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Agglutinin isolated from the red marine alga *Hypnea cervicornis* J. Agardh reduces inflammatory hypernociception: Involvement of nitric oxide

Jozi G. Figueiredo [a,1], Flavio S. Bitencourt [b,1], Thiago M. Cunha [c], Patrícia B. Luz [b], Kyria S. Nascimento [a], Mario R.L. Mota [b], Alexandre H. Sampaio [d], Benildo S. Cavada [a], Fernando Q. Cunha [c], Nylane M.N. Alencar [b,*]

[a] Department of Biochemistry and Molecular Biology, Federal University of Ceara, 60451-970, Fortaleza-CE, Brazil
[b] Department of Physiology and Pharmacology, Federal University of Ceara, Cel. Nunes de Melo 1127, 60430-270, Fortaleza-CE, Brazil
[c] Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo,14049-900, Ribeirão Preto-SP, Brazil
[d] Department of Fisheries Engineering, Federal University of Ceara, Fortaleza-CE, Brazil

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ABSTRACT

Hypnea cervicornis agglutinin (HCA), a lectin isolated from the red marine alga has been previously shown to have an antinociceptive effect. In the present study in rats, mechanisms of action of HCA were addressed regarding mechanical hypernociception induced by carrageenan, ovalbumin (as antigen), and also by prostaglandin E_2 in rats. The lectin administered intravenously inhibited carrageenan- and antigen-induced hypernociception at 1, 3, 5 and 7 h. This inhibitory effect was completely prevented when lectin was combined with mucin, demonstrating the role of carbohydrate-binding sites. The inhibition of inflammatory hypernociception by HCA was associated with the prevention of neutrophil recruitment to the plantar tissue of rats but was not associated with the inhibition of the release of pro-hypernociceptive cytokines (TNF-α, IL-1β and CINC-1). HCA also blocked mechanical hypernociception induced by PGE_2, which was prevented by the administration of nitric oxide synthase inhibitors. These results were corroborated by the increased circulating levels of NO metabolites following HCA treatment. These findings suggest that the anti-hypernociceptive effects of HCA are not associated with the inhibition of pro-inflammatory cytokine production. However, these effects seem to involve the inhibition of neutrophil migration and also the increase in NO production.

1. Introduction

Lectins are non-immune proteins with the ability to reversibly bind to carbohydrates and to decipher cell glycocodes (Gabius and Gabius, 2002). These proteins can be found in all kingdoms of life ranging from viruses to animals and algae to plants (Sharon and Lis, 1989). Marine alga lectins are especially interesting for biological applications because they have generally lower molecular weights as compared to most plant lectins. An additional benefit could be that small algal lectin molecules may be expected to be less antigenic than the larger plant lectins (Rogers and Hori, 1993). Furthermore, they possess great stability due to several disulfide bridges and show high specificity for complex carbohydrates and glycoconjugates, especially for mucins (Ainouz et al., 1995; Nagano et al., 2005; Sampaio et al., 1998).

Pain is an important symptom of inflammatory disease. The sensitization of primary afferent nociceptors is a common denominator of all kinds of inflammatory pain, leading to states of hyperalgesia and/or allodynia, better described as hypernociception in animal models (Millan, 1999; Parada et al., 2003). Hypernociception is induced by the direct action of inflammatory mediators, such as prostaglandins and sympathetic amines, on peripheral nociceptors (Ferreira and Nakamura, 1979; Khasar et al., 1999; Nakamura and Ferreira, 1987). These direct-acting hyperalgesic mediators are ultimately released in the inflamed tissue following a cascade of cytokines (TNF-α, IL-1β and chemokines) released by the resident and migratory cells (Cunha et al., 1992, 2005; Verri et al., 2006). The pro-nociceptive role of cytokines and chemokines seems to be mediated by the recruitment of neutrophils, which in the last instance is responsible for the release of direct-acting mediators such as prostaglandins (Cunha et al., 2008a).

It has been recently shown that an agglutinin (HCA) isolated from the red marine alga *Hypnea cervicornis* has anti-inflammatory and antinociceptive properties (Bitencourt et al., 2008; Nascimento et al., 2006). This antinociceptive effect probably occurred through peripheral mechanisms. Additionally, HCA inhibited the neutrophil migration and did not show visible signs of toxicity in mice. However, the exact mechanism of action involved remains to be elucidated. In the present study, we investigated the effect of HCA on inflammatory hypernociception induced by different stimuli, and examined the possible mechanism involved in this effect as well.

* Corresponding author. Tel.: +55 85 33668339; fax: +55 85 33668333.
E-mail address: nylanemaria@yahoo.com.br (N.M.N. Alencar).
[1] Co-first authors.

