Anti-inflammatory and Analgesic Effect of Plumbagin through Inhibition of Nuclear Factor-κB Activation

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ABSTRACT

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (PL) is a naturally occurring yellow pigment found in the plants of the Plumbaginaceae, Droseraceae, Ancistrocladaceae, and Dioncophyllaceae families. It has been reported that PL exhibits anticarcinogenic, anti-inflammatory, and analgesic activities. However, the mechanism underlying its anti-inflammatory action remains unknown. In the current study, we investigated and characterized the anti-inflammatory and analgesic effects of PL orally administered in a range of dosages from 5 to 20 mg/kg. We also examined the role of nuclear factor κB (NF-κB) and proinflammatory cytokines and mediators in this effect. The results showed that PL significantly and dose-dependently suppressed the paw edema of rats induced by carrageenan and various proinflammatory mediators, including histamine, serotonin, bradykinin, and prostaglandin E₂. PL reduced the number of writhing episodes of mice induced by the intraperitoneal injection of acetic acid, but it did not reduce the writhing episode numbers induced by MgSO₄ in mice or prolong the tail-flick reaction time of rats to noxious thermal pain. Mechanistic studies showed that PL effectively decreased the production of the proinflammatory cytokines interleukin 1β, interleukin 6, and tumor necrosis factor α. It also inhibited the expression of the proinflammatory mediators inducible nitric-oxide synthase and cyclooxygenase 2, whereas it did not inhibit the expression of cyclooxygenase 1. Further studies demonstrated that PL suppressed inhibitor of κBα phosphorylation and degradation, thus inhibiting the phosphorylation of the p65 subunit of NF-κB. This study suggests that PL has a potential to be developed into an anti-inflammatory agent for treating inflammatory diseases.

Introduction

Inflammation has been demonstrated at the root of almost all chronic diseases, such as cancer, cardiovascular diseases, and autoimmune diseases, and huge efforts and resources are dedicated to the development of anti-inflammatory drugs. Nuclear factor κB (NF-κB) plays a critical role in the pathogenesis of inflammation, and a variety of drugs designed to treat human inflammatory disease are focused on the inhibition of NF-κB activation (Tak and Firestein, 2001). Under normal conditions, NF-κB is present in the cytoplasm as an inactive heterotrimer consisting of three subunits: p50, p65, and inhibitor of κBα (IκBα). Upon external stimuli, such as mitogens, inflammatory cytokines, ultraviolet irradiation, ionizing radiation, viral proteins, bacterial lipopolysaccharides, and reactive oxygen species, IκBα undergoes phosphorylation that is mediated through the activation of the IκB kinase (IKK) complex (Ducut Sigala et al., 2004) and ubiquitination-dependent degradation by the 26S proteasome, thus exposing nuclear localization signals on the p50–p65 heterodimer, leading to nuclear translocation and binding to DNA. The binding of NF-κB with DNA results in the transcription of the NF-κB-regulated genes (Aggarwal, 2004) and induces the transcription of proinflammatory mediators, such as inducible nitric-oxide synthase (iNOS), cyclooxygenase (COX) 2, tumor necrosis factor (TNF) α, interleukin (IL) 1β, and IL-6 (Baeuerle and Baltimore, 1996). These mediators play important roles in the mediation, propagation, and extension of a local or systemic inflammatory process and can cause further activation of NF-κB, subsequently increasing further production of these proinflammatory mediators via positive feedback mechanisms (Sonis, 2002). Inhibition of these mediators is beneficial for the treatment of inflammatory diseases and has become an important strategy for suppressing inflammation as is the case in nonsteroidal anti-inflammatory drugs (Appleby et al., 1994; Bogdan, 2001).

