Antiinflammatory and Antihyperalgesic Activity of C-Phycocyanin

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BACKGROUND: C-phycocyanin (C-PC), a biliprotein found in blue green algae, such as *Spirulina platensis*, is often used as a dietary nutritional supplement due to its various therapeutic values. In addition, the antiinflammatory activity of C-PC partly through inhibition of proinflammatory cytokine formation, inducible nitric oxide synthase (iNOS) and cyclooxygeanase-2 (COX-2) expression has been demonstrated in many *in vitro* and *in vivo* studies. However, whether C-PC also has antihyperalgesic activity in inflammatory nociception has not been investigated.
METHODS: Using a carrageenan-induced thermal hyperalgesia model, we evaluated the effect of C-PC on nociception by measuring paw withdrawal latency. To clarify the mechanisms involved, the expression of iNOS and COX-2 and the formation of nitrate and tumor necrosis factor-α (TNF-α) in the rat paw were determined.
RESULTS: Pre- or posttreatment with C-PC (30 or 50 mg/kg, IP) significantly attenuated carrageenan-induced inflammatory nociception and the induction of iNOS and COX-2 at the late phase, (4 h) accompanied by an inhibition of the formation of TNF-α, prostaglandin E_2, nitrate and myeloperoxidase activity.
CONCLUSIONS: Based on these results, it is suggested that the inhibition of NO and prostaglandin E_2 over-production through suppressing iNOS and COX-2 induction and attenuation of TNF-α formation and neutrophil infiltration into inflammatory sites by C-PC may contribute, at least in part, to its antihyperalgesic activity.
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It has been a well accepted concept that all pain, whether acute or chronic, peripheral or central, originates from inflammation and the inflammatory response.[1] The carrageenan-induced inflammatory response in the rat hindpaw, acting as an acute model of inflammation, has been widely used for screening novel antiinflammatory drugs.[2] The mechanisms of carrageenan-induced inflammatory nociception involve a complex chain of events, in which various mediators, including histamine, serotonin, proinflammatory cytokines, nitric oxide (NO), cyclooxygenase (COX)-derived products and sympathomimetic amines, are released.[3] In addition, it is proposed that carrageenan-induced hyperalgesia is associated with a cascade of cytokine release. The first cytokine released is tumor necrosis factor-α (TNF-α), which triggers the release of interleukin (IL)-1β, IL-6, synthesis of prostaglandins and sympathetic amines, suggesting that TNF-α plays an early and crucial role for the subsequent inflammatory responses.[4] This concept is confirmed by the fact that injection of antiserum neutralizing endogenous TNF-α markedly abolishes the response to carrageenan.[4] Furthermore, over-production of inflammatory prostaglandins, such as prostaglandin E_2 (PGE_2) and NO, mainly derived from COX-2 and inducible NO synthase (iNOS) respectively, are also critical mediators accounting for the pathogenesis of inflammatory nociception.[5–8] Another pathogenic mediator, reactive oxygen species (ROS), has been reported to play an important role in the maintenance of carrageenan-induced paw edema.[9]

C-phycocyanin (C-PC), a biliprotein found in blue green algae, such as *Spirulina platensis*, is used in many countries as a dietary supplement due to its various beneficial activities, including hepatoprotective, antiaggregatory, neuroprotective, and ROS-scavenging actions observed in various experimental models.[10–12] In addition, the antiinflammatory activity of C-PC, including attenuation of lipopolysaccharide-induced iNOS expression and TNF-α formation through suppressing nuclear factor-κB activation in RAW 264.7 macrophages, and inhibition of COX-2 activity and leukotriene B_4 formation, has been demonstrated *in vitro*.[13–15] Based on the importance of inflammation in nociception and its antiinflammatory characteristics, we propose that C-PC may also

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exert antihyperalgesic activity against inflammatory nociception through inhibition of these proinflammatory mediators. Although several protective effects of C-PC have been reported, there is no information on carrageenan-evoked inflammatory nociception. In the present study, we first demonstrated that C-PC exhibits antihyperalgesic activity in carrageenan-evoked thermal hyperalgesia and that the inhibition of TNF-α, NO, and PGE_2 formation may be involved.

