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ORIGINAL ARTICLE

Antiinflammatory and antinociceptive effects in mice of a sulfated polysaccharide fraction extracted from the marine red algae *Gracilaria caudata*

Luciano de Sousa Chaves[1,3], Lucas Antonio Duarte Nicolau[1], Renan Oliveira Silva[1], Francisco Clark Nogueira Barros[3], Ana Lúcia Ponte Freitas[3], Karoline Sabóia Aragão[2], Ronaldo de Albuquerque Ribeiro[2], Marcellus Henrique Loiola Ponte Souza[2], André Luiz dos Reis Barbosa[1], and Jand-Venes Rolim Medeiros[1]

[1]*Biotechnology and Biodiversity Center Research (BIOTEC), Laboratory of Experimental Physiopharmacology (LAFFEX), Federal University of Piauí-CMRV, Parnaíba, PI, Brazil,* [2]*Department of Physiology and Pharmacology, Federal University of Ceará, Laboratory of Pharmacology of Inflammation and Cancer (LAFICA), Fortaleza, CE, Brazil, and* [3]*Department of Biochemistry and Molecular Biology, Federal University of Ceará, Laboratory of Proteins and Carbohydrates of Marine Algae, Fortaleza, CE, Brazil*

Abstract

Many algal species contain relatively high concentrations of polysaccharide substances, a number of which have been shown to have anti-inflammatory and/or immunomodulatory activity. In this study, we evaluated the anti-inflammatory and antinociceptive effects in mice of a sulfated polysaccharide fraction (PLS) extracted from the algae *Gracilaria caudata*. The antiinflammatory activity of PLS was evaluated using several inflammatory agents (carrageenan, dextran, bradykinin, and histamine) to induce paw edema and peritonitis in Swiss mice. Samples of the paw tissue and peritoneal fluid were removed to determine myeloperoxidase (MPO) activity or TNF-α and IL-1β levels, respectively. Mechanical hypernociception was induced by subcutaneous injection of carrageenan into the plantar surface of the paw. Pretreatment of mice by intraperitoneal administration of PLS (2.5, 5, and 10 mg/kg) significantly and dose-dependently reduced carrageenan-induced paw edema ($p < 0.05$) compared to vehicle-treated mice. Similarly, PLS 10 mg/kg effectively inhibited edema induced by dextran and histamine; however, edema induced by bradykinin was unaffected by PLS. PLS 10 mg/kg inhibited total and differential peritoneal leukocyte counts following carrageenan-induced peritonitis. Furthermore, PLS reduced carrageenan-increased MPO activity in paws and reduced cytokine levels in the peritoneal cavity. Finally PLS pretreatment also reduced hypernociception 3–4 h after carrageenan. We conclude that PLS reduces the inflammatory response and hypernociception in mice by reducing neutrophil migration and cytokines concentration.

Keywords: Sulfated polysaccharide, marine algae, antiinflammatory, antinociceptive, edema

Introduction

The search for natural products with pharmacological properties has led to the discovery of pharmacologically active substances with significant applications in the experimental and therapeutic arenas.[1–5] Presently, about 25–30% of all active principles used as therapeutic treatments are extracted from natural products.[6] The plant kingdom is responsible for the majority of chemical diversity reported in the literature to date and has contributed considerably to the research and discovery of new drugs of natural origin, as well as to the supply of therapeutically active compounds.[7–11] Natural marine products have also been the focus for discovery of new products of pharmacological and biomedical interest.[12–19]

Marine algae are rich sources of sulfated polysaccharides, which are recognized as having a number of biological activities, including anticoagulant,[20]

Address for Correspondence: Jand-Venes Rolim Medeiros BIOTEC/LAFFEX/UFPI, Av. São Sebastião, nº 2819, CEP 64202-020, Parnaíba, PI, Brazil. Tel.: +55-86-99862374/+55-86-33234750. Fax: +55-86-33235406. E-mail: jandvenes@ufpi.edu.br

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gastroprotective,[21] antiinflammatory,[20] and antinociceptive activities,[22] which might give them relevance in pharmaceutical applications. However, these last two research fields have not been well explored, despite the wealth of our marine flora.