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ABBREVIATIONS: NF-κB, nuclear factor κB; p-NF-κB, phosphorylated NF-κB; PL, plumbagin; COX, cyclooxygenase; IκBα, inhibitor of κBα; IKK, IκB kinase; iNOS, inducible nitric-oxide synthase; PGE₂, prostaglandin E₂; TNF, tumor necrosis factor; IL, interleukin.
Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (PL) is a naturally occurring yellow pigment found in the plants of the Plumbaginaceae, Droseraceae, Ancistrocladaceae, and Dioncophyllaceae families. Available reports on PL have focused mainly on its anticancer activity and the underlying mechanisms. Animal and cell studies have demonstrated that PL has anticancer, antiproliferative, chemopreventive, radiosensitizing, and antimetastatic activities (Prasad et al., 1996; Singh and Udupa, 1997; Devi et al., 1998; Sugie et al., 1998; Wang et al., 2008). Mechanistic studies revealed that these activities of PL are related to its ability to modulate the NF-κB activation pathway, which in turn induces S-G2/M cell cycle arrest through the induction of p21 (an inhibitor of cyclin-dependent kinase) (Jaiswal et al., 2002), changes redox status of cell (Srinivas et al., 2004), and inhibits the enzyme NADPH oxidase (Ding et al., 2005).

The anti-inflammatory and analgesic activities of leaves of Plumbago zeylanica and plumbagin have been reported by Sheeja et al. (2010) in bioassay-guided isolation of anti-inflammatory and antinociceptive compound from this plant. In Sheeja et al.’s report, plumbagin inhibited carrageenan-induced rat paw edema, prolonged hot plate reaction time in mice, and shortened duration of pain response in formalin-induced nociception. However, the mechanism underlying the anti-inflammatory action of plumbagin remains unknown. Because NF-κB plays a pivotal role in inflammation and PL has the ability to modulate NF-κB in cancer cells, we therefore hypothesize that PL could suppress experimental inflammation through the inhibition of NF-κB activation.

In this article, the anti-inflammatory activity of PL was examined in the rat paw edema models induced by commonly used carrageenan and other phlogistic agents, and the role of NF-κB pathway and the proinflammatory mediators COX, iNOS, TNF-α, IL-1β, and IL-6 were examined. In addition, the analgesic activity of PL was investigated in inflammatory and noninflammatory pain models. This research will provide a solid foundation for the use of PL as an anti-inflammatory agent for therapeutic purposes.

Materials and Methods

Experimental Animals. Male ICR mice weighing 17 to 23 g and male SD rats weighing 200 to 250 g were purchased from the Laboratory Animal Services Center, the Chinese University of Hong Kong, Hong Kong. The animals were acclimated for 1 week under a 12-h light and 12-h dark cycle at a room temperature of 22°C ± 1°C. Chow diet and water were provided ad libitum. Rats and mice were fasted 24 h before experiment. After completion of experimental testing, animals were injected with Dorminal, which contains 20% pentobarbital, and then sacrificed by cervical dislocation. Animal care and treatment procedures conformed to the Institutional Guide lines and Animal Ordinance (Department of Health, Hong Kong Special Administrative Region).

Drugs and Reagents. Plumbagin (purity 99%), indomethacin, aspirin, carrageenan, Tween 80, histamine, serotonin, prostaglandin E₂ (PGE₂), bradykinin, acetic acid, and magnesium sulfate (MgSO₄) were purchased from Sigma-Aldrich (St. Louis, MO). Rotundine, an analgesic drug derived from a medical plant in China, was purchased from Shanghai Shiqiao Pharmaceutical Co., Ltd., Guangzhou, China. Morphine hydrochloride injection was purchased from North-east Pharmaceutical Group Co. (Shenyang, China). Plumbagin, indomethacin, and rotundine were dissolved in 100% ethanol and then resuspended with 0.5% carboxymethyl-cellulose for animal oral administration (final concentration of ethanol: 10%). Vehicle was prepared in the same method without drugs added.