METHODS

Animals

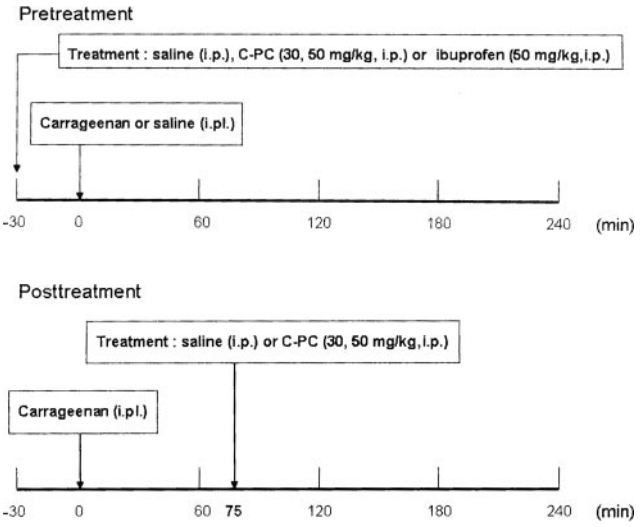
Male Sprague-Dawley rats (200–250 g) purchased from the National Animal Center (Taipei, Taiwan) were used in this study, which was approved by the local institutional animal care and use committee. Animals were housed in standard environment and maintained on tap water and rat chow (Rodent Diet 5010, LabDiet) *ad libitum* throughout the investigation.

Carrageenan-Evoked Thermal Hyperalgesia

This test was performed as described in our previous study.[16] First, rats were allowed 30 min to acclimatize to the device before testing. Acute inflammation was then produced by the subplantar (i.pl.) administration of 100 μL of 2% (w/v) λ-carrageenan (Sigma, St. Louis, MO) dissolved in normal saline into the right hindpaw of each rat. The hyperalgesia was assessed by placing the hindpaw above a radiant heat source and measuring paw withdrawal latency to evaluate thermal hyperalgesia every 60 min for 240 min after injection of carrageenan with a commercially available device (7370 Plantar Test, UGO Basile, Comerio, Italy). Data were calculated as a mean of three repeated measurements.

Experimental Design

In additional groups, rats were treated with either normal saline (0.2 mL, IP), C-PC (30 or 50 mg/kg, IP, C-PC with a purity of A_{620}/A_{280} >3.5, Sigma, St. Louis, MO) or ibuprofen (50 mg/kg, IP, Sigma, St. Louis, MO) at 30 min before or 75 min after the injection of carrageenan. The experimental design scheme is shown below. After injection of carrageenan for 4 h, the paw exudates and paw tissues were collected to measure the levels of PGE_2, nitrate, and TNF-α, COX-2 and iNOS expression and myeloperoxidase (MPO) activity. Rats receiving saline (IP) at 30 min before injection of saline (i.pl.) acted as the control group and the saline (IP) and carrageenan (i.pl.) injected rats acted as the carrageenan group. At 4 h after injection of carrageenan, the levels of TNF-α, PGE_2 and nitrate in paw exudates and the expression of COX-2 and iNOS in paw tissues were measured. Each group contained 5–6 rats.



Measurement of Cytokines, Nitrate, and PGE_2 Production in Paw Exudates

To obtain paw exudates, the hindpaws of rats were cut at the level of the calcaneus bone and centrifuged at 400*g* for 15 min at 4°C to collect the edematous fluid.[16] The levels of cytokines and PGE_2 in paw exudates were then measured by enzyme immunoassay kits, respectively (Genzyme Corporation, Cambridge). The concentrations of nitrate in paw exudates were measured by a Sievers Nitrite Oxide Analyzer (Sievers 280 NOA, Sievers, Boulder, CO).

Western Blot Analysis

Soft tissues were removed from rat paws and homogenized in a lysis solution containing 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonate (CHAPS), 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 1 μM pepstatin, and 10 μM leupeptin to obtain supernatant by centrifugation at 12,000*g* for 20 min. Proteins (50 μg) were then applied on 10% sodium dodecylsulphate-polyacrylamide minigel using a standard method. The proteins were transferred to polyvinylidene difluoride membranes and Western blotting was performed by adding an anti-COX-2, anti-iNOS (Transduction Lab, Lexington, KY) or anti-β-actin (Santa Cruz, San Francisco, CA) antibodies overnight at 4°C followed by incubation with horseradish peroxidase-conjugated secondary antibody. The ECL reagent (Amersham International Plc., Buckinghamshire, UK) was used to detect the protein bands and the relative density of iNOS and COX-2 was quantified by densitometry.