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2. Materials and methods

2.1. Marine alga

Specimens of the red alga *Hypnea cervicornis* J. Agardh (Rhodophyta, Gigartinales, Hypneaceae) were collected from Pacheco Beach on the Atlantic coast of Ceará State, Brazil. The algal sample was identified by Dr. A.H. Sampaio and W.R.L. Farias at the Fisheries Engineering Department using as reference the works of Taylor (1960) and Joly (1965). The material was cleaned to remove epiphytes, transported to the laboratory within 1 h after collection, and stored at −20.0 °C until use.

2.2. Lectin preparation

The frozen alga was ground to a fine powder in liquid nitrogen before being submitted to lectin extraction and isolation. Isolation was performed by ion exchange chromatography (DEAE Sephacel), and lectin purity assessed by mass spectrometry (MALDI-TOF) and N-terminal sequencing (Applied Biosystems Precise instrument) (Nagano et al., 2005; Nascimento et al., 2006). The purified lectin was stored at −20.0 °C until use.

2.3. Animals

Wistar rats (180–220 g) were housed in temperature-controlled rooms (22–25 °C) with access to water and food *ad libitum*. Testing sessions took place during the light phase (between 08:00 A.M. and 5:00 P.M.). Experiments were carried out according to the Guide for the Care and Use of Laboratory Animals of the U.S. Department of Health and Human Services (NIH publication no. 85-23, revised 1985) and approved by the Institutional Animal Care and Use Committee of the Federal University of Ceará (n 60/09), Fortaleza, Brazil.

2.4. Drugs and reagents

Carrageenan (Cg; lambda type IV), OVA, bovine submaxillary mucin (mucin), aminoguanidine and L-NAME were purchased from Sigma Chemical Co. (St Louis, MO, USA). All drugs and the lectin were dissolved in sterile 0.9% (w/v) NaCl (saline). All other chemicals were of analytical grade.

2.5. Experimental protocols

2.5.1. Mechanical hypernociception

The term hypernociception was used to define the decrease in nociceptive withdrawal threshold (Cunha and Verri, 2007). Mechanical hypernociception was evaluated in rats as previously reported (Vivancos et al., 2004). Briefly, in a quiet room, rats were placed in acrylic cages (12×20×17 cm) with wire grid floors, 15–30 min before the beginning of the test. The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer adapted with a 0.7-mm^2 and 0.5-mm^2 polypropylene tip for rats and mice, respectively (Electronic von Frey; IITC Life Science, Woodland Hills, CA). A tilted mirror placed under the grid provided a clear view of the rat hind paw. The investigator was trained to apply the tip in between the five distal footpads with a gradual increase in pressure. The stimulus was automatically discontinued and its intensity recorded when the paw was withdrawn. The end point was determined by the removal of the paw in a clear flinch response after paw withdrawal. The animals were tested before and after treatments. A different investigator performed each test, as in the case of the preparation of solutions and treatment of the animals. The results are expressed by the Δ withdrawal threshold (in grams, g) which was calculated by subtracting the average of the last three measurements after the treatments from the average of three measurements before the treatments.

2.5.1.1. Carrageenan-induced inflammatory hypernociception. All rat groups tested were treated intravenously (i.v.) 15 min before carrageenan (Cg) with the following in a volume of 0.1 ml: HCA (0.1, 1 and 10 mg/kg; as described by Bitencourt et al., 2008) or mucin (10 mg/kg) or HCA (1 mg/kg) combined with mucin (10 mg/kg) or sterile saline (0.9% NaCl, w/v). In this protocol where the lectin was administered in combination with mucin (lectin-binding sugar), mucin and lectin alone or combined were previously incubated overnight at 37 °C before administration. Hypernociception was measured at 0, 1, 3, 5, 7, 12 and 24 h after Cg (100 μg/paw; 100 μl; as described by Cunha et al., 2000) or saline i.pl. (negative control) injection into the hind paws. The Cg dose administered was the lowest dose that evoked maximal acute mechanical hypernociception.

2.5.1.2. Procedures for active immunization and ovalbumin immunization in rats. Rats were immunized as previously described (Cunha et al., 2003). Briefly, ovalbumin (OVA; 200 μg) emulsified in complete Freund's adjuvant (CFA) plus saline, 1:1, was injected subcutaneously into rats on day 0. The rats were injected subcutaneously at two different sites on their back with OVA (200 μg) emulsified in incomplete Freund's adjuvant plus saline (1:1) on days 7 and 14 (OVA-immunized). Control rats (sham-immunized) received the same emulsions without OVA. Rats were challenged on day 21 by intraplantar (i.pl.) injection of 100 μg of OVA. At this time, rats were treated 15 min before i.pl. injection of OVA with i.v. HCA (1 mg/kg) or saline. Hypernociception was determined at 0, 1, 3, 5, 7, 12 and 24 h after OVA administration. CFA was used as adjuvant in this immunization protocol because it induces a mainly Th1-driven response and augments the efficiency of the immunization procedure (Canetti et al., 2001).