Induction of Acute Inflammation in Rat Hind Paws. The acute inflammation in the hind paws of rats was induced by subcutaneous injection of phlogistic agents, including carrageenan, histamine, serotonin, bradykinin, or PGE₂ into the right hind paws of rats according to our previous methods (Zhou et al., 2006). In brief, two distinct schemes of treatment have been adopted. PL (5, 10, 20 mg/kg), indomethacin (reference drug, 10 mg/kg), or vehicle was orally administrated 1 h before inflammation induction for the prophylactic scheme. At induction, each rat was injected with freshly prepared solutions of carrageenan (0.1 ml, 1% w/v), histamine (0.05 ml, 1% w/v), serotonin (0.05 ml, 1% w/v), bradykinin (0.05 ml, 1% w/v), or PGE₂ (20 μg in 0.05 ml) in physiological saline (0.9% w/v NaCl) into subplantar tissues of the right hind paw of rats. In the therapeutic scheme, the animals received PL (5, 10, and 20 mg/kg), indomethacin (reference drug, 10 mg/kg), or vehicle 60 min after the injection of carrageenan. The left hind paws without injection were used as controls. The volumes (ml) of both hind paws of each animal were measured with a plethysmometer (7150; UGO Basile, Comerio, Italy) at 1 h before inflammation induction and at different time intervals designed from 0.5 to 6 h after injection of the phlogistic agents. The percentage of increase in paw volume (paw edema) of the right hind paws of each rat at each time point were calculated by the following equation: percentage of increase = (A − B/B) × 100, where A represents the paw volumes at different time points after injection, and B represents the paw volume before injection. At the end of experiment, the paws injected with carrageenan were collected to obtain paw exudates. Three to five samples of paw exudates were randomly selected for Western blot analysis.

Visceral Nociceptive Model Induced by Acetic Acid and MgSO₄ in Mice. The abdominal writhing test induced by chemical stimulation of acetic acid was performed in mice as described originally by Siegmund et al. (1957). In brief, PL (5, 10, and 20 mg/kg), aspirin (200 mg/kg), or vehicle was orally administrated 2 h before acetic acid injection. After intraperitoneal injection of 0.2 ml of acetic acid (0.8% w/v) in physiological saline, the animals were isolated for observation. The numbers of abdominal writhing syndrome/events, which consisted of the contraction of abdominal area with extension of hind legs, were accurately recorded during a 15-min period in each animal.

The abdominal writhing test induced by MgSO₄ was performed in mice as described originally by Gyires and Torma (1984) with minor modifications. In brief, 1 h before MgSO₄ intraperitoneal injection (120 mg/kg, 10 ml/kg), PL (5, 10, and 20 mg/kg), or vehicle was orally administrated, and morphine (10 mg/kg) was administrated subcutaneously. After intraperitoneal injection of MgSO₄, the animals were isolated in transparent cages for observation. After the first writhing movement appeared the animals were kept under observation for 5 min, and the number of writhing movements was counted during this period.

Central Nociceptive Model Induced by Radiant Heat Stimulation in Rats. The antinociceptive effects of PL and the reference drug, expressed as the time required for rat tail flick after exposure to a source of radiant heat, were evaluated according to the description of Zhou et al. (2006). In brief, animals were placed in a Plexiglas box that allowed their tails to be free, and then the box was placed on IITC model 336 tail-flick analgesia meter (IITC Inc., Woodland Hills, CA) with the tail occluding a slit over a photocell for radiant heat stimulation generated by a power lamp mounted in a reflector. The tail-flick response was elicited by applying radiant heat to the point 1/3 of length away from the tip of the tail. The apparatus was arranged so that when the operator turned on the lamp a timer was activated. When the rat felt pain and flicked its tail, light fell on the photocell such that the timer was automatically stopped. The intensity of the heat stimulus in the tail-flick test was adjusted so that the animal flicked its tail within 3 to 5 s. A 20-s cutoff time was set to prevent tail tissue from damage. Before the experiments, the heat...
stimulation latency of all animals was tested, and those with a response time to heat stimulation <2 s or >6 s were excluded. The tail-flick response was measured at 1, 2, and 3 h after oral administration of PL (5, 10, and 20 mg/kg), rotundine (100 mg/kg) as reference drug, or the vehicle.