MPO Activity Assay

Soft tissues from paws were removed and washed with sterile normal saline and homogenized in ice-cold 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH = 6.0, 5 mL hexadecyltrimethylammonium bromide/g tissue) by using a homogenizer (Pro model 200, Monroe, CT), and then sonicated and centrifuged at 15,000*g* for 15 min at 4°C. The supernatant was mixed 1:30 (supernatant: assay buffer) and read at

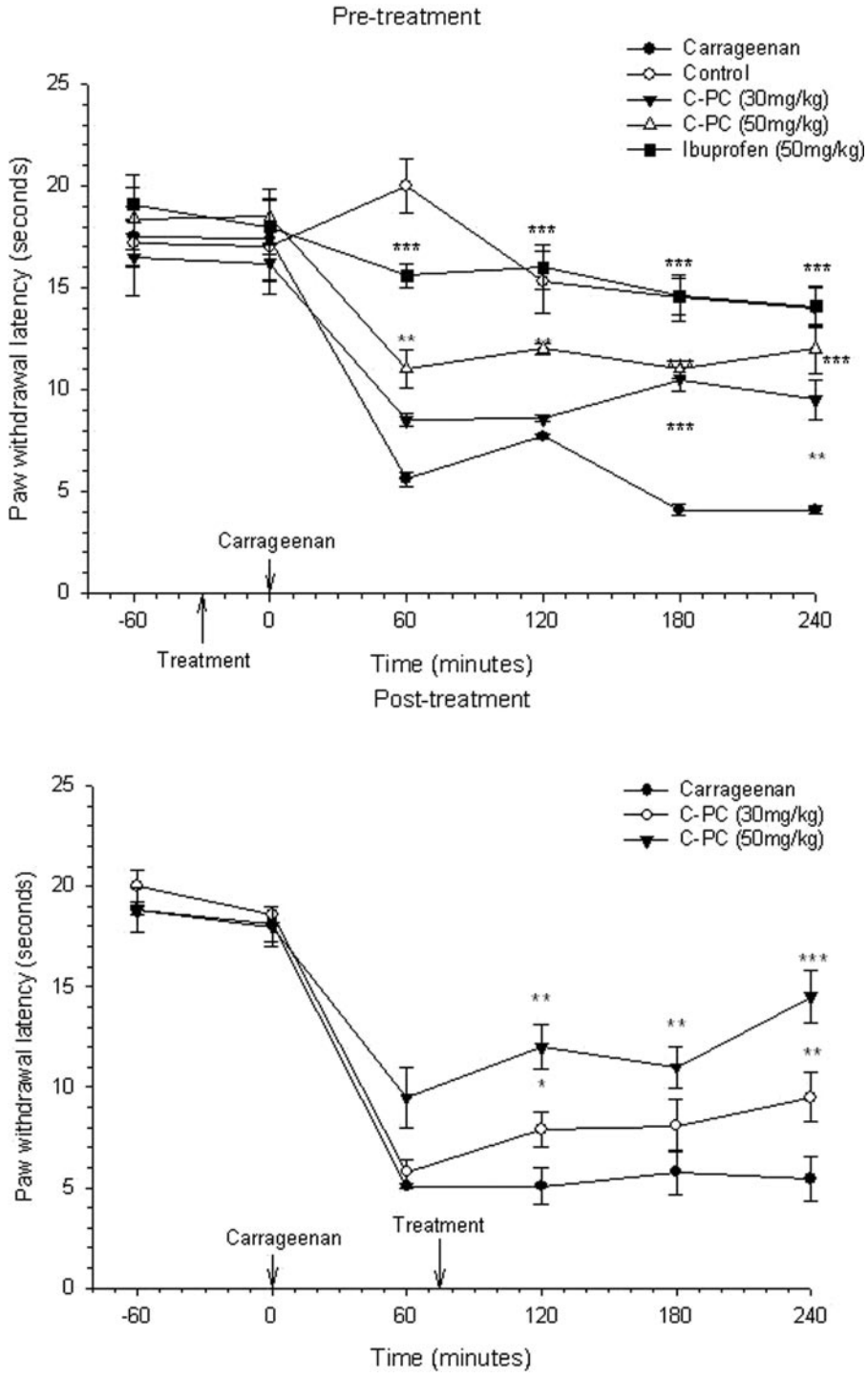


Figure 1. Effect of C-phycocyanin (C-PC) on carrageenan-evoked paw thermal hyperalgesia. Saline (0.2 mL, IP), C-PC (30 or 50 mg/kg, IP) or ibuprofen (50 mg/kg, IP) was administered 30 min before or 75 min after injection of carrageenan (1 mg/paw, i.pl.). Paw withdrawal latencies of rat hindpaws were assessed at specific times. Rats receiving saline (IP) at 30 min before injection of saline (i.pl.) acted as the control group and the saline (IP) and carrageenan (i.pl.)-injected rats acted as the carrageenan groups ($n = 6$ in each group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs carrageenan group.