The inflammatory process consists of diverse physiological and pathological processes. A key characteristic of the inflammatory reaction is the migration of leukocytes from the blood into the tissues, which occurs in a sequence of steps. Locally produced inflammatory mediators, including TNF-α and IL-1β, activate vascular endothelial cells and upregulate key adhesion molecules that mediate tethering, rolling, cell adhesion, and extravasation of leukocytes. The leukocytes then migrate towards the site of tissue inflammation. Acute and chronic inflammatory reactions can cause irreversible tissue damage and induce organ failure.[23–25] Interestingly, there are no marine-derived antiinflammatory natural products in clinical development at this time.[24]

Considering that marine algae are important sources of new chemical substances with potential therapeutic effects, this study sought to evaluate the antiinflammatory and antinociceptive effects of a sulfated polysaccharide fraction extracted from the algae *Gracilaria caudata*.

Methods

Extraction of the polysaccharide fraction of *Gracilaria caudata*

Specimens of the red algae *G. caudate* were collected in August 2008 from the Atlantic coast northeast of Brazil (Fleixeira Beach, Trairi-Ceará). After collection, the algae were cleaned of epiphytes, washed with distilled water, and stored at –20°C. For extraction of polysaccharides, 5 g of dried *G. caudate* tissue was ground into a fine powder and stirred for 2 h in distilled water (1.5% w/v) at 100°C. After filtration and concentration of the solution, polysaccharides were precipitated with ethanol (1:3 v/v) and the precipitate was washed with acetone and dried under hot air. The polysaccharide fraction was then redissolved in distilled water (1.5% w/v) and the process of precipitation, washing, and drying was repeated.

Chemicals

λ-Carrageenan, indomethacin, bradykinin, dextran, histamine and captopril were purchased from Sigma Chemical Co., St Louis, MO, USA. Heparin and Evans blue dye were provided by Merck, Brazil. All other chemicals were analytical reagent grade. All drugs and sulfated polysaccharide fraction (PLS) were dissolved in sterile 0.9% (w/v) NaCl (saline).

Animals

Male Swiss mice (20–30 g) were housed in cages in temperature-controlled rooms, and food and water were available *ad libitum*. Animals were fasted for 18–24 h before the experiments. All animal treatments and surgical procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals (National Institute of Health, Bethesda, MD, USA) and were approved by the appropriate ethics committee (protocol Nº 0066/10).

Carrageenan-induced paw edema

The paw edema experiments were adapted and carried out as previously described.[27] The animals were randomly divided into six groups (n = 5) and edema was induced by injection of 50 μL of a 0.3% (w/v) suspension of carrageenan (300 μg/paw) in 0.9% sterile saline into the right plantar aponeurosis. Test articles were administered intraperitoneally (i.p.) 30 min before injection of carrageenan. Animals received: sterile saline 0.9% (group I untreated control); indomethacin 10 mg/kg (group II reference control); or PLS 2.5, 5, or 10 mg/kg (groups III, IV, and V, respectively). The contralateral paw received 50 μL of sterile saline and served as a control. The volume of the right hind paw was measured with an Ugo Basile plethysmometer at 0, 1, 2, 3, and 4 h after carrageenan treatment (V_t). The effect of pretreatments were calculated as percent inhibition of edema relative to the paw volume of the saline-treated controls using the following formula:[28]

$$\%\,\text{inhibition of edema} = \frac{\left(V_t - V_0\right)_{\text{control}} - \left(V_t - V_0\right)_{\text{treated}}}{\left(V_t - V_0\right)_{\text{control}}} \times 100$$

Effect of PLS on paw edema induced by different inflammatory agents

To induce edema with different inflammatory agents, animals received 50 μL injections of dextran (300 μg/paw), bradykinin (3.0 nmol/paw), or histamine (50.0 nmol/paw) into one hindpaw, as adapted from Barbosa et al. (2009) and Vasconcelos et al. (2011).[29,30] The contralateral paw received 50 μL of sterile saline as a control. In the experiment with bradykinin, the animals were pretreated with captopril (5 mg/kg, i.p.) 1 h prior to bradykinin induction to prevent bradykinin degradation. Edema was measured as described above.

Myeloperoxidase activity

Myeloperoxidase is an enzyme found primarily in neutrophil azurophilic granules that has been used extensively as a biochemical marker for granulocyte infiltration into tissues, including paw tissue.[31] Here, we measured myeloperoxidase (MPO) activity to evaluate neutrophil accumulation in the mouse paw. Briefly, 50–100 mg of paw tissue was homogenized at 50 mg/mL in potassium buffer containing 0.5% hexadecyltrimethylammomium bromide (HTAB). The homogenate was centrifuged at 40,000*g* for 7 min at 4°C. The pellet was resuspended and MPO activity was assayed by measuring the change in absorbance at 450 nm using *o*-dianisidinedihydrochloride and 1% hydrogen peroxide. MPO activity is reported as units/mg of tissue. A unit of MPO activity was defined

as that converting 1 μmol of hydrogen peroxide to water in 1 min at 22°C.