2.6. Cytokine measurements

At 3 h after Cg intraplantar injection, animals were terminally anesthetized, and the plantar tissues (≅0.5 cm^2) were removed from the injected and control paws (saline). The time point for cytokine measurements was based on the time-course determined for cytokines induced Cg. It was demonstrated that cytokine production peaks at 3 h after Cg (Cunha et al., 2000). The samples were triturated and homogenized in 500 μl of the appropriate buffer (phosphate-buffered saline containing 0.05% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 kallikrein international units of aprotinin A) followed by centrifugation for 10 min at 2000×*g*. The supernatants were stored at −70 °C until further analysis. The levels of TNF-α, IL-1β and CINC-1/CXCL1 were determined using sandwich ELISA. ELISA kits for TNF-α, IL-1β and CINC-1/CXCL1 were from the National Institute for Biological Standards and Control (Potters Bar, UK). Briefly, sheep polyclonal anti-rat TNF-α, IgG-raised TNF-α (2.0 μg/ml), sheep polyclonal anti-rat IL-1β, IgG-raised IL-1β (2.0 μg/ml), sheep polyclonal anti-rat CINC-1, or IgG-raised CINC-1/CXCL1 (3.0 μg/ml), diluted in 50 μl of PBS buffer, was used to coat the microtiter plates (Nunc Maxisorb, Roskilde, Denmark). Nonspecific binding sites were blocked by incubating the plates with PBS containing 2% BSA for 90 min at 37 °C. After incubation (4 °C, overnight) and washing the plates in assay buffer (0.01 M phosphate, 0.05 M NaCl, 0.1% Tween 20, pH 7.2), 50 μl of standard (CINC-1, TNF-α, IL-1β or sample) were added to each well and incubated overnight at 4 °C. After washing the plates, 50 μl of either rabbit polyclonal anti-rat TNF-α (1:2000 with assay buffer plus 1% normal sheep serum), rabbit anti-rat IL-1β (1:2000) or sheep polyclonal anti-rat CINC-1 IgG (diluted 1:500) biotinylated antibody were added, and the plates were incubated for 30 min at 37 °C and then for 1 h at room temperature. After the second incubation, the plates were washed again, and 50 μl of avidin–peroxidase conjugate (1:5000 dilution — DAKO) were added to all the wells, and the plates were incubated for 30 min with 50 μl of substrate (40 μg/well; orthophenylenediamine dihydrochloride, Sigma). After

color development, the reaction was stopped with the addition of sulfuric acid (1 M). Absorbance was measured at 490 nm. These ELISA methods consistently detected TNF-α, IL-1β and CINC-1 levels over 20 pg/ml and did not cross-react with other cytokines. We have previously standardized the ELISA for the quantification of these cytokines and have detected that the plate absorbance (690 nm) does not produce any statistical difference in the results. The results are expressed as picograms (pg) of each cytokine per paw. As a control, the concentrations of these cytokines were determined in animals injected with saline.

2.7. Determination of myeloperoxidase activity

Myeloperoxidase (MPO) activity was used as a measure of neutrophil accumulation in the rats' plantar tissues, based on a kinetic-colorimetric assay as previously described (Cunha et al., 2008b). Approximately 0.5 cm^2 of plantar tissue was harvested 3 h after the intraplantar injection of inflammatory stimuli. Samples were collected in 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide (HTAB) and kept at −80 °C. Just before the assay, the tissue was homogenized using a Polytron (PT3100) and centrifuged at 13,000×*g* for 4 min. To prepare the solution for analysis, 10 µl of supernatant were mixed with 200 µl of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml *O*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. MPO activity was measured by the absorbance of the solution at 450 nm (Spectra max), taking three readings in 1 min. MPO activity was determined using a standard curve for neutrophils obtained from rat blood (Bradley et al., 1982). The results were presented as number of neutrophils × 10^6/mg tissue (MPO activity).

2.8. Hypernociception induced by prostaglandin E_2 (PGE_2)

PGE_2 was injected (100 ng/paw; 100 µl; as described by Ferreira et al., 1978a,b, 1988) into the rats pretreated 15 min before with i.v. administration of HCA (1 mg/kg) or saline. Hypernociception was measured as described above at times 0 and 3 h after injection of prostaglandin or saline (negative control) into the hind paws (i.pl.).

2.9. Measurement of NO concentration in serum of rats treated with HCA

Nitrite (NO_2^-) concentrations were determined in serum obtained from the animals 3 h after intraplantar injection of Cg or i.v. injection of HCA. HCA (1 mg/kg) or saline was administered intravenously 15 min before Cg. Other groups of animals only received HCA or saline without the injection of Cg. Nitric oxide production was determined using the Griess method (Green et al., 1982) by measuring nitrite concentrations in an ELISA plate reader at 540 nm, and the results were expressed as micromoles of nitrite.