460 nm. The assay buffer consisted of 100 mM potassium phosphate, pH 6.0, 0.083 mL H_2O_2 (Sigma; 30% stock diluted 1:1000) and 0.834 mL o-dianisidine hydrochloride (Sigma; 10 mg/mL). MPO activity was calculated and expressed as ΔA_{460}/min/mg protein.

Statistical Analysis

Data are expressed as mean ± SEM. The difference among groups was assessed using a one-way analysis of variance with *post hoc* analysis via Scheffe test. P values <0.05 were considered statistically significant.

RESULTS

Effect of C-PC on Carrageenan-Evoked Thermal Hyperalgesia

Injection of carrageenan into the rats' right hindpaws evoked thermal hyperalgesia with a significant decrease of withdrawal latency compared with that of the control group. Pretreatment with C-PC (30 or 50 mg/kg, IP) at 30 min before injection of carrageenan inhibited hyperalgesia from 1 h to 4 h compared with results in the carrageenan group (Fig. 1). Potent antihyperalgesic activity from ibu-

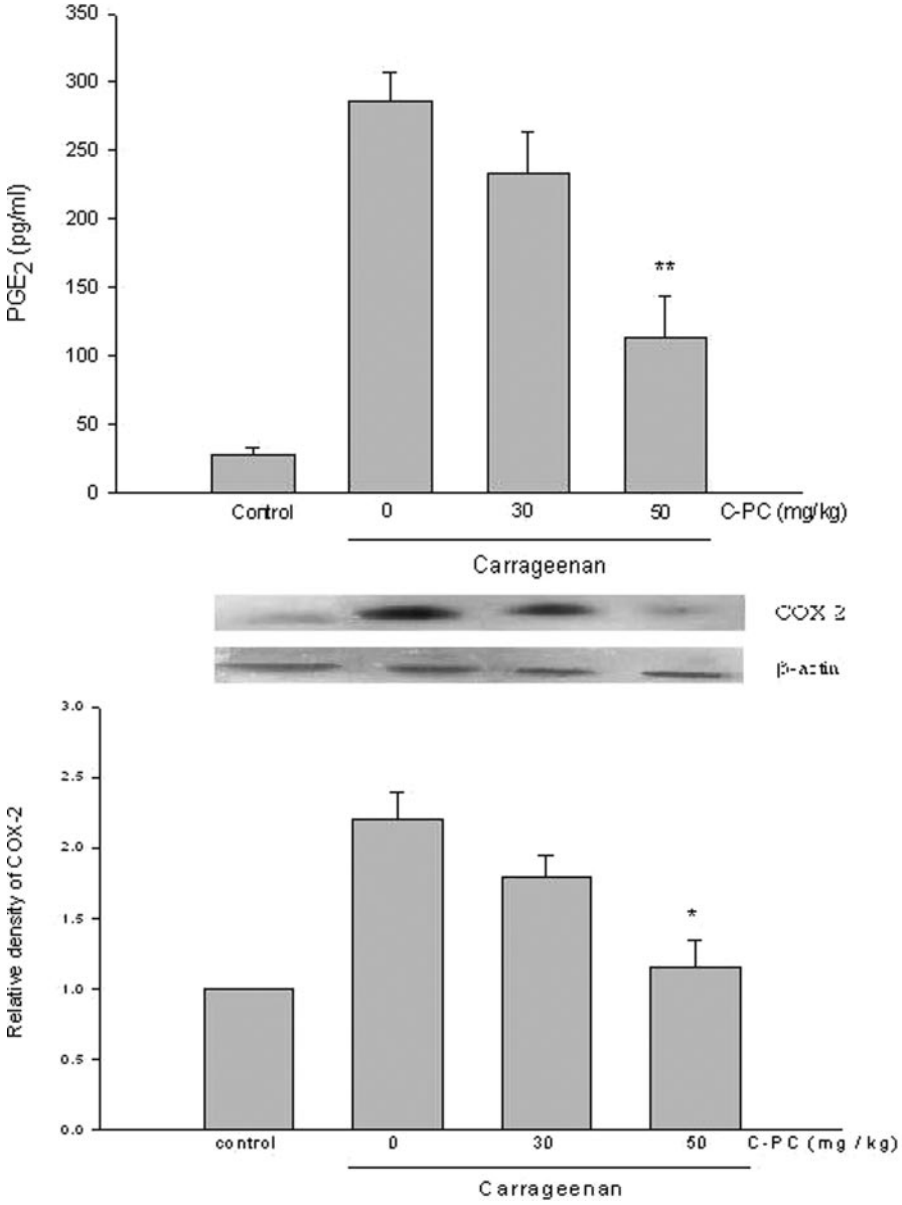


Figure 2. Effect of C-phycocyanin (C-PC) on prostaglandin E_2 (PGE_2) formation and cyclooxygeanase-2 (COX-2) expression in carrageenan-injected paws. C-PC (30 or 50 mg/kg, IP) was administered 30 min before injection of carrageenan and the levels of PGE_2 in the paw exudates (upper) and the COX-2 protein expression of the tissue of paws (bottom) were measured at 4 h after injection of carrageenan. The relative density of COX-2 was quantified by densitometry and the density of control group was set as 1. $^*P < 0.05$, $^{**}P < 0.01$ vs carrageenan group ($n = 5$ in each group).