Evaluation of neutrophil migration

For the determination of neutrophil migration into the peritoneal cavity, mice were injected i.p. with 250 μL sterile saline, indomethacin 10 mg/kg, or PLS 10 mg/kg. Thirty minutes later, the animals were injected i.p. with 250 μL carrageenan (300 μg/mouse). Mice were euthanized 4 h later and the peritoneal cavity was washed with 1.5 mL of heparinized phosphate buffered saline (PBS) to harvest peritoneal cells. The volumes recovered were similar in all experimental groups and were equivalent to ~95% of the injected volume. Total cell counts were performed in a Neubauer chamber, and differential cell counts (100 cells total) were carried out on cytocentrifuge slides stained with hematoxylin and eosin. The results are presented as the number of neutrophils per milliliter of peritoneal exudate. Aliquots of the peritoneal exudates were stored at –70°C for later analysis of cytokine content.

Cytokine measurements

The levels of TNF-α and IL-1β in peritoneal exudates were evaluated using sandwich ELISAs. ELISA kits for TNF-α and IL-1β were from the National Institute for Biological Standards and Control (Potters Bar, UK). Briefly, polyclonal anti-mouse TNF-α or antimouse IL-1β antibodies (4.0 μg/mL, DuoSet Development kit; R&D Systems catalog # DY501 and DY510, respectively), or polyclonal antimouse IL-1β antibody (2.0 μg/mL), were diluted in PBS and added to microtiter plates at 50 μL/well (NuncMaxisorb, Roskilde, Denmark). Nonspecific binding sites were blocked with PBS containing 2% BSA for 90 min at 37°C. Plates were washed in assay buffer (0.01 M phosphate, 0.05 M NaCl, 0.1% Tween 20, pH 7.2), and 50 μL of peritoneal exudate or standard TNF-α or IL-1β was added to the wells and incubated overnight at 4°C. After washing the plates, 50 μL/well of biotinylated polyclonal rabbit antimouse TNF-α (1:1000 dilution in assay buffer containing 1% normal sheep serum) or rabbit antimouse IL-1β (1:1000 dilution) was added and plates were incubated for 30 min at 37°C. The plates were washed again, and 50 μL/well of HRP-streptavidin (1:5000 dilution; Dako, Carpinteria, CA, USA) was added for 1 h at room temperature. The plates were washed, and wells were incubated for 15 min with 50 μL of substrate (40 μg/well *o*-phenylenediaminedihydrochloride; Sigma-Aldrich). After color development, the reaction was stopped by the addition of 1 M sulfuric acid and absorbance was measured at 490 nm. The ELISAs detected TNF-α and IL-1β levels up to 4000 pg/mL and did not cross-react with other cytokines. The results are expressed as picogram of cytokine per milliliter peritoneal exudate.

Mechanical hypernociception

The term hypernociception (increased nociception) was used to describe the behavioral response induced by mechanical pressure in rats and mices. Hyperalgesia was induced by a subcutaneous injection of carrageenan (300 μg/paw) into the plantar surface of the mice hindpaw and measured by the paw pressure test described by Cunha et al. (2004).[32] A digital analgesiometer (Insight®, Brazil) with a cone-shaped, rounded tip paw-presser was used to apply a linearly increasing force to the right hindpaws of the mice. The nociceptive threshold was measured in the right paw and determined by the average of 3 consecutive trials recorded before (zero time), and 3 and 4 h after carrageenan (50 μL; 300 μg/paw) injection (peak effect). Hyperalgesia was calculated from the difference between these two averages (Δ of nociceptive threshold) and expressed in grams. To reduce stress, the mice were habituated to the apparatus 1 day prior to the experiments.

Toxicological studies

The mice were daily treated with PLS 100 mg/kg intraperitoneally or by gavage for 7 days (adapted from Bitencourt et al., 2008).[33]

Statistical analysis

Results are expressed as mean ± SEM from at least five animals per group and statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Newman–Keulspost hoc test, when appropriate. Statistical significance was set at $p < 0.05$.

Results

Carrageenan-induced paw edema

Table 1 show that carrageenan induced severe paw edema within an hour of injection, which was maintained until

Table 1. Effect of PLS on carrageenan-induced paw edema in mice.