2.10. Investigation of the role of nitric oxide in the antinociceptive action of HCA

Thirty minutes before the injection of Cg, the rats were treated subcutaneously (s.c.) with saline or with NOS inhibitors, either L-arginine analog N-nitro-L-arginine methyl ester (L-NAME; nonspecific NOS inhibitor) or aminoguanidine (Amino; selective inhibitor of iNOS), at a dose of 50 mg/kg. At this time, rats were treated 15 min before Cg with i.v. HCA (1 mg/kg) or saline. Hypernociception was assessed 3 h after injection of Cg (100 µg/paw; 100 µl) or saline i.pl. as described above.

2.11. Statistical analysis

All results were expressed as mean ± S.E.M. for groups of five animals. Statistical evaluation was carried out by analysis of variance (ANOVA) followed by Bonferroni's test. A *p* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of HCA on mechanical hypernociception induced by carrageenan and antigen in immunized rats

HCA at doses of 0.1, 1 and 10 mg/kg significantly reduced Cg-induced mechanical hypernociception by 38%, 51% and 73%, respectively. This inhibitory effect was blocked when the lectin was combined with its binding sugar mucin (Fig. 1A). Moreover, mucin, administered alone to rats, did not modify the hypernociceptive response induced by Cg (Fig. 1A). There was a significant occurrence of hypernociception 1 h after Cg injection, reaching a maximum in 3 h and decreasing thereafter (5–24 h), compared to the negative group (saline) (Fig. 1B). HCA (1 mg/kg) treatment also inhibited Cg-induced hypernociception by 50%, 48%, 40% and 47% determined at 1, 3, 5 and 7 h, respectively after stimulus injection (Fig. 1B). All assays were carried out at 1 h and 24 h after the administration of the inflammatory mediator (Cg).

In the inflammatory reaction to antigen challenge, the animals given ovalbumin (OVA) at a dose of 100 µg/paw already showed a significant reaction 1 h after OVA injection, and this reaction peaked 3 h later, declining thereafter and reaching the control level 12 and

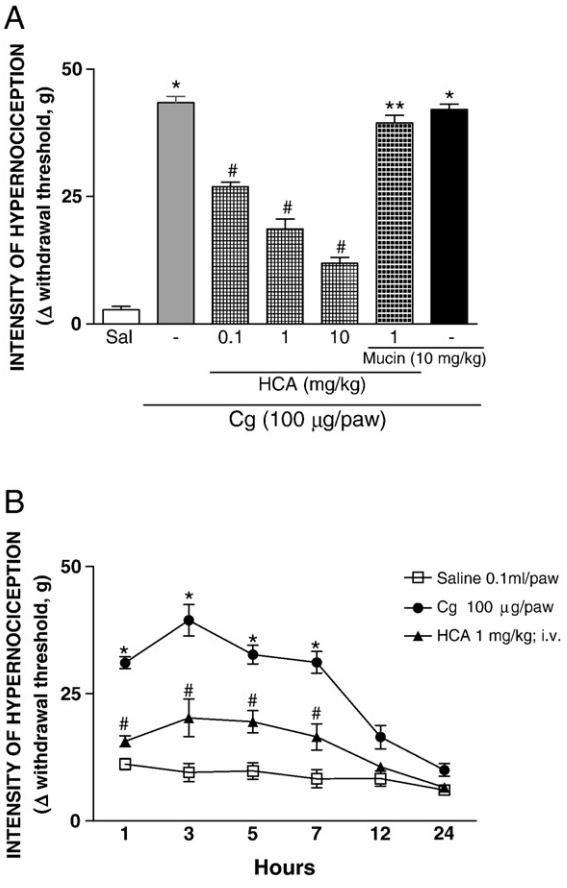

Fig. 1. Effect of HCA on hypernociception induced by carrageenan (Cg). (A) Rats were pretreated 15 min before Cg with i.v. HCA (0.1, 1 and 10 mg/kg) or mucin (10 mg/kg) or HCA (1 mg/kg) combined with mucin or saline (0.1 ml). Hypernociception was assessed 3 h after Cg injection. (B) Animals were treated 15 min before Cg with i.v. HCA (1 mg/kg). Hypernociception was measured at 1, 3, 5, 7, 12 and 24 h after Cg or saline (negative control) i.pl. injections into the hind paws. Results are shown as the mean ± S.E.M. (n = 5). * $p<0.05$ when compared with saline (Sal) group. # $p<0.05$ when compared with the Cg group. **$p<0.05$ when compared with HCA at a dose of 1 mg/kg. ANOVA followed by Bonferroni's test.