profen (50 mg/kg, IP), a nonsteroidal antiinflammatory drug (NSAID), was also observed. Similarly, posttreatment with C-PC at 75 min after injection of carrageenan also significantly reduced hyperalgesia (Fig. 1). The withdrawal latencies of the contralateral left hindpaw (no injection in this paw) remained constant at basal levels (18.5 ± 1.5 s) through the entire experiment (data not shown).

Effect of C-PC on PGE_2 Formation and COX-2 Expression

Treatment with a higher dose of C-PC (50 mg/kg, IP) at 30 min before injection of carrageenan caused an inhibition of PGE_2 formation and COX-2 protein expression in carrageenan-injected paws at 4 h compared with results in the carrageenan group (Fig. 2).

Effect of C-PC on Nitrate Formation and iNOS Expression

Treatment with C-PC (30 or 50 mg/kg, IP) at 30 min before injection of carrageenan resulted in a reduction of nitrate formation and iNOS protein expression in carrageenan-injected paws at 4 h compared with results in the carrageenan group (Fig. 3).

Effect of C-PC on TNF-α and IL-10 Formation

Pretreatment with C-PC (30 or 50 mg/kg, IP) significantly inhibited the carrageenan-induced rise of TNF-α formation in paw exudates at 4 h compared with results of the carrageenan group (Fig. 4). However, C-PC had no significant effect on IL-10 formation (data not shown).

Effect of C-PC on MPO Activity

The carrageenan-induced increase of MPO activity in paws was also significantly suppressed by treatment with C-PC (30 or 50 mg/kg, IP) (Fig. 5).

DISCUSSION

Although a previous study has shown that C-PC reduces carrageenan–induced paw edema,[17] the effect of