		Paw edema (mL)			
Treatment	Dose (mg/kg)	1 h	2 h	3 h	4 h
Control (Cg)		0.074 ± 0.009	0.102 ± 0.016	0.104 ± 0.009	0.110 ± 0.008
Saline		0.014 ± 0.007	0.016 ± 0.007	0.004 ± 0.002	0.006 ± 0.002
Indomethacin	10	0.066 ± 0.008 (10.81)	0.048 ± 0.009 (52.94)	0.074 ± 0.008 (28.84)	0.048 ± 0.017** (53.36)
PLS	2.5	0.045 ± 0.016 (39.18)	0.042 ± 0.009** (58.82)	0.022 ± 0.005** (78.84)	0.068 ± 0.012 (38.18)
	5	0.026 ± 0.009** (64,86)	0.044 ± 0.011** (56.86)	0.058 ± 0.009** (44.230)	0.050 ± 0.006** (54.54)
	10	0.028 ± 0.008** (62.16)	0.022 ± 0.009** (78.43)	0.030 ± 0.006** (71.15)	0.016 ± 0.009** (85.45)

Values of paw edema are expressed in mean ± SEM ($n = 5$). % Inhibition of paw edema is indicated in parenthesis. Control Cg = carrageenan.
*$p < 0.01$ compared with control (one way ANOVA followed by Newman-Keuls' test).
**$p < 0.01$ compared with control (one way ANOVA followed by Newman-Keuls' test).

4 h after injection. Paw edema was significantly decreased by indomethacin ($p < 0.05$) throughout the experimental period, with a maximal inhibition of 53.36%. Similarly, PLS (5 and 10 mg/kg) induced a long-lasting inhibition of paw edema between 1 and 4 h. At 4 h, the animals pretreated with 5 and 10 mg/kg PLS showed a reduction in edema of 54.54 and 85.45%, respectively, compared to the carrageenan group. Because a PLS dose of 10 mg/kg afforded the most protection against edema induced by carrageenan, this dose was selected for the study of the possible mechanisms involved in PLS-mediated antiinflammatory and antinociceptive effects.

Effect of PLS on paw edema induced by different inflammatory agents

Figure 1 shows that injection of carrageenan (paw volume: 0.104 ± 0.09 mL; Figure 1A), dextran (0.101 ± 0.10 mL; Figure 1B), or histamine (0.095 ± 0.12 mL; Figure 1D) increased edema over time, peaking approximately 3 h after injection. By contrast, paw volume in the control saline-injected group was 0.07 ± 0.04 mL. Pretreatment of animals with PLS (10 mg/kg) effectively inhibited edema at 3 h after injection of carrageenan (71.15% inhibition; Figure 1A), dextran (45.2%; Figure 1B), or histamine (59.6%; Figure 1D). By contrast, PLS did not inhibit paw edema evoked by bradykinin (Figure 1C).

MPO activity

Figure 2 shows the increase in MPO activity in paw tissue from animals injected with carrageenan (20.3 ± 4.1 U/mg of tissue). Pretreatment of mice with PLS at 10 mg/kg reduced the MPO activity to 8.4 ± 1.3 U/mg of tissue, which was similar to the effect of the indomethacin reference control treatment (9.1 ± 0.5 U/mg of tissue).

Effect of PLS on carrageenan-induced peritonitis

PLS was tested in the carrageenan-induced peritonitis model to investigate its effect on leukocyte migration. In this model, the total leukocyte count in the peritoneal cavity of control, carrageenan-injected animals was $7.475 \times 10^6 \pm 0.725 \times 10^6$ cells/mL (Figure 3A). Administration of PLS (10 mg/kg, i.p.) significantly reduced the peritoneal leukocyte count to $4.975 \times 10^6 \pm 0.286 \times 10^6$ cells/mL). The same dose of PLS also significantly reduced neutrophil migration into the peritoneal cavity compared with the control group ($1.918 \times 10^6 \pm 0.417 \times 10^6$ cells/mL vs. $5.880 \times 10^6 \pm 0.586 \times 10^6$ cells/mL; Figure 3B). This result is consistent with the fact that neutrophils are the most abundant cells in primary inflammatory exudates.

