis. TNFα is one of the agents initially released in response to carrageenan and is a cytokine marker of acute inflammation. Thus, the observed results corroborate our edema measurements and leukocyte counts, clarifying, at least in part, the mechanism of the anti-inflammatory activity of RipII.

Carrageenan is a flogistic agent that induces the migration of leukocytes by an indirect mechanism [49], unlike fMLP, which is a peptide with a recognized direct chemotactic activity on the neutrophils [50].

Our results showed that administration of fMLP induces a substantial cell infiltrate and that pretreatment with RipII decreased the number of leukocytes in the peritoneum compared to the control group, similarly to dexamethasone. This observation indicates that RipII diminishes leukocyte migration by an indirect mechanism, probably reflecting its ability to reduce the inflammatory processes including release of TNFα and IL-1β. RipII would thus partially suppress the effects of these mediators on leukocytes and endothelial cells. We do not understand the mechanism behind of the capacity to RipII decrease the IL-1β only in the lower dose, but without any doubt the RipII is able to reduce the interleukin release and the decreasing in TNFα *per is* capable to explain at least in part the action of RipII as anti-inflammatory substance In addition, RipII may directly prevent leukocyte stimulation by fMLP. This tripeptide leads to leukocyte chemotaxis, adhesion, phagocytosis and the release of superoxide anions by activation of formyl peptide receptors, which are pertussis toxin-sensitive G-protein coupled receptors [51,52]. Thus, the ability of RipII to reduce the neutrophil influx directly induced by fMLP could be due to antagonism of fMLP receptors or interference with their signaling.

In the assessment of the reduction of vascular permeability by RipII, it was shown that RipII at both doses significantly reduced the concentration of protein, compared to the control group, similar to the effects of dexamethasone, confirming its anti-inflammatory activity. This result demonstrates the potential of RipII to influence the inflammatory process both in cellular and vascular events. The effects of RipII on histamine-induced paw oedema suggest that this agent may inhibit histamine-activated processes.

Free radicals and related reactive species are strongly involved in several pathologic and physiologic processes, including cancer, cell death, inflammation, and pain. [53]. Thus, we assessed the antioxidant potential of RipII by testing its ability to prevent oxidative damage to lipids induced by a free-radical source. Vast evidence has recently implicated that intracellular ROS production plays a key role in modulation of release of other mediators of inflammation. This is related mainly to the constitutive expression of NAD(P)H oxidases (termed NOXs- non-phagocytic oxidases) in various tissues [54,55]. ROS produced by this family of enzymes can regulate adhesion molecule expression on endothelium and inflammatory cells, thus affecting cell recruitment to the sites of inflammation [56,57].

An indicative method, extensively used, of evaluating lipid peroxidation is analysis of tissue thiobarbituric acid reactive substance (TBARS). The reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form a colored complex (TBA-MDA) that can be quantified spectrophotometrically from its visible absorbance (Em,x 532 nm) is the basis of the commonest method used to assess lipid peroxidation in biological materials [58]. The elevated levels of TBARS were significantly decreased after the treatment of RipII. This substance may protect the formation of free radicals, which might reduce the inflammation.

We next investigated the antioxidant potential of RipII against levels of nitrate. Nitric oxide (NO) and reactive oxygen species exert multiple modulating effects on inflammation and play a key role in the regulation of immune responses. They affect virtually every step of the development of inflammation. NO has been shown to increase the production of pro-inflammatory prostaglandins in in vitro [59–61], ex vivo [62] and in vivo studies [63,64], potentially by S-nitrosation of cysteine residues in the catalytic domain of cyclo-oxygenase (COX) enzymes [65,66]. The RipII was able to reduce the levels of nitrate.

In addition, the antioxidant action of RipII observed in the TBARS and NO assays suggests that this substance may protect against oxidative damage to membrane polyunsaturated fatty acids, such as arachidonic acid, which is a very important component in the response to pain the cyclooxygenase pathway.

In conclusion, RipII, a substance isolated from the green fruit of *A. riparia*, is a molecule with interesting anti-inflammatory activity potentially due its ability to decrease TNF-α and IL-1β production and its histamine antagonism. Furthermore, the inhibition of migration of polymorphonuclear neutrophils implicated in the inflammatory process may also play a role in its biologic activities. Given the increased interest in obtaining natural products with therapeutic potential, RipII should be further examined and possibly used to produce drugs for the treatment of inflammatory diseases.

6. Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2013.07.007.

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