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Anti-inflammatory activity of the non-peptidyl low molecular weight radical scavenger IAC in carrageenan-induced oedema in rats

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Abstract

Objective In this research we investigated the anti-inflammatory activity of a non-peptidyl low molecular weight radical scavenger (IAC) in an acute and chronic animal model of inflammation.

Methods For this purpose the effect of IAC (10, 25, 50 mg/kg) was tested in rats on the associated behavioral responses to subsequent inflammatory and noxious challenges, such as hind paw oedema induced by intra-plantar injection of carrageenan and granuloma induced by subcutaneous implant of a cotton pellet, using indometacin (2.5 mg/kg) as reference drug. Moreover, the serum level of several cytokines was tested in the animal treated (or not) with IAC (50 mg/kg) both in the absence and presence of carrageenan-induced inflammation.

Key findings IAC showed a significant anti-inflammatory activity in both in acute and chronic models of inflammation. In addition IAC down regulated significantly the serum levels of interleukin (IL) 2 and IL6 whereas it increased the serum concentration of IL1α and glutathione.

Conclusion Although it remains to be elucidated whether or not the antioxidant property of IAC is directly responsible for the modulation of the tested cytokines, these results suggest IAC to be a possible candidate for a novel anti-inflammatory compound

Keywords carrageenan; cytokines; IAC; inflammation; interleukins

Introduction

The bis (1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)-decandioate, IAC, is a new non-peptidyl low molecular weight radical scavenger able to give a fast reaction with the majority of radical species involved in the oxidative stress.[1] This compound has been reported to cross cell membranes and to distribute in the biological environment and to exert a protective effect *in vitro* and *in vivo*.[2–5] Of course, its intrinsic property might be of particular interest in any process, such as inflammation, where it is present as an over-production of reactive oxygen species (ROS). Indeed, it is well known that the systemic inflammatory response is associated with the production of ROS, nitric oxide (NO), which in turn deplete endogenous glutathione (GSH), mediating cytotoxicity.[6–9] Upon injury or damage, an increase in microvascular permeability is an early event that leads to oedema formation during inflammation. After this change, many other mechanisms are activated, contributing to the amplification of the inflammatory response and tissue damage leading to the induction of hyperalgesia.[10] The factors that mediate these responses are mainly characterized by inflammatory lipid metabolites, ROS/NO and by cytokines, which are major determinants of the systemic responses to inflammation.[10–14] Cytokines, such as interleukins IL1, IL2 and IL6, are involved in extensive networks that account for synergistic as well as antagonistic interactions and exhibit both negative and positive regulatory effects on various target cells.[10,12,15–17] They are pleiotropic molecules that elicit their effects locally or systemically in an autocrine or paracrine manner.

Several natural or synthetic compounds acting on oxidative stress or on cytokine modulation, such as IL1, have been reported to exert an anti-inflammatory effect in a carrageenan-induced oedema animal model.[18–23] In a recent paper we clearly showed that IAC protects

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gastric mucosa in an animal model of indometacin-induced ulcer, through local increase of PGE_2 levels and its antioxidant properties.[24] In addition it has been demonstrated that IAC significantly decreased colonic damage and inflammation and tumour necrosis factor-α (TNF-α) levels in 2,4-dinitrobenzene sulphonic acid (DNBS)-colitis.[3,4]

It seems reasonable that a molecule that possesses the ability to regulate these cytokines and ROS without affecting prostaglandin regulation would be a good candidate as an anti-inflammatory agent.

The aim and the novelty of this research was to investigate the ability of IAC to exert anti-inflammatory activity in an animal model of acute carrageenan-induced inflammation or chronic cotton pellet-induced granuloma through cytokine modulation. For this purpose we measured the serum levels of pro-inflammatory cytokines IL-1α, IL-2, IL-4, IL-6 and GSH in rats submitted to intraplantar injection of carrageenan to induce paw oedema.