Effect of PLS on TNF-α and IL-1β levels in carrageenan-induced peritonitis

Figure 4A shows that the intraperitoneal administration of carrageenan induced a marked increase in TNF-α and IL1-β concentrations in peritoneal exudate fluid. The TNF-α level in the peritoneal cavity of control animals was 81.39 ± 6.30 pg/mL, which was

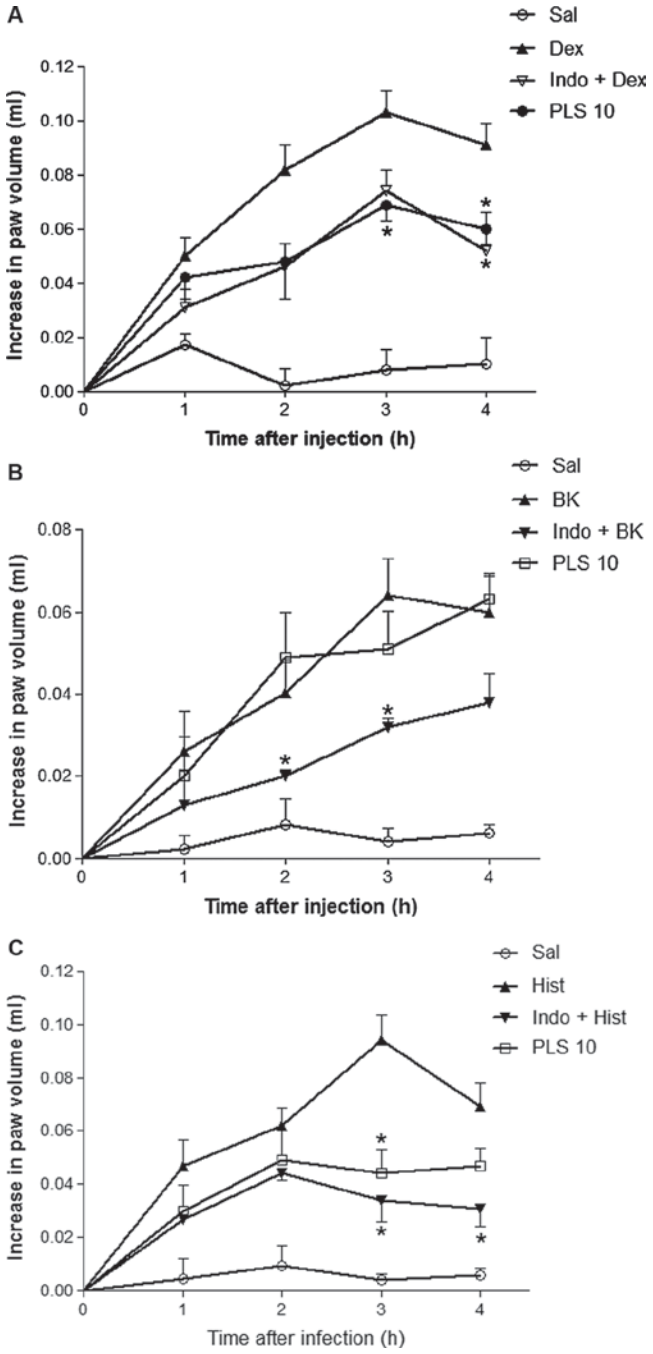

Figure 1. Effects of the sulfated polysaccharide fraction of *Gracilaria caudata* on paw inflammation induced by different inflammatory agents. Edema was induced by: panel A, dextran (Dex); panel B, bradykinin (BK); and panel C, histamine (Hist). Animals were pretreated with PLS (10 mg/kg i.p.), saline (Sal; control), or indomethacin (Indo; 10 mg/kg, i.p.). Each point represents the mean ± SEM. of five animals. *$p < 0.05$ significantly different to the control group. Statistical analysis was performed by ANOVA followed by the Newman-Keuls post hoc test.

increased to 190.33 ± 23.25 pg/mL after carrageenan injection. Pretreatment of animals with PLS (10 mg/kg, i.p.) significantly reduced TNF-α levels to 80.53 ± 12.94 pg/mL (Figure 4A). In addition, PLS treatment decreased the concentration of peritoneal IL-1β compared to the carrageenan control group (372.5 ± 158.6 pg/mL vs. 1412.0 ± 186.4 pg/mL; Figure 4B).