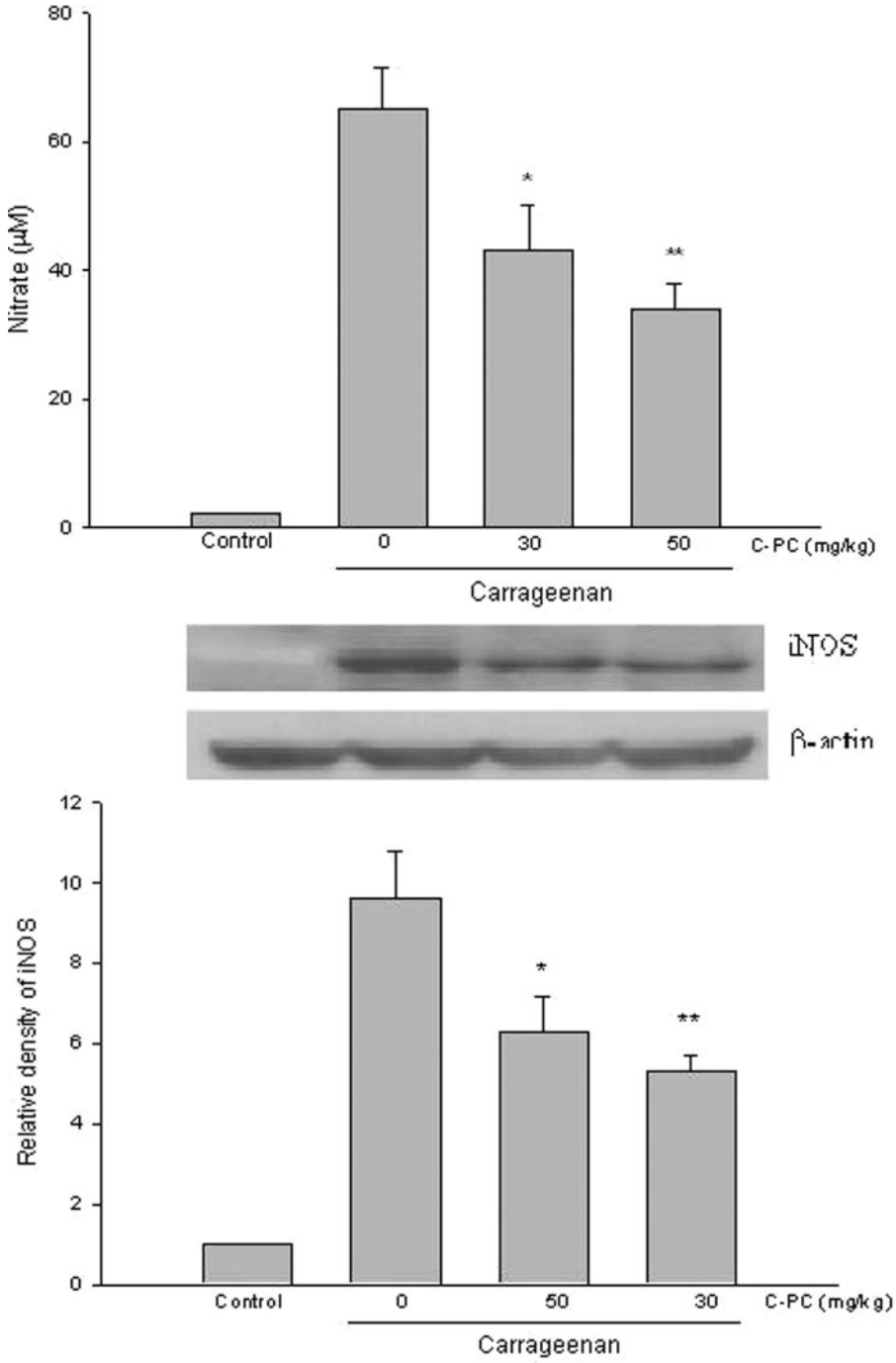


Figure 3. Effect of C-phycocyanin (C-PC) on nitrate formation and inducible nitric oxide synthase (iNOS) expression in carrageenan-injected paws. C-PC (30 or 50 mg/kg, IP) was administered 30 min before injection of carrageenan and the levels of nitrateinthe paw exudates (upper) and iNOS protein expression of the tissue of paws (bottom) were measured at 4 h after injection of carrageenan. The relative density of iNOS was quantified by densitometry and the density of control group was set as 1. $*P < 0.05$, $**P < 0.01$ vs carrageenan group ($n = 5$ in each group).

C-PC on inflammatory nociception has not been reported. Thus, the present study is the first to evaluate whether C-PC may also exert antihyperalgesic activity and further investigate the possible antiinflammatory mechanisms involved in a rat model of carrageenan-evoked thermal hyperalgesia. In this model, the development of edema and nociception in the rat hindpaw was described as a biphasic event.[18] The initial phase observed during the first hour was attributed to a release of histamine and serotonin; the second phase (4 h after carrageenan injection) was due to a release of proinflammatory mediators, including prostaglandin-like substances.[3] In this study, we first demonstrated that pre- or posttreatment with C-PC significantly attenuates carrageenan-evoked thermal hyperalgesia, suggesting that C-PC may have preventive and therapeutic activity on inflammatory nociception. In addition, we also found that C-PC exhibited significantly antihyperalgesic activity both in early and late phases. Since histamine plays an important role in the development of carrageenan-induced vascular permeability and edema in the early phase, the result suggests that C-PC may have an immediate inhibitory effect on histamine release. This hypothesis was supported by C-PC suppression of compound 48/80 (a histamine releaser)-induced histamine release from rat peritoneal mast cells.[19] Accordingly, C-PC may also affect the function of mast cells that are an important resource for