Methods

Animals

Male Sprague–Dawley rats (Harlan Laboratories, Udine, Italy), 180–200 g, were used in these experiments. They were housed two per cage and maintained at a temperature of 22 ± 1°C and humidity 65%, with a 12-h light–dark cycle. Pellet food and water were freely available. All procedures involving animals were performed in accordance with the Italian law (D.L. n. 116/1992) and European legislation (EEC n. 86/609). The experimental design received the approval of the Bioethical Committee of Italian Institute of Health.

Drug administration

IAC was kindly supplied by Medestea Research & Production S.p.A. (Torino, Italy). It was solubilized in saline and administered intraperitoneally at a dose of 2 ml/kg. Carrageenan, indometacin and phenylquinone were purchased from Sigma-Aldrich (Milan, Italy). Indometacin, dissolved in saline and sonicated for 30 min, was administered intraperitoneally at a dose of 2 ml/kg. Phenylquinone, dissolved in ethanol 5%, was intraperitoneally injected at a dose of 2 mg//2 ml/kg. Ketamine hydrochloride (Ketavet 100; Farmaceutici Gellini S.p.a., Peschiera Borromeo, Italy) diluted in saline (50%) was intraperitoneally administered at a dose of 2 ml/kg to anaesthetize rats for cotton pellet implant and removal.

Carrageenan-induced paw oedema

Paw inflammation was induced by injecting 0.1 ml of 1% (w/v) carrageenan in saline into the plantar region of the right hind-foot of rats, according to the method described by Winter *et al*.[25] Carrageenan injection was followed by the oral administration of water (5 ml per rat) to assure uniform hydration of the rats and to reduce the variability of oedema formation. Paw volume was measured using a plethysmometer (Ugo Basile, Milan, Italy) immediately after carrageenan injection (t = 0) and 2, 4, 6, 8 and 24 h later. The inflammatory response was given by the percentage increase in paw volume over time 0. Five groups of eight rats each were intraperitoneally administered with IAC dosed at 10, 25 and 50 mg/kg or indometacin, used as positive control, dosed at 2.5 mg/kg, or vehicle (saline solution), 30 min before carrageenan injection. The doses of IAC and indometacin were chosen taking in account previous results obtained by Vasina *et al*.[3] and by Zanoli *et al*.[26]

Cotton pellet-induced granuloma

The effects of IAC and indometacin on the proliferation phase of inflammation were studied by the cotton-pellet granuloma test, according to the method described by Schiatti *et al*.[27] Each rat received a subcutaneous implant of a cotton pellet, 10 ± 0.5 mg, in the dorsal region, under ketamine anaesthesia. IAC (25 and 50 mg/kg), indometacin (2.5 mg/kg) or vehicle (saline 2 ml/kg) were daily administered intraperitoneally to four groups of six rats each for seven consecutive days starting from the day of cotton pellet implantation. Rats were anaesthetized on the eighth day and cotton pellets were removed surgically and made free from extraneous tissues. The moist pellets were weighed and then dried at 60°C for 18 h; thereafter dried pellets were weighed again.

Cytokines assays

Sprague–Dawley male rats were treated with IAC (50 mg/kg) or indometacin (2.5 mg/kg) 30 min before the intraplantar injection of carrageenan. They were killed by decapitation 4 h after carrageenan treatment. Trunk blood was collected into centrifuge tubes and serum was prepared by centrifugation (3000 rev/min, 20 min, 4°C) and stored frozen until assayed.

Serum concentrations of IL-1α, IL-2, IL-4 and IL-6 were determined using enzyme-linked immune-sorbent assay (ELISA) kits (Biosource International California, Camarillo, CA, USA) following the manufacturer's instruction. Briefly, 100 μl of standard recombinant rat cytokine or 50 μl of standard diluent buffer and 50 μl sample were added to each well on the plates coated with corresponding capture antibodies and incubated for 2 h at 37°C. After that the plates were washed and 50 μl of rat IL1-α, IL-2 or IL-4 biotin conjugates or 100 μl IL-6 biotin conjugate was added to each well and incubated for 1 or 2 h at room temperature then washed four times. Finally 100 μl streptavidin-HRP was added to each well at room temperature. After 30 or 45 min, the plates were washed and 100 μl of the stabilized chromogen added. The reaction was terminated with 100 μl stop solution and the optical density measured at 450 nm with a microplate reader (Multiskan MCC/340; Lab System, Helsinski, Finland).