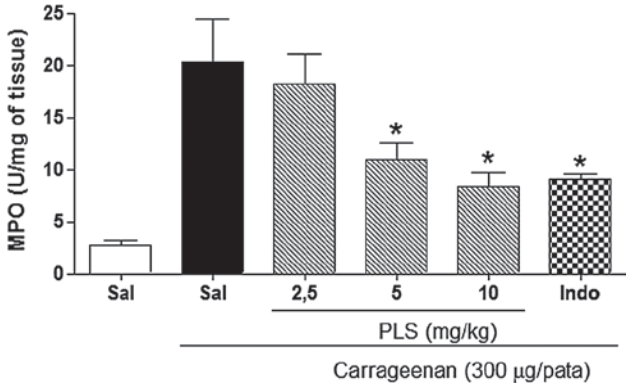

Figure 2. Effect of PLS on paw tissue myeloperoxidase activity induced by carrageenan. Mice were injected s.c. with saline (Sal) or carrageenan (Cg). Thirty minutes earlier, animals were administered indomethacin (10 mg/kg, i.p.) or different doses of PLS (2.5, 5, and 10 mg/kg, i.p.). The control group (Sal) was treated only with saline. Paw tissue MPO activity was determined after 4 h. Results are expressed as the mean ± SEM for at least five animals per group. *$p < 0.05$ compared to the Sal plus Cg group. Statistical analysis was performed by ANOVA followed by the Newman-Keuls post hoc test.

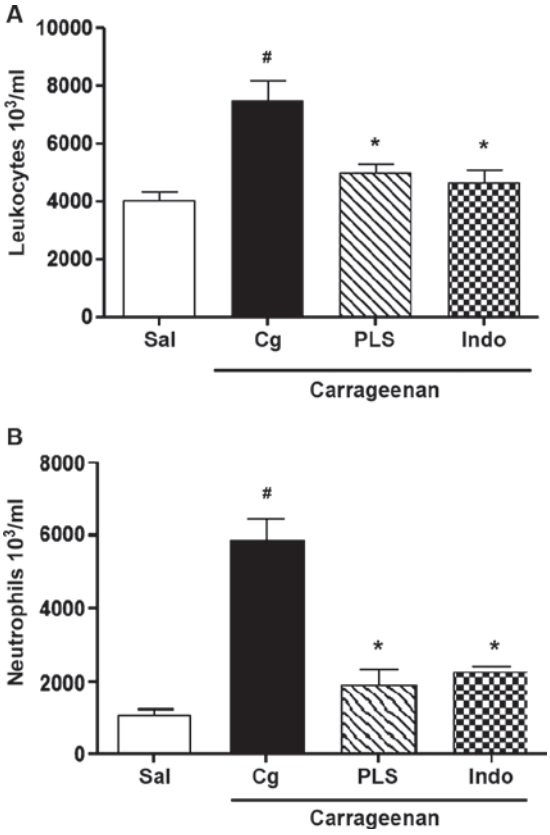

Figure 3. Antiinflammatory effect of PLS on carrageenan-induced peritonitis in mice. Mice were injected i.p. with 250 µL of saline, indomethacin (10 mg/kg i.p.), or PLS (10 mg/kg) and 30 min later were injected i.p. with 300 µg carrageenan. Neutrophil migration was evaluated 4 h later. The white bars represent the peritoneal neutrophils in animals injected i.p. with saline (control group). (A) Total counts and (B) differential counts. The values are means ± SEM. *$p < 0.05$ compared to the carrageenan group; #$p < 0.05$ compared to the saline group. Statistical analysis was performed by ANOVA followed by the Newman-Keuls post hoc test.