**Western Blot Analysis of IκBa, p-NF-κB p65, iNOS, COX-1, COX-2, TNF-α, IL-1β, and IL-6 Protein Expression.** To obtain paw exudates, the rats were sacrificed by diethyl ether asphyxiation. Then, each hind paw injected with carrageenan was cut at the level of the calcaneus bone, and several transversal cuts were made with a scalpel. Each paw was then centrifuged at 10,000 g for 10 min at 4°C to collect tissue exudates (edema fluid). For iNOS, COX-1, COX-2, TNF-α, IL-1β, and IL-6 protein analysis, the edema fluid was vortexed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na3VO4, and 1 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na2EDTA, 1 mM β-mercaptoethanol, 5 mM Tris-HCl, 2 mM sodium fluoride, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.2) for 1 min. The RIPA or lysis buffer suspensions were immediately centrifuged at 14,000 g for 20 min at 4°C, and the supernatant was gently collected. The contents of total protein in the supernatants were determined by using a protein kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts (100 μg) of protein were boiled in sample loading buffer for 5 min before loading on 10% SDS-polyacrylamide gel for electrophoresis and transferred onto Immobilon-P membrane for blotting (pore size 0.45 μm; Millipore Corporation, Billerica, MA). The nonspecific binding sites on the membrane were blocked with 5% nonfat milk in Tris-buffered saline plus 0.1% Tween 20 overnight, and then the membranes were incubated with specific primary antibodies, including IκBa (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p-NF-κB p65 (Santa Cruz Biotechnology, Inc.), iNOS (BD Biosciences, San Jose, CA), COX-1 (Cayman Chemical, Ann Arbor, MI), COX-2 (Cayman Chemical), TNF-α (Cell Signaling Technology), IL-1β (Santa Cruz Biotechnology, Inc.), IL-6 (Abcam Inc., Cambridge, MA), and β-actin (Santa Cruz Biotechnology, Inc.). Membranes were subsequently incubated with peroxidase-conjugated secondary antibodies in 5% nonfat milk in Tris-buffered saline plus 0.1% Tween 20 for 1 h at room temperature. The membrane was washed six times, and the immunoreactive proteins were detected by enhanced chemiluminescence using hyperfilm and enhanced chemiluminescence reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the manufacturer’s instructions. Band intensities were quantified by using a densitometer analysis system and expressed as an arbitrary unit (Quantity One software, Bio-Rad Laboratories).

**Statistical Analysis.** All values are expressed as means ± S.E.M. Statistical significance of the difference was assessed by repeated measures analysis of variance, followed by a post hoc test using the least significant difference method for acute inflammation and tail-flick test. Statistical significance of the difference was assessed by one-way analysis of variance followed by a post hoc test with the least significant difference method for other tests. p values lower than 0.05 were considered statistically significant.

**Results**

**Plumbagin Inhibited Paw Edema Induced by Different Phlogistic Agents in Rats.** Figure 1A shows the prophylactic effect of PL on the inhibition of the acute paw edema in rats evoked by carrageenan injection into the subplantar tissues of right hind paws. The maximum phlogistic response of carrageenan was observed at 4 to 6 h after the injection in the control animals. The paw volumes from PL-treated animals with dosages of 10 and 20 mg/kg at 2 to 6 h after induction of paw edema showed marked decreases in comparison with the data of nontreated animals at the same time points. The reference drug, indomethacin, also significantly suppressed the paw edema. Rats treated with 5 mg/kg PL did not differ from the control group. These results indicate that the antiacute inflammatory effect of PL in rats was dose-dependent. Figure 1B shows the therapeutic effect of PL on inhibition of the acute paw edema in rats evoked by carrageenan. The paw volumes from PL-treated animals with dosages of 10 and 20 mg/kg showed marked decreases in comparison with the data of nontreated animals. Indomethacin also demonstrated significant suppression to the paw edema. For the normal group, a very slight increase in paw edema was observed, and the immunoreactive proteins were detected by enhanced chemiluminescence using hyperfilm and enhanced chemiluminescence reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the manufacturer’s instructions. Band intensities were quantified by using a densitometer analysis system and expressed as an arbitrary unit (Quantity One software, Bio-Rad Laboratories).
edema was found at 4 h after 0.1-ml saline injection (2.67 ± 2.53%; n = 3).