Glutathione assay

Total GSH was assessed in rat serum samples using the Glutathione Assay Kit (Sigma, St Louis, MO, USA). Briefly, samples were first de-proteinized with 5% 5-sulfosalicylic acid solution, centrifuged and then assayed for GSH. The procedure is based on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by GSH to 5-thio-2-nitrobenzoic acid (TNB) and the glutathione disulfide (GSSG) formed is recycled by glutathione reductase and NAPPH. The yellow product, TNB, was measured spectrophotometrically at 412 nm.

Statistical analysis

Data were expressed as mean ± SEM. The biochemical assays and quantitative measurements were performed in

duplicate for each experiment, which was repeated at least three times to ensure reproducibility. Comparison of multiple means was performed with one-way analysis of variance with Bonferroni's and Dunnett's post-hoc test or Fischer's exact test, as indicated in the figures (Graph-Pad 5 Software Inc, San Diego, CA, USA). *P*-values <0.05 were considered significant.

Results

Carrageenan-induced paw oedema

The effect of IAC and the reference drug, indometacin, on paw oedema induced by 1% w/v carrageenan is shown in Figure 1. The intraperitoneal administration of IAC at a dose of 10 mg/kg, 30 min before carrageenan, significantly ($P < 0.05$) reduced the oedema at 4 h after carrageenan injection. At higher doses (25 and 50 mg/kg) IAC was able to significantly ($P < 0.01$) reduce the oedema at 2, 4, 6 and 8 h after the carrageenan injection. At all tested doses the anti-inflammatory effect of IAC still persisted at 24 h after the carrageenan injection. The oedema was strongly inhibited by the intraperitoneal pre-treatment with indometacin (2.5 mg/kg) ($P < 0.01$ vs vehicle group).

Cotton pellet-induced granuloma

IAC at both dosages (25 and 50 mg/kg), as well as indometacin (2.5 mg/kg), inhibited the granuloma formation surrounding the pellets compared with the vehicle-treated group (Table 1). In all treatment groups the weight of both the moist and the dried cotton pellet were significantly reduced ($P < 0.01$) in comparison with the vehicle-treated group.

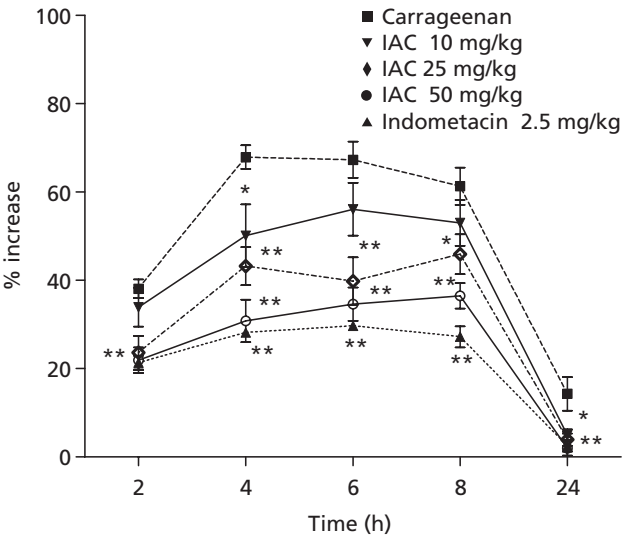

Figure 1 Influence of IAC and the reference drug indometacin on the inflammatory response to carrageenan. IAC dosed at 10, 25 and 50 mg/kg and indometacin dosed at 2.5 mg/kg were intraperitoneally administered 30 min before intraplantar carrageenan injection; The paw volume was recorded at time 0, immediately after carrageenan injection and 2, 4, 6, 8 and 24 h later. The percentage of increase over the 0 time volume was calculated. Each value is the mean ± SEM ($n = 8$ rats/group). *$P < 0.05$, **$P < 0.01$ vs vehicle-treated group (one-way analysis of variance followed by Dunnett's post test)