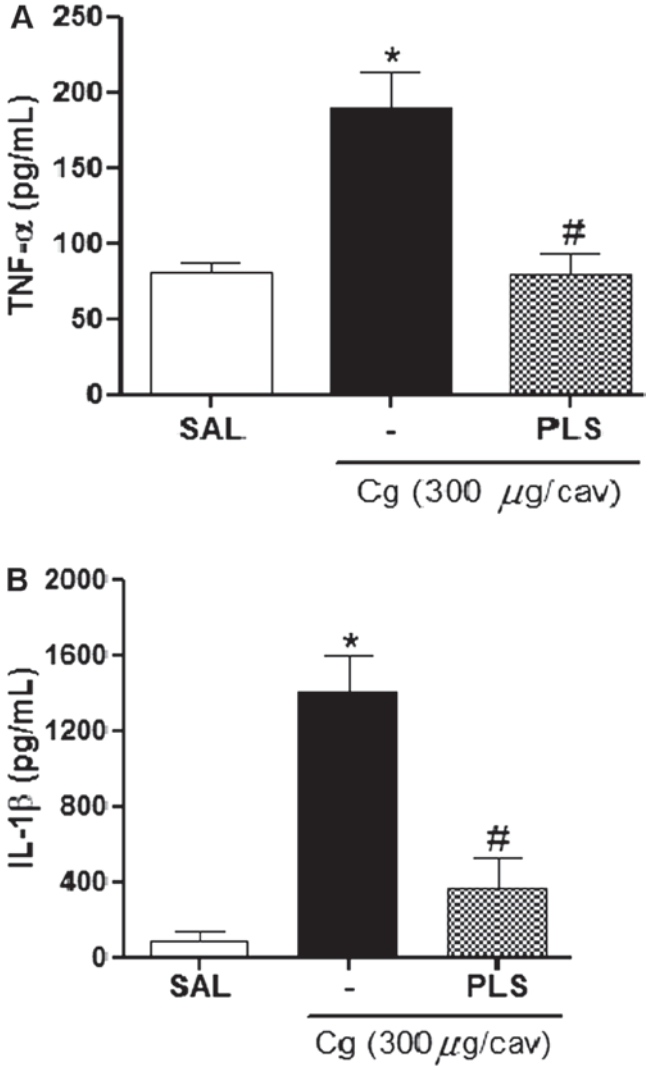

Figure 4. Effect of PLS on carrageenan-induced cytokine production in peritonitis. Levels of TNF-α (panel A) or IL-1β (panel B) in the peritoneal cavities were measured 4 h after carrageenan injection. Mice were orally administered PLS (10 mg/kg), and 1 h later 250 µL (300 µg/cav) carrageenan was injected i.p. Each point represents the mean ± SEM of five animals. *$p < 0.05$ compared to saline-treated animals; #$p < 0.05$ compared to the carrageenan group. Statistical analysis was carried out using one-way ANOVA followed by the Newman-Keuls post hoc test.

Effect of PLS on carrageenan-induced hypernociception

As shown in Figure 5, pretreatment of mice with PLS 10 mg/kg significantly decreased hypernociception between 3 and 4 h after carrageenan administration.

Toxicological studies

Polysaccharides are known to be well tolerated in experimental animals such as rats and mice. Subchronic daily treatment of mice with PLS (100 mg/kg) for 7 days significantly increased the animal body mass, but did not affect the wet weight of important organs such as liver, heart, or kidney. The macroscopic and microscopic appearance of these organs, and that of the stomach, was normal. Renal function, hepatic function, and numbers of circulating leukocytes were unaffected by PLS treatment.

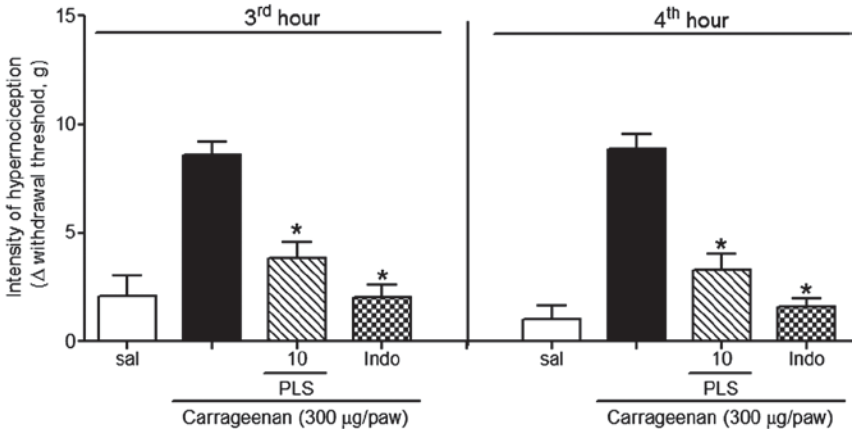

Figure 5. Effect of PLS 10 mg/kg on carrageenan-induced hypernociception. Carrageenan (300 µg/paw) was injected and paw hypernociception was measured after 3 and 4 h (panel A and B). Animals were pretreated with PLS (10 mg/kg i.p.), saline (Sal; control), or indomethacin (Indo; 10 mg/kg, i.p.). *$p < 0.05$ compared to the saline group. Statistical analysis was performed by ANOVA followed by the Newman-Keuls post hoc test.