In the case of histamine-, serotonin-, bradykinin-, and PGE₂-induced rat paw edema, all measurements were conducted at time intervals of 0.5, 1, 2, 3, and 4 h after injection of the above phlogistic agents. Figure 2 shows that PL can dose-dependently inhibit the acute inflammatory responses evoked by histamine, serotonin, PGE₂, or bradykinin. However, Fig. 2 also shows that the anti-inflammatory effect of PL, although dose-dependent, also varies according to inflammatory agents. In rat paw edema induced by histamine, bradykinin, and PGE₂, the paw volumes from PL-treated animals with dosages of 10 and 20 mg/kg showed marked decrease throughout the experiments (Fig. 2, A, C, and D), whereas, in the paw edema induced by serotonin, only a higher dose (20 mg/kg) significantly reduced edema (Fig. 2B). Indomethacin (10 mg/kg) also showed anti-inflammatory effects in all animals (Fig. 2).

Plumbagin Alleviated Pain Induced by Acetic Acid but Not MgSO₄ and Radiant Heat Stimulation. The writhing assay induced by peritoneal injection of acetic acid in mice was used in the study. Figure 3A shows the numbers of the abdominal writhing episodes evoked by intraperitoneal injection of acetic acid in mice and the antinociceptive effect of PL. It can be seen that treatment with PL could dose-dependently reduce the number of writhing episodes of mice in comparison with those of vehicle-treated animals, whereas the reference drug, aspirin, had a stronger effect than PL did.

Figure 3B shows the numbers of the abdominal writhing episodes evoked by intraperitoneal injection of MgSO₄ in mice. It can be seen that treatment with PL did not reduce the number of writhing episodes of mice in comparison with that of vehicle-treated animals, whereas the reference drug, morphine, had a significant effect in reducing MgSO₄-induced writhing episodes.

Table 1 shows that the tail-flick reaction time of the control animals was approximately 5 s at 1, 2, and 3 h after orally taking the vehicle. Rats treated with PL did not differ from the control group, whereas rotundine, a positive analgesic agent (Zhou et al., 2006), prolonged the reaction time of the animals and demonstrated significant antinociceptive action.

Fig. 2. Effect of PL on histamine-induced (A), serotonin-induced (B), bradykinin-induced (C), and PGE₂-induced (D) paw edema of rats. PL at dosages of 5 (○), 10 (▲), and 20 (▲) mg/kg, the reference drug indomethacin at dosage of 10 mg/kg (△), and the vehicle (□) were orally administrated to rats 1 h before the injection of histamine, serotonin, bradykinin, or PGE₂. Each point represents the mean ± S.E.M. (n = 8–9); p value, compared with the vehicle control animals at the corresponding time point. For histamine-induced paw edema, the basal paw volume at 0 h for vehicle, indomethacin, and PL at 5, 10, and 20 mg/ml is 1.47 ± 0.03, 1.54 ± 0.03, 1.47 ± 0.03, 1.57 ± 0.02, and 1.51 ± 0.03 ml, respectively. For serotonin-induced paw edema, the basal paw volume at 0 h for vehicle, indomethacin, and PL at 5, 10, and 20 mg/ml is 1.38 ± 0.02, 1.40 ± 0.03, 1.39 ± 0.03, 1.37 ± 0.01, and 1.44 ± 0.03 ml, respectively. For bradykinin-induced paw edema, the basal paw volume at 0 h for vehicle, indomethacin, and PL at 5, 10, and 20 mg/ml is 1.45 ± 0.03, 1.49 ± 0.02, 1.46 ± 0.02, 1.51 ± 0.02, and 1.39 ± 0.03 ml, respectively. Data represent the mean ± S.E.M.
Plumbagin Suppressed Elevation of iNOS and COX-2 Expression Induced by Carrageenan in Paw Edema Fluid. It can be seen in Fig. 4 that PL dose-dependently attenuated the protein expression of iNOS and COX-2 in carrageenan-injected paw tissues. Approximately 45 and 50% reduction in iNOS protein expression was achieved by treatment with PL at dosages of 10 and 20 mg/kg, respectively, whereas no difference was found at the dosage of 5 mg/kg. Approximately 60 to 70% reduction in COX-2 protein expression was achieved by treatment with PL at all three dosages. Examination of COX-1 protein expression showed that the level of expression was not suppressed by administration of PL at any dosage. These results suggest that PL may have a selective inhibitory effect on COX-2 and iNOS protein expression. However, indomethacin at a dosage of 10 mg/kg demonstrated significant inhibition on iNOS, COX-1, and COX-2 protein expression. No significant increase in iNOS and COX-2 protein expression was found in normal animals in which no carrageenan was injected into the paw.