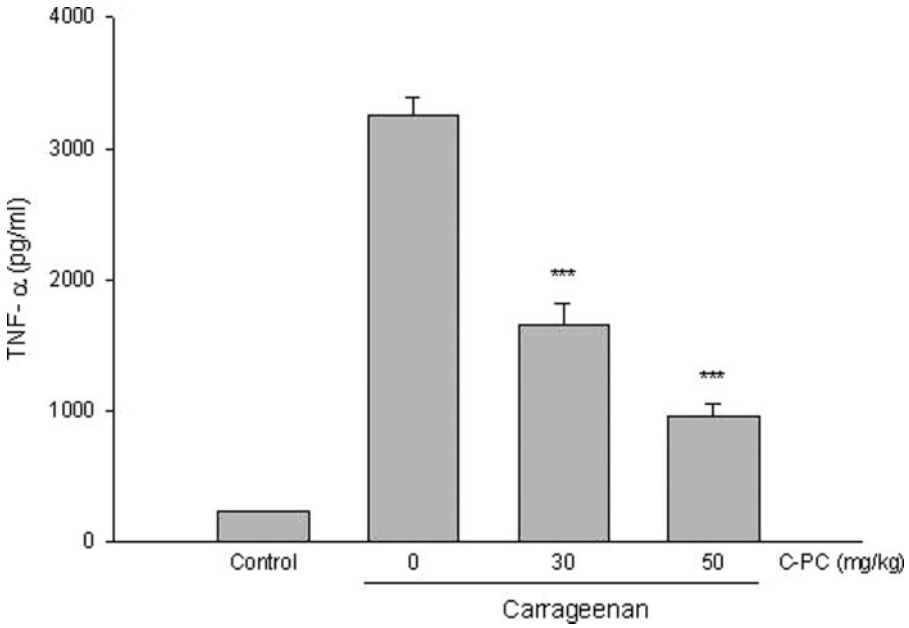


Figure 4. Effect of C-phycocyanin (C-PC) on tumor necrosis factor-α (TNF-α) formation in carrageenan-injected paws. C-PC (30 or 50 mg/kg, IP) was administered 30 min before injection of carrageenan and the levels of TNF-α in paw exudates were measured at 4 h after injection of carrageenan. $^{***}P < 0.001$ vs carrageenan group ($n = 6$ in. each group).

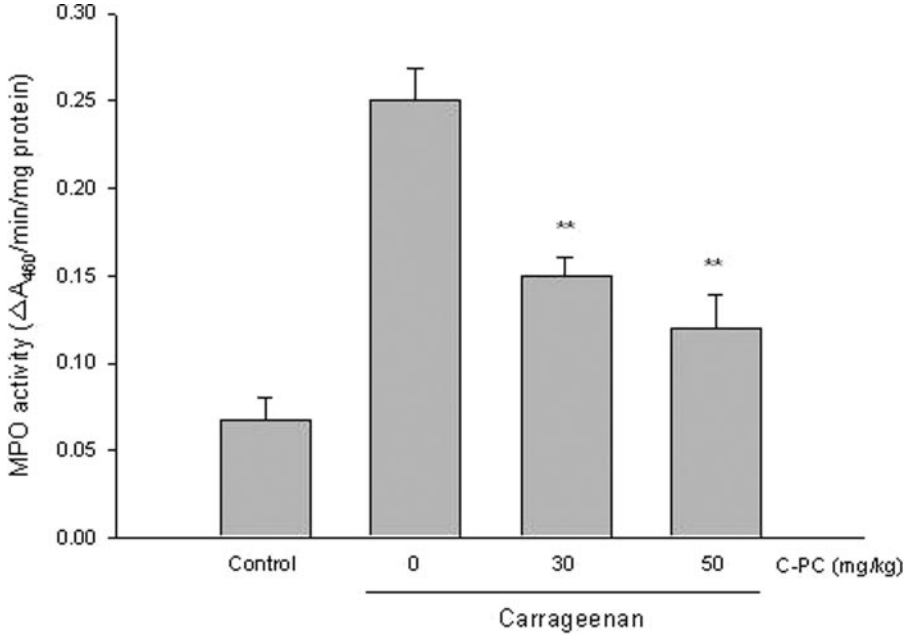


Figure 5. Effect of C-phycocyanin (C-PC) on myeloperoxidase (MPO) activity in carrageenan-injected paws. The C-PC (30 or 50 mg/kg, IP) was administered 30 min before injection of carrageenan. Paw tissue was then removed for MPO activity determination at 4 h after injection of carrageenan. $^{**}P < 0.01$ vs carrageenan group ($n = 6$ in each group).

synthesis and release of prostaglandin D2, leukotrienes, ROS and cytokines, such as TNF-α.[20] In this model, TNF-α plays an early and crucial role for the subsequent inflammatory response through stimulating the production of COX products and IL-8 that induce local production of sympathetic amines.[2,21] Thus, inhibition of TNF-α formation by C-PC in carrageenan-injected paws may contribute to its antihyperalgesic activity. However, C-PC had no effect on IL-10 (an antagonist cytokine) formation, suggesting that its antiinflammatory activity may be not mediated by IL-10 production.