Discussion

Natural products derived from algae exhibit a wide range of pharmacological activities and are used in folk medicine all over the world. Over the years, natural products have contributed enormously to the development of important therapeutic drugs currently used in modern medicine.[34,35] In the present study, we sought to investigate, by the use of pharmacological tools and molecular procedures, the possible anti-inflammatory and antinociceptive properties of a sulfated polysaccharide fraction extracted from the marine red algae *Gracilaria caudata*. Our results clearly demonstrate that PLS has anti-inflammatory and antinociceptive effects in models of inflammation (paw edema and peritonitis) and nociception (Von Frey test) in mice.

To evaluate antiinflammatory activity, PLS was first tested in the paw edema model. Carrageenan-induced edema has a biphasic inflammatory response, initially involving effects on vascular permeability by diverse mediators such as histamine, serotonin, and bradykininas well as TNF-α, IL-1β, IL-2, and IFN-γ.[35,36] Collectively, these mediators stimulate the molecular inflammatory response and trigger nociception.[34,35] Subsequently, there is an increase in nitric oxide and prostaglandins, and an intense neutrophil infiltrate.[35–39] Because the second phase (edema) of inflammation induced by carrageenan is sensitive to most clinically effective antiinflammatory drugs, this assay is useful for studying the antiedematous effects of natural products.[40] On the other hand, dextran is a proinflammatory agent that promotes release of vasoactive amines, such as histamine and serotonin, causing an osmotic edema, characterized by an increase in vascular permeability with low levels of protein and neutrophils.[41] Hence, the present investigation demonstrated that PLS significantly reduced biphasic carrageenan-induced edema. PLS also inhibited edema induced by dextran and histamine, but did not inhibit that induced by bradykinin. Based on these reports, it could be concluded that the anti-edematogenicaction of PLS might be due to differential inhibition of the mediators involved in the inflammatory events.

The paw inflammatory response induced by carrageenan was accompanied by an intense neutrophil infiltrate.[35,36] MPO activity is a commonly used indicator of neutrophil infiltration and in this study, we demonstrated that PLS (5 and 10 mg/kg) inhibited neutrophil infiltration, as measured by the MPO activities in the mouse paws. These results suggest that the antiedematogenic response of PLS is related to inflammatory events involving neutrophil migration.

To confirm that antiinflammatory effect of PLS involved neutrophil migration, we tested PLS in a carrageenan-induced peritonitis model. Consistent with the results of the paw edema assays, we observed that PLS also decreased leukocyte migration to the peritoneal cavity of these animals. Carrageenan induces neutrophil migration into the rat peritoneal cavity by an indirect mechanism, via activation of macrophages.[42] In the current study, we have shown for the first time that PLS administration prevented carrageenan induced increases in the peritoneal levels of TNF-α and IL-1β. Such increases incytokine levels might result in plasma protein extravasation and cellular infiltration into the inflammatory site.[43,44] TNF-α and IL-1β are potent proinflammatory cytokines that have multiple effects, including activation of inflammatory cells, induction of several inflammatory proteins, cytotoxicity, edema formation, and neutrophil migration.[26,45,46] Based on these reports, it could be inferred that the antiinflammatory effectof PLS might occur through inhibition of the cytokines involved in carrageenan-induced peritonitis.

It is clear that there is a strong association between the inflammatory process and the development of pain. Several studies have demonstrated that inhibition of neutrophil migration reduces hypernociception induced by different inflammatory stimuli.[31,47] The induction of inflammatory hypernociception is primarily driven by the

sensitization of primary nociceptive neurons, an effect that is caused by nociceptor-sensitizing mediators. Our results demonstrate that pretreatment with PLS decreased carrageenan-induced hypernociception. Carrageenan-induced hypernociception depends on the concomitant release and action of cytokines, which induce the subsequent release of IL-1β and prostanoids. On the other hand, PGE2, stimulated by cytokines, acts on nociceptive neurons by a direct mechanism. In this study we demonstrated that PLS diminished the levels of TNF-α and IL-1β in the peritoneal cavity.[31] Hence, it is possible that PLS decreased the inflammatory response by reducing cytokine release and, subsequently, inflammatory hypernociception. Several studies have demonstrated that algal extracts and sulfated polysaccharide isolates from algae have antinociceptive activity, and diminish leukocyte migration and cytokine levels.[47–49] Similarly, published reports have demonstrated that the edema induced by carrageenan is dependent on cytokine production by resident cells and on neutrophil infiltration.[48–50] Thus, we conclude that PLS reduces the inflammatory response and hypernociception by decreasing neutrophil migration and cytokine concentrations.

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Declaration of interest

The authors report no declarations of interest.

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