Plumbagin Reversed Change of IkBa and p-NF-kB p65 Expression Induced by Carrageenan in Paw Edema Fluid. The appearance of IkBa in cell lysate was investigated by immunoblot analysis. A basal level of IkBa was detectable in the normal animals in which no carrageenan was injected into the paw. At 4 h after carrageenan administration, the IkBa level was substantially reduced (Fig. 5) in the control group. Pretreatment with PL prevented carrageenan-mediated IkBa degradation. In fact, the IkBa band intensity of PL in the pretreatment group remained approximately 50% compared with the normal animals at 4 h after carrageenan administration (Fig. 5). In carrageenan-treated animals, the level of p65 NF-kB subunit was increased compared with the normal animals (Fig. 5). However, administration of PL (20 mg/kg) significantly reduced p65 band intensity. The reference drug indomethacin at 10 mg/kg also reversed the change of IkBa and p65 NF-kB subunit induced by carrageenan in the paw edema fluid of rats.

Plumbagin Inhibited Elevation of TNF-α, IL-1β, and IL-6 Expression Induced by Carrageenan in Paw Edema Fluid. The proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the carrageenan-injected paw edema fluid were examined by immunoblot analysis (Fig. 6). A basal level of TNF-α and IL-6 was detectable in the normal group, whereas IL-1β was not detected in normal rat paw fluid. Four hours after injection, carrageenan induced an obvious increase in the expression of TNF-α and IL-1β and a mild increase of IL-6 of paw edema fluid. Both PL and indomethacin attenuated the increased expression of TNF-α, IL-1β, and IL-6.

### Discussion

Although PL has been reported to have several biological functions, mostly related to anticarcinogenic activity (Sugie et al., 1998; Wang et al., 2008), the anti-inflammatory and analgesic effect and the underlying mechanism of PL have not yet been investigated. In the present study, we have provided the first evidence showing the anti-inflammatory and analgesic effects of PL in vivo through inhibition of NF-kB activation.

The pharmacological results of our current studies revealed that PL elicited significant anti-inflammatory activities in the carrageenan model in both prophylactic and therapeutic schemes. PL administered before or after the carrageenan injection can still inhibit the paw edema (Fig. 1). In fact, carrageenan-induced paw edema is one of the most commonly used models for inflammation investigation. This model has been widely accepted as a useful phlogistic tool for new anti-inflammatory agent screening. Development of paw edema of rats induced by carrageenan is highly correlated with the early exudative stage of inflammation (Ozaki, 1990). After carrageenan injection, a sudden elevation of paw volume can be observed that is correlated with vascular permeability induced by the action of histamine and serotonin (Vinger et al., 1987). Inflammation begins to be severe at approximately 1 h after induction, and paw edema gradually elevates to a peak 4 to 6 h after induction, which is the second phase caused by the liberation and overproduction of bradykinin, prostaglandins, and kinins in paw tissue accompanied by leukocyte migration (Vinger et al., 1987). The inflammatory pattern in our present study is in close accordance with
previous reports, whereas the dose-dependent inhibition of inflammation by PL from 1 to 6 h after the induction of inflammation suggests that PL may act in both the earlier and later phases of inflammation.