It has been reported that, during the development of carrageenan-evoked inflammatory nociception, peripheral constitutive COX-1 and constitutive NOS play a primary role in the early phase (1 h); in the late phase (4 h), in which COX-2 and iNOS are fully activated. The over production of prostaglandins and NO mainly synthesized by COX-2 and iNOS is a key mediator for the maintenance of inflammation.[8,22,23] Furthermore, overproduction of NO may react with superoxide anion to form more cytotoxic peroxynitrite, which is often seen in carrageenan-injected paws.[24] Blocking COX-2 induction and PGE_2 formation or iNOS-derived NO formation has been demonstrated to exert a protective effect against inflammatory nociception and sepsis.[6,16,25] In this study, C-PC significantly inhibited the carrageenan-induced increase of PGE_2 and nitrate production accompanied by a suppression of COX-2 and iNOS expression in rat paws at the late phase, suggesting that C-PC may be a selective inhibitor for COX-2 and iNOS. Thus, it was possible that attenuation of over-production of NO and PGE_2 by C-PC, through suppressing iNOS and COX-2

induction, may be associated with its beneficial effect against inflammatory nociception.

Inflammation often causes neutrophil activation and infiltration into the damaged tissues. The infiltrated neutrophils may also be an important source of various proinflammatory mediators, including cytokines and oxygen-derived free radicals.[26] The increased MPO activity, an indicator of neutrophil infiltration observed in carrageenan-injected paws was significantly reduced by C-PC, suggesting that suppression of neutrophil infiltration may be another possible mechanism accounting for its antiinflammatory activity. In addition, research done in different models of inflammation has indicated that C-PC exerted antiinflammatory activity, not only in the acute models of inflammation, but also in a subchronic model, such as the cotton pellet granuloma in rats.[17]

The mitogen-actived protein kinases (MAPKs) family includes extracellular signal-regulated kinases (ERK), p38, and c-Jun N-terminal kinases. The p38 activated in dorsal root ganglion nociceptor neurons by peripheral inflammation has been implicated in the maintenance of inflammatory heat hyperalgesia.[27] It has been demonstrated that carrageenan-induced inflammation also triggers phosphorylation of spinal p38 MAPK.[28] Moreover, p38 MAPK regulates the synthesis of cytokines, including TNF-α and IL-1β as well as the induction of COX-2 and iNOS, and the blocking p38 MAPK phosphorylation resulting in a marked suppression in inflammatory edema and hyperalgesia.[29,30] These findings indicate that inhibition of p38 MAPK may have antihyperalgesic activity. A recent study has shown that C-PC attenuated ischemia-reperfusion induced cardiac dysfunction through its antiapoptotic action by inhibiting the activation of p38 MAPK and enhancing the activation of ERK1/2.[31] Thus, it is possible that the antihyperalgesic activity of C-PC may also be associated with the modulation of p38 MAPK and ERK1/2.

The standard antiinflammatory drug, ibuprofen, used as a positive control in the experiment, also had potent antihyperalgesic activity. Although NSAIDs, including ibuprofen and indomethacin, are the most commonly used remedy for treating inflammation, they often induce several serious adverse effects including gastrointestinal (GI) toxicity such as GI bleeding, resulting from platelet dysfunction.[32] Furthermore, the formation of some proinflammatory cytokines, such as TNF-α, is also modulated by endogenous prostaglandins *in vivo*[33] and treatment with ibuprofen may lead to a pronounced elevation of serum levels of TNF-α and IL-6 accompanied by higher mortality in murine endotoxic shock.[34] Thus, it is proposed that the adverse GI effects and exacerbated inflammatory responses caused by NSAIDs may be, in part, due to their nonselective inhibition of COX.[35] Our previous and present results have indicated that C-PC is a selective inhibitor of COX-2 and iNOS, which helps to prevent or reduce the adverse effects of NSAIDs. It has been reported that the LD_{50} of indomethacin is 12 mg/kg PO in rats.[36] However, there is no enhanced mortality in rats, and alterations in behavior or in organs, even at the high dose of C-PC (3 g/kg, PO),[17] suggesting that C-PC is much safer than traditional NSAIDs. Based on these characteristics, C-PC may be a better choice than NSAIDs for treating inflammatory nociception.

In this study, we first demonstrate that C-PC attenuates carrageenan-evoked thermal hyperalgesia. Furthermore, we propose that the antihyperalgesic mechanisms of C-PC may be associated with the inhibition of NO and PGE_2 over-production through suppressing iNOS and COX-2 induction. In addition, the inhibition of TNF-α formation and neutrophils infiltration in inflammatory sites may be also involved. These findings suggest that C-PC may be a potential therapeutic drug for reducing inflammatory nociception.

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