Serum cytokine modulation

The important activity elicited by IAC in the carrageenan animal model described above, led us to investigate whether the compound was able to modulate cytokines serum level in the acute model of inflammation. Four hours following the treatments, the screening performed on the major cytokines involved in inflammatory processes outlined a scenario where IL-1α, IL-2 and IL-6 play a major role. Indeed, as shown in Figure 2a, carrageenan injection (1% w/v) significantly reduced ($P < 0.05$) IL-1α serum level (122.6 ± 14 pg/ml, $n = 8$) compared with the cytokine concentration measured at the same time in the serum of vehicle-treated rats (151.5 ± 17 pg/ml, $n = 8$).

Indometacin (2.5 mg/kg) was able to modulate the IL-1α values in the same way, down-regulating dramatically the concentration of this cytokine compared with all other treated groups (77.5 ± 11 pg/ml, $n = 8$, $P < 0.001$). Surprisingly, as clearly shown in Figure 2a, IAC (50 mg/kg) was able to increase significantly the IL-1α level in comparison with the carrageenan ($P < 0.01$) and indometacin ($P < 0.001$) treated groups, bringing the value close to that of the control group (163.9 ± 8 pg/ml; $n = 8$). A different effect was elicited by the inflammatory insult on the IL-2 concentration. The injection of carrageenan (1% w/v) into the hind paw induced a significant elevation ($P < 0.001$) of the IL-2 serum level, compared with that found in the vehicle group (i.e. carrageenan: 1044 ± 19 mg/kg, $n = 8$; vehicle: 26.6 ± 8 pg/ml $n = 8$). Both IAC and indometacin strongly and significantly ($P < 0.001$) reduced the IL-2 serum level in comparison with the carrageenan group, bringing it to 176.7 ± 20 pg/ml ($n = 8$) and 204.6 ± 94 pg/ml ($n = 8$), respectively (Figure 2b). The injection of carrageenan (1% w/v) into the hind paw significantly ($P < 0.001$) increased serum IL-6 concentration to 112.6 ± 21 pg/ml ($n = 8$) in comparison with the vehicle-treated rats, in which it reached only 14 ± 3 pg/ml ($n = 8$). In contrast, IAC (50 mg/kg) and indometacin (2.5 mg/kg) were able to restore the level close to that of the control, decreasing significantly ($P < 0.001$) the IL6 values in comparison with those following carrageenan injection to

Table 1 Effect of IAC on cotton pellet-induced granuloma in rats

Treatment (mg/kg)	Weight of cotton pellet (mg)	
	Moist	Dried
Vehicle	150.5 ± 13.6	23.0 ± 2.1
Indometacin 2.5	79.1 ± 7.1**	11.9 ± 0.9**
IAC 25	87.5 ± 8.3**	11.6 ± 1.4**
IAC 50	83.8 ± 5.6**	11.4 ± 1.5**

IAC dosed at 25 and 50 mg/kg and indometacin (INN) dosed at 2.5 mg/kg were daily administered for seven days, starting from the day of cotton pellet implantation. On the eighth day rats were anaesthetized and the pellets were removed and weighed. Values are the mean ± SEM ($n = 6$ rats/group). **$P < 0.01$ vs vehicle-treated group (analysis of variance followed by Dunnett's post test)