Because PL can inhibit both the first and second phase in carrageenan-induced edema, these results suggest that the anti-inflammatory activity of PL could be related to the impairment of proinflammatory mediators in the cyclooxygenase pathway, because most of the nonsteroidal anti-inflammatory drugs inhibit the production of proinflammatory mediators, including eicosanoids. Thus, different inflammatory mediators, i.e., histamine, serotonin, prostaglandin E₂, and bradykinin, were used for paw edema study, so as to

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**Fig. 4.** Effects of PL on iNOS, COX-2, and COX-1 protein expression in carrageenan-induced paw edema fluid of rats. PL at the dosages of 5, 10, and 20 mg/kg, the reference drug indomethacin (indo) at dosage of 10 mg/kg, and vehicle were orally administrated 1 h before the carrageenan injection. Animal without carrageenan injection served as normal control. At 4 h after the injection, paws were removed and paw edema fluid was collected by centrifugation. Protein expressions of iNOS, COX-2, and COX-1 in the paw edema fluid were detected by Western blot analysis using β-actin as the loading control. Each bar represents the mean ± S.E.M. (n = 3–5). *, P < 0.05; ***, P < 0.001, compared with the vehicle control animals.

**Fig. 5.** Effects of PL on IκBα and p-NF-κB p65 protein expression in carrageenan-injected paw edema fluid of rats. The experiment was performed as described in Fig. 4. Protein expressions of IκBα and p-NF-κB p65 in the paw edema fluid were detected by Western blot analysis using β-actin as the loading control. Indo, indomethacin. Each bar represents the mean ± S.E.M. (n = 3–5). *, P < 0.05; ***, P < 0.001, compared with the vehicle control animals.

**Fig. 6.** Effects of PL on TNF-α, IL-6, and IL-1β in carrageenan-injected paw edema fluid of rats. The experiment was performed as described in Fig. 4. Protein expressions of TNF-α, IL-6, and IL-1β in the paw edema fluid were detected by Western blot analysis using β-actin as the loading control. Indo, indomethacin.
further elucidate the anti-inflammatory effect of PL. The results show that PL had marked dose-dependent inhibitory effect with different pharmacological intensities on various inflammatory models induced by histamine, serotonin, prostaglandin $E_2$, or bradykinin. The results suggest that the underlying anti-inflammatory mechanisms of PL are possibly associated with the inhibition of either the synthesis, the release, or the actions of those proinflammatory mediators.

Analgesic effect usually accompanies anti-inflammation. We therefore examined the analgesic effect of PL with three nociceptive animal models: the tail-flick test of rats evoked by radiant heat stimulation (noxious thermal pain) and acetic acid- and MgSO$_4$-induced abdominal writhing assay in mice. The tail-flick test is more sensitive in centrally acting analgesics, whereas the acetic acid-induced abdominal writhing assay is commonly used for detecting both central and peripheral analgesia (Dewey et al., 1970; Fukawa et al., 1980; Won et al., 2006; Schmidt et al., 2009). Acetic acid was injected into the peritoneal cavity of mice to cause nociception in abdomen caused by the release of various substances that excite pain nerve endings (Raj, 1996). The MgSO$_4$-induced abdominal writhing assay is used as a model of non-inflammatory, prostaglandin-independent pain reaction. With the tail-flick test model, it was found that PL did not have a significant ability of prolonging the response latencies to the treatment of noxious thermal pain (Table 1). In the writhing response model, PL showed an ability of diminishing the numbers of writhing episodes in a dose-dependent manner, indicating a significant inhibition of the acetic acid-induced visceral nociception, whereas it did not inhibit MgSO$_4$-induced pain. These results suggest that the analgesic effect of PL is prostaglandin-dependent and PL might not be effective for the treatment of noxious thermal pain. It can be speculated that PL could inhibit the cyclooxygenase pathway as shown in Fig. 4, thus further interfering with the mechanism of transduction in primary afferent nociceptors in a prostaglandin-dependent manner (Fields, 1987).

In the past two decades, a number of anti-inflammatory botanical-derived medicines have been developed, but only a few of them have been studied with the goal of elucidating the molecular mechanisms of their actions (Surh et al., 2001). To address this issue, we first evaluated the anti-inflammatory and analgesic properties of PL and then determined the molecular mechanisms relevant to these actions, focusing on several key molecular targets, including IκBα, NF-κB, COX-2, iNOS, and the proinflammatory cytokines TNF-α, IL-1β, and IL-6.