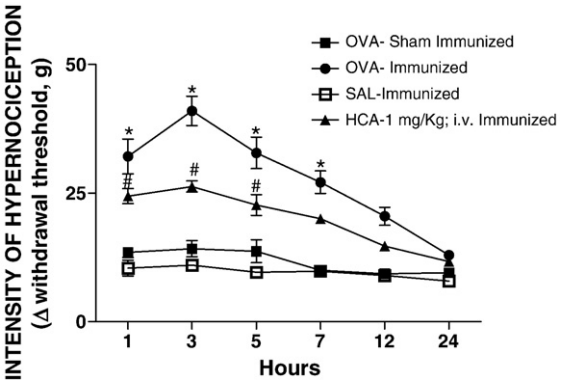

Fig. 2. The role of HCA in OVA-induced mechanical inflammatory hypernociception. Rats were pretreated 15 min before ovalbumin (OVA; 100 μg/paw) with i.v. HCA (1 mg/kg). Control rats were injected with saline (0.1 ml/paw; SAL-Immunized) and OVA (100 μg/paw; OVA-Immunized). Mechanical hypersensitivity was evaluated at 1, 2, 5, 7, 12 and 24 h after intraplantar injection of OVA in rats at a dose of 100 μg/paw. Results are shown as the mean ± S.E.M. (n = 5) *p<0.05 when compared with the OVA-Sham Immunized and SAL-Immunized groups. #p<0.05 when compared with the OVA-Immunized group. ANOVA followed by Bonferroni's test.

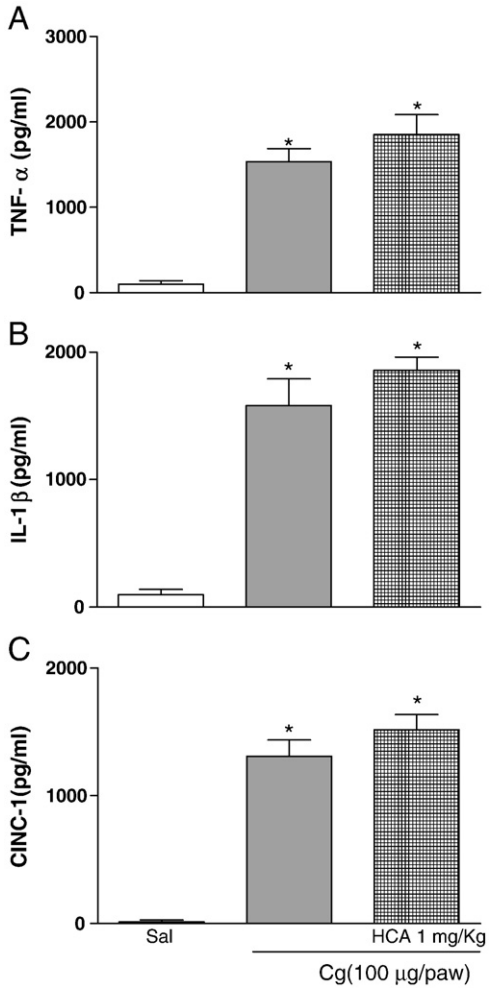

Fig. 3. Effect of HCA on concentrations of TNF-α, IL-1β and CINC-1 in Cg-induced hypernociception. Rats were pretreated 15 min before Cg with i.v. HCA (1 mg/kg). Cytokines were assessed 3 h after Cg injection. Results are shown as the mean ± S.E.M. (n = 5). *p<0.05 when compared with the saline (Sal) group. ANOVA followed by Bonferroni's test.

24 h later, when compared with saline-injected rats. Pretreatment with HCA (1 mg/kg) decreased OVA-induced mechanical inflammatory hypernociception by 24%, 36% and 31% in immunized animals at 1, 3 and 5 h, respectively, compared to OVA-immunized rats (Fig. 2).

3.2. HCA does not inhibit the production of cytokines

Intraplantar injection of Cg induced a significant increase in the production of TNF-α, IL-1β, CINC-1 in the paw skin of rats when compared to saline-injected rats. The administration of HCA (1 mg/kg) 15 min before Cg injection did not inhibit the release of TNF-α, IL-1β and CINC-1 into paw exudate at 3 h after Cg injection, when compared to vehicle-treated rats (Fig. 3).

3.3. Effect of HCA on levels of myeloperoxidase

HCA (1 mg/kg) given 15 min before the i.pl. injection of Cg (100 μg/paw) or OVA (100 μg/paw) into immunized rats reduced neutrophil accumulation assessed by measuring paw tissue myeloperoxidase activity at 3 h after stimulus (Fig. 4A and B). This reduction was approximately 58% in both experiments.