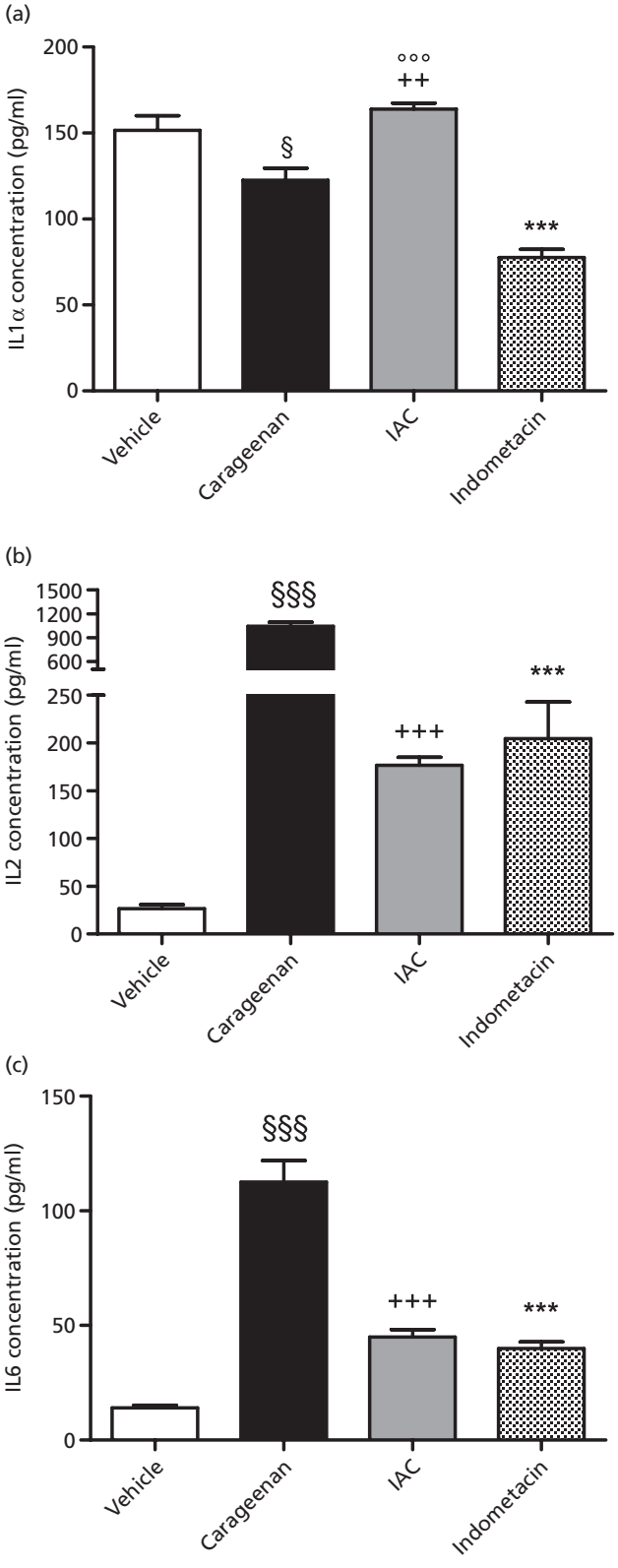

Figure 2 Effect of IAC and indometacin on cytokine serum level in carrageenan-induced hind paw oedema in rats. Serum concentration of IL-1α (a), IL-2 (b) and IL-6 (c) were evaluated 4 h after 1% w/v carrageenan or saline solution injection (vehicle) pre-treatment (30 min before carrageenan injection) (or not) with IAC 50 mg/kg (i.p) or indometacin 2.5 mg/kg (i.p.). Each column represents the mean ± SEM of eight rats. IL-1α: §$P < 0.05$ carrageenan vs vehicle; $^{++}P < 0.01$ IAC vs carrageenan; $^{\circ\circ\circ}P < 0.001$ IAC vs indometacin and $^{***}P < 0.001$ indometacin vs carrageenan; IL-2: §§§ $P < 0.001$ carrageenan vs vehicle; $^{+++}$ $P < 0.001$ IAC vs carrageenan and *** $P < 0.001$ indometacin vs carrageenan; IL-6: §§§ $P < 0.001$ carrageenan vs vehicle; $^{+++}$ $P < 0.001$ IAC vs carrageenan and *** $P < 0.001$ indometacin vs carrageenan (one-way analysis of variance followed by Bonferroni's post test)