It has been widely accepted that the formation of proinflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) and the overproduction of vasoactive mediators (e.g., nitric oxide by iNOS or eicosanoids via COX-2) play important roles in the pathophysiology of inflammation. The expression of inducible genes leading to the formation of these proteins relies on transcription factors, which are either controlled by (other) inducible genes and, hence, require de novo protein synthesis or alternatively, by so-called “primary transcription factors.” Among the latter, NF-κB has received a considerable amount of attention because of its unique mechanism of activation, active role in cytoplasmic/nuclear signaling, and rapid response to pathogenic stimulation. Activation of NF-κB is centrally involved in the local or systemic inflammation (Rutten and Thieme, 1997). Binding of NF-κB to the respective binding sequence on genomic DNA encoding for iNOS, COX-2, TNFα, IL-1β, and IL-6 results in a rapid and effective transcription of these genes (Collart et al., 1990; Xie et al., 1994). The anticarcinogenic, apoptotic, and radiosensitizing effects described previously suggest that PL mediates its effects by suppressing NF-κB activation that is caused by the interaction of PL with the cysteine residue of both IKK and p65 directly (Sandur et al., 2006). In the current study, we found that PL indeed suppressed IκBα phosphorylation and degradation and, as a result, inhibited phosphorylation of the p65 subunit of NF-κB in carrageenan-induced paw edema in vivo. This resulted in suppression of NF-κB-regulated reporter gene transcription and gene products involved in inflammation, i.e., TNF-α, IL-1β, IL-6, COX-2, and iNOS in this study.

As far as the COXs are concerned, COX-2 is the predominant cyclooxygenase isoform in all stages of inflammation, including facilitation of the production of proinflammatory prostanooids and inflammatory prostaglandins (Vane and Botting, 1998). Inflammation is induced or potentiated by the overproduction of prostaglandins (Harris et al., 2002), whereas selective COX-2 inhibitors can suppress inflammatory conditions through inhibition of the inflammatory prostaglandins (Dannenberg et al., 2001). NO is crucially involved in the regulation of the COX pathway and can modulate eicosanoid production by acting at several levels (Mollace et al., 2005). Thus, in inflammatory conditions where both the iNOS and COX-2 systems are induced there is a NO-mediated induction of COX-2 leading to increased formation of proinflammatory prostaglandins. This results in an exacerbated inflammatory response (Mollace et al., 2005). Thus, inhibition of NO production by the suppression of the enzyme activity of iNOS is one of the major pathways for anti-inflammatory effect. Moreover, Salvemini et al. (1996) reported that peripheral or central administration of iNOS inhibitors could effectively inhibit carrageenan-induced hyperalgesia in rats, which means that NO produced by iNOS is involved in the maintenance of the carrageenan-evoked inflammatory response. In the present studies, we demonstrated that PL can significantly suppress the de novo expressions of inducible NOS and COX-2 enzymes, but it cannot inhibit the COX-1 enzyme, which is believed to be one of the mechanisms by which PL reduces carrageenan-induced paw edema of rats.

Other than being regulated by NF-κB, proinflammatory cytokines TNF-α, IL-1β, and IL-6 help to propagate the extension of a local or systemic inflammatory process by activating NF-κB, forming a positive feedback mechanism to exacerbate the inflammatory process (Sunis, 2002). IL-1β is a potent proinflammatory cytokine that exerts its effects by binding to its receptor (IL1-R1) on the plasma membrane. This binding induces phosphorylation of the IKK complex, a crucial step in NF-κB activation based on the recruitment of the IL-1 receptor-associated kinase (Wang et al., 2001). Like IL-1β, TNF-α is a potent proinflammatory cytokine that plays a crucial role in inflammation (Tracey and Cerami, 1993). It binds to its cellular receptor TNF receptor 1, which triggers signaling cascades that activate NF-κB and activator protein 1 transcription factors. It has also been demonstrated that IL-6 can induce activation of NF-κB in the intestinal epithelia (Wang et al., 2003). Therefore, the inhibition of the production of TNF-α, IL-1β, and IL-6 by PL
described in the present study could also likely be attributed to the inhibitory effect of PL on the activation of NF-κB.

In conclusion, PL inhibits NF-κB, resulting in a decrease of the proinflammatory cytokines TNF-α, IL-1β and IL-6, COX-2, and iNOS expression, thus giving its anti