3.4. HCA inhibits mechanical hypernociception induced by prostaglandin E_2

The injection of prostaglandin E_2 (PGE_2) into the hind paw of rats evoked a significant hypernociceptive effect measured 3 h after injection. Pretreatment 15 min before PGE2 injection with HCA

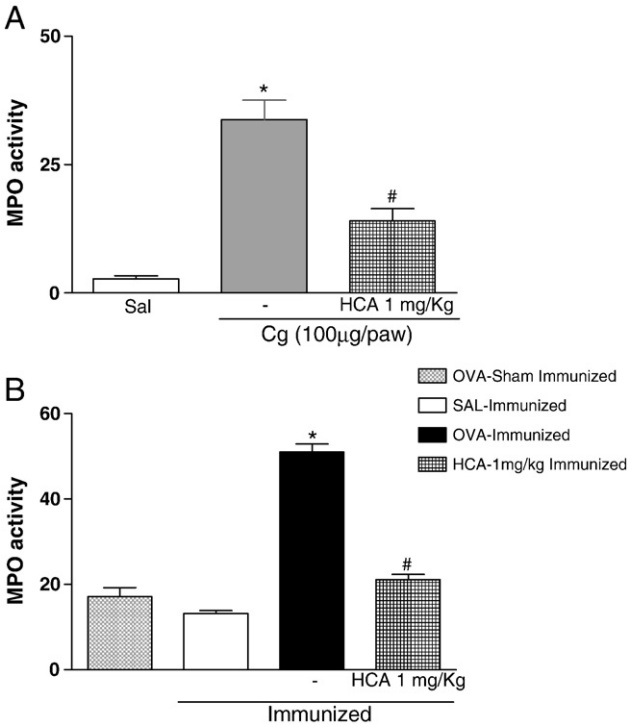

Fig. 4. Effect of pretreatment with HCA lectin on the influx of neutrophils into the hind paw of rats after Cg (A) or OVA (B) administration. (A) Saline (−) or HCA (1 mg/kg) was injected i.v. and, 15 min later, Cg (100 μg/paw) was injected into the hind paws. Saline (Sal) was injected into the hind paw (0.1 ml). (B) Rats were pretreated 15 min before ovalbumin (OVA; 100 μg/paw) with i.v. HCA (1 mg/kg). Control rats were injected with saline (0.1 ml/paw; SAL-Immunized) and OVA (100 μg/paw; OVA-Immunized). Myeloperoxidase activity in the hind paw was used as an index of neutrophil influx, and it was measured 3 h after Cg or OVA. Results are shown as the mean ± S.E.M. (n = 5) *p<0.05 when compared with the control groups. #p<0.05 when compared with the Cg or OVA-Immunized groups. ANOVA followed by Bonferroni's t test.

(1 mg/kg; i.v.) inhibited hypernociception caused by PGE_2 (Fig. 5). This inhibition was approximately 40% when compared to the PGE_2 group.

3.5. Anti-hypernociceptive effect of HCA is mediated by increase in nitric oxide production

The role of nitric oxide (NO) in the antinociceptive role of HCA was assessed by treating the animals with L-NAME, a nonselective nitric oxide synthase inhibitor, and aminoguanidine, a selective inducible nitric oxide inhibitor. It was observed that L-NAME and Amino prevented the antinociceptive effects of HCA in Cg-induced hypernociception (Fig. 6A). Corroborating this finding, HCA (1 mg/kg), when administered without the injection of Cg, significantly induced NO production (determined in serum) compared with animals pretreated with sterile saline. Furthermore, the increase in NO levels induced by HCA was confirmed when the lectin was administered with the phlogistic agents (Cg) compared to animals that received only intraplantar Cg (Fig. 6B).

4. Discussion

In the present study, we demonstrated an anti-hypernociceptive effect of the lectin isolated from the red marine alga *Hypnea cervicornis* in different models of inflammatory hypernociception. The lectin was able to inhibit inflammatory hypernociception induced by carrageenan, antigen and also the direct-acting mediator PGE_2. This anti-hypernociceptive effect of the lectin was not associated with the inhibition of pro-inflammatory cytokine production, but seemed to involve the inhibition of neutrophil migration and also the direct blockade of hypernociception through an increase in nitric oxide production.

The inhibitory activity of HCA was completely prevented when combined with i.v. injection of mucin (carbohydrate-binding). These data suggest that carbohydrate-binding sites on the HCA molecule are important for its anti-hypernociceptive action. Our group previously demonstrated anti-inflammatory effects for various lectins, each involving different carbohydrate determinants in leukocyte recruitment (Alencar et al., 2005, 1999; Assreuy et al., 1997, 1999; Mota et al., 2006; Neves et al., 2007; Santi-Gadelha et al., 2006). Mucin-like glycoproteins seem to play a key role in leukocyte–endothelium interaction, probably due to the presence of carbohydrate-binding selectins (Ley, 2002; Panés et al., 1999; Tedder et al., 1995).