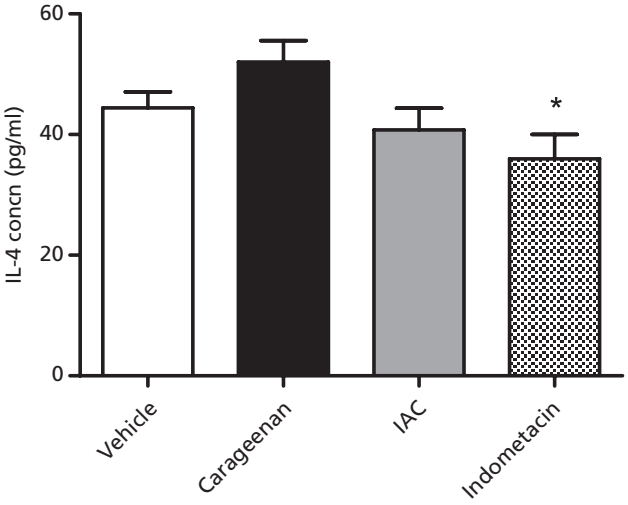

Figure 3 Effect of IAC and indometacin on IL-4 serum concentration in rats. Level of IL-4 were evaluated 4 h after 1% w/v carrageenan or saline solution (vehicle) injection pre-treatment (30 min before carrageenan injection) (or not) with IAC 50 mg/kg (i.p.) or indometacin 2.5 mg/kg (i.p.). Each column represents the mean ± SEM of eight rats. *$P < 0.05$ indometacin vs carrageenan (one-way analysis of variance followed by Bonferroni's post test)

44.9 ± 8.31 pg/ml ($n = 8$) and 39.9 ± 7.43 pg/ml ($n = 8$), respectively (Figure 2c). Interestingly, compared with saline injection, carrageenan (1% w/v) with or without IAC (50 mg/kg) pre-treatment, did not produce significant modulation in IL-4 serum level concentration (Figure 3). However, the pre-treatment with indometacin significantly reduced ($P < 0.05$) the IL-4 serum level (35.9 ± 9 pg/ml, $n = 8$) when compared with the carrageenan-treated group (52 ± 8 pg/ml, $n = 8$).

IAC *per se* did not alter significantly the pattern of interleukins tested (data not shown).

Modulation of glutathione levels

At 4 h after intraplantar injection of carrageenan (1% w/v), serum GSH level was significantly decreased ($P < 0.05$) compared with vehicle group. The pre-treatment with IAC 50 mg/kg and indometacin 2.5 mg/kg (i.p.) significantly increased ($P < 0.001$) serum GSH content compared with 1% w/v carrageenan-injected rats. Moreover, as clearly shown in Figure 4, the antioxidant compound IAC elicited a significant rise in GSH serum concentration in comparison with the indometacin-treated group ($P < 0.001$).

Discussion

Bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)-decandioate has been reported to exert anti-inflammatory properties in a

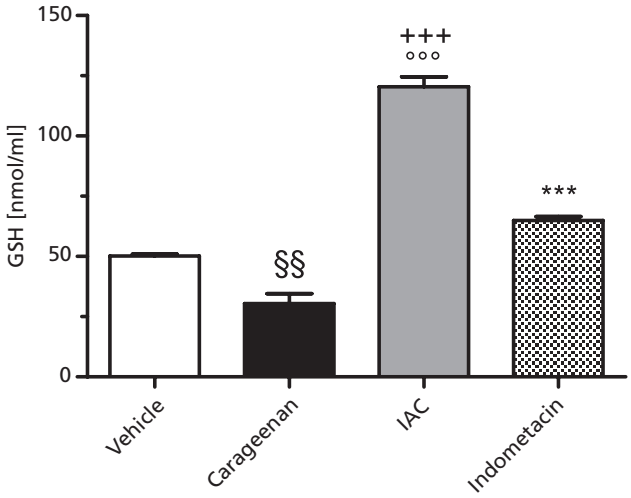

Figure 4 Effect IAC and indometacin on serum glutathione content in carrageenan-induced rat hind paw oedema. Both IAC (50 mg/kg) and indometacin (2.5 mg/kg) were administered intraperitoneally, 30 min before intraplantar carrageenan treatment (1% w/v). The concentration of GSH was assessed 4 h after carrageenan treatment. Each point represents the mean ± SEM of eight rats. §§$P < 0.01$ carrageenan vs Vehicle group; $^{+++}P < 0.001$ IAC vs carrageenan, $^{\circ\circ\circ}P < 0.001$ IAC vs indometacin; ***$P < 0.001$ indometacin vs carrageenan (one-way analysis of variance followed by Bonferroni's post test)

broad spectrum of inflammatory diseases.[2,3,28] Most of the authors ascribed this effect mainly to the antioxidant property of the compound and at present there is no evidence regarding a direct involvement of IAC in pro-inflammatory interleukin modulation. Recently we reported a significant anti-ulcer effect of a non-peptidyl low molecular weight radical scavenger, bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)-decandioate, in an indometacin-induced gastric ulcer model. This finding prompted us to evaluate the potential anti-inflammatory property of IAC. To this aim we tested the effect of IAC on the associated behavioral responses to subsequent inflammatory and noxious challenges, such as carrageenan-induced hind paw oedema and cotton pellet granuloma. In addition, we tested the effect of the compound on serum cytokines and GSH concentrations in an acute model of inflammation. The results obtained clearly showed a significant anti-inflammatory property of IAC both in acute and chronic models of inflammation. In particular the activity of the compound on carrageenan-induced paw oedema was already present 2 h after carrageenan treatment and lasted for 24 h. Interestingly, the effect of IAC on the serum level of the pro-inflammatory cytokines IL-2 and IL-6 was quite similar to that of the reference drug indometacin, although this effect was reached with much higher doses. Indeed both interleukins were strongly inhibited by IAC 4 h after carrageenan treatment. Instead, on IL-1α serum level, IAC exerted an opposite effect to that of the reference drug, increasing significantly the amount of IL1α. While it seems reasonable that indometacin, as an anti-inflammatory drug acting on PGE_2, was able to reduce all the measured cytokines, generally involved in the inflammatory processes,[18–31] apparently the up-regulation of IL1α mediated by IAC could be considered a pro-inflammatory effect. This is a very interesting issue that requires more experiments and investigation, although Alebouyeh *et al*.[32] reported an important role elicited by IL-1α in the anti-inflammatory effect of morphine in carrageenan-induced paw oedema. The evidence that IAC was not able to modulate significantly the serum level of IL-4, a cytokine which is mostly expressed during allergies and not in this model of inflammation might suggest that the compound could act selectively on interleukin modulation, probably through the modulation of ROS but certainly not through PGE_2 inhibition. Indeed, indometacin, which is known to decrease PGE_2 levels and therefore to inhibit a number of cytokines in PGE_2-related fashion,[33] reduced significantly the value of IL-4 compared with carrageenan treatment alone.

It is well known that GSH, ROS and eNOS-derived NO, play a critical role in acute inflammation and in particular in the early changes of vascular permeability.[34] Previous reports describe IAC as a free radical scavenger, protecting against inflammation through its intrinsic antioxidant properties in both *in vivo* and *in vitro* models.[3–5,28] Our results regarding the ability of this compound to increase significantly the serum GSH levels compared with the carrageenan-treated groups, suggest that IAC might provide protection from inflammation through its antioxidant properties, indicating a clear activity of the molecule in the early stages of inflammation.

In conclusion, our research clearly demonstrated that this new molecule possesses a wide spectrum of anti-inflammatory activity both in acute and chronic models of inflammation tested. In addition, in the present model of acute inflammation we provided evidence, for the first time, that IAC was able to modulate pro-inflammatory cytokines. Although further experiments are necessary to determine the exact role of IAC in the modulation of pro-inflammatory interleukins and in particular whether its antioxidant properties could be partially or completely responsible for such activity, these results suggest its potential as a novel, promising, anti-inflammatory compound.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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