Under the experimental conditions used in the present study, we previously demonstrated that carrageenan or antigen (OVA immunization in rats) induces mechanical hypernociception in rats, activating

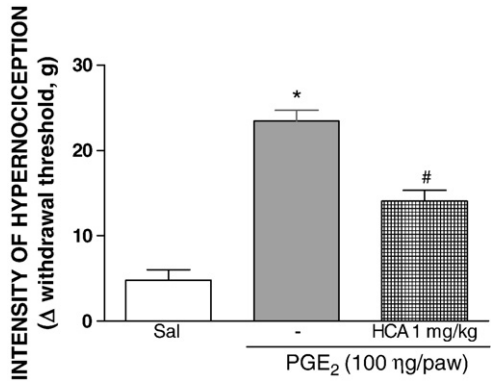

Fig. 5. Effect of HCA on hypernociception induced by prostaglandin E_2 (PGE_2). Rats were pretreated 15 min before PGE_2 with i.v. HCA (1 mg/kg). Hypernociception was assessed 3 h after PGE_2 injection. Results are shown as the mean ± S.E.M. (n = 5) *p<0.05 when compared with the saline (Sal) group. #p<0.05 when compared with the PGE_2 group. ANOVA followed by Bonferroni's test.

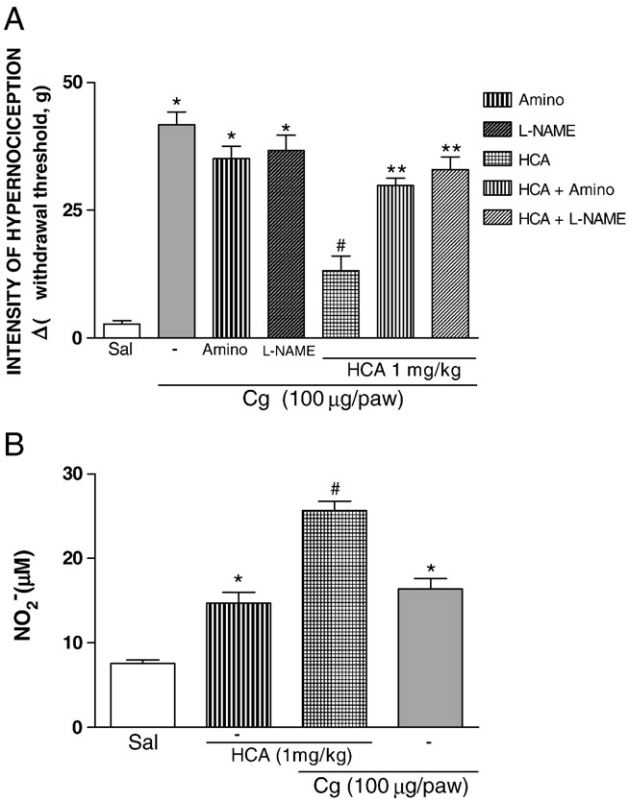

Fig. 6. Anti-hypernociceptive effect of HCA is mediated by increase in nitric oxide production: NOS inhibitors prevented this effect. (A) The antinociceptive effect of HCA (1 mg/kg; i.v.) was determined in animals pretreated with L-NAME (50 mg/kg s.c.) and aminoguanidine (50 mg/kg; s.c.; Amino). Hypernociception was assessed 3 h after Cg injection. (B) The level of NO was determined in animals, pretreated with HCA (1 mg/kg; i.v.) or sterile saline, that did or did not receive Cg. Results are shown as the mean ± S.E.M. (n = 5). *p<0.05 when compared with the saline (Sal) group. #p<0.05 when compared with the Cg group. **p<0.05 when compared with the HCA group. ANOVA followed by Bonferroni's test.

a sequential cytokine cascade. This cascade begins with TNF-α which stimulates two distinct pathways: a) IL-1β which in turn activates cyclooxygenase to produce prostanoids and b) CXCL1/CINC-1 production which stimulates the release of sympathetic amines (Verri et al., 2006). Prostanoids and sympathetic amines are ultimately responsible for nociceptor sensitization (Cunha et al., 2005). Therefore, drugs that inhibit cytokine production, such as corticosteroids, thalidomide (Ribeiro et al., 2000), pentoxifylline (Vale et al., 2004) and natural products (e.g., sequiterpene lactones, Valerio et al., 2009) are antinociceptive in inflammation models. Taking these findings into account, we examined whether the antinociceptive effect of HCA depends on inhibition of cytokine production. However, in these experimental models, HCA treatment, at a dose that inhibited mechanical hypernociception, did not alter Cg-induced cytokine production (TNF-α, IL-1β and CINC-1) in the paw skin tissue, suggesting that, at the dose tested, cytokine modulation is not involved in the mechanism of action of HCA's antinociceptive effect.

While important to the genesis of inflammatory hypernociception by triggering the production of direct-acting mediators (prostaglandins and sympathetic amines), the hypernociceptive activity of cytokines is also largely dependent on neutrophil recruitment (Cunha et al., 2008a; Levine and Gordon, 1984; Ting et al., 2008). Since we had previously demonstrated that HCA and other lectins show an inhibitory effect on neutrophil migration, we examined whether the inhibition of neutrophil migration could be a target for HCA antinociceptive action. In support of this hypothesis, HCA almost completely blocked Cg- and OVA-induced neutrophil migration, as determined by the reduced myeloperoxidase activity in the paw tissues, suggesting that HCA-

induced antinociception probably depends on inhibition of neutrophil migration.

A great number of *in vivo* studies have shown that peripherally injected PGE_2 produces hyperalgesia and allodynia, both in experimental animals and in humans (Ferreira, 1972; Kuhn and Willis, 1973). This nociceptive effect seems to be related to the ability of PGE_2 to sensitize peripheral terminals of small diameter and high threshold, including primary afferent fibers sensitive to thermal, chemical and mechanical stimuli (Kumazawa et al., 1993; Mizumura et al., 1993; Schaible and Schmidt, 1988). In fact, PGE_2 acts directly on receptors present on nociceptor membranes, triggering their sensitization (Cunha et al., 1992). Therefore, hypernociception elicited by PGE_2 is independent of the production of other inflammatory mediators or recruitment of cells such as neutrophils (Cunha et al., 2008a,c). The fact that HCA treatment also inhibited PGE_2-induced hypernociception, implies that HCA directly reduces nociceptor sensitization or that HCA can even induce an endogenous mediator through this action. It is important to mention that analgesics that act by inhibiting COX (i.e., NSAID drugs), cytokine production (e.g., thalidomide) or neutrophil migration (e.g., fucuidin) are not able to affect PGE_2-induced hypernociception (Cunha et al., 2008c; Ribeiro et al., 2000).

One possible endogenous mediator involved in HCA's action is nitric oxide. Indeed, the direct blockade of ongoing hypernociception induced by PGE_2 can be achieved by the use of peripheral opioids, dipyrone, diclofenac and other substances that activate the L-arginine/nitric oxide antinociceptive pathway (Duarte et al., 1992; Ferreira, 1993; Ferreira et al., 1991). Furthermore, there are studies in the literature showing that lectins such as *concanavalin A* can induce NO production *in vivo* and *in vitro* (Kesherwani and Sodhi, 2007; Sodhi et al., 2007). For instance, Lima et al. (2004) showed that the lectin of the red marine alga *Bryothamnion triquetrum* induces endothelium-dependent relaxation of the rat aorta via nitric oxide release, and Gadelha et al. (2005) demonstrated that the lectin of the marine alga *Canavalia maritime* was able to exert a concentration-dependent relaxant action in isolated aortic rings, resulting in a release of nitric oxide. Here, we observed that the antinociceptive effect of HCA on Cg-induced hypernociception is prevented by treatment with nitric oxide synthase inhibitors, suggesting that nitric oxide may participate in the HCA effect. Supporting this notion, rats treated only with HCA or HCA and the intraplantar injection of the phlogist agent carrageenan showed an increase in the level of nitric oxide metabolites. Nitric oxide reduces hyperalgesia due to the activation of the arginine/NO/cGMP pathway, causing direct blockade of acute and persistent hypernociception by opening $K^+_{(ATP)}$ channels via the stimulation of protein kinase G. This mechanism has been demonstrated for analgesics, where morphine and dypirone are able to activate this pathway (Sachs et al., 2004).

At this stage, it is important to mention that the inhibitory effect of HCA on neutrophil migration could also be mediated by the induction of nitric oxide production. Actually, there is a great body of evidence showing that pharmacological inhibition of NO synthesis or genetic deletion of NO synthases enhances leukocyte–endothelial cell interaction in various organs and tissues during the inflammatory process (Akimitsu et al., 1995; Dal-Secco et al., 2003, 2006; Hickey, 2001). Furthermore, the administration of nitric oxide donors reduces leukocyte infiltration in different models of inflammation (Al-Swayeh et al., 2000; Keeble and Moore, 2002).

In summary, this study is the first to demonstrate that HCA exhibits a potent antinociceptive effect on mechanical inflammatory hypernociception evoked by Cg, PGE_2 and OVA. Furthermore, we propose that the antinociceptive mechanisms of HCA may be associated with the inhibition of neutrophil migration and NO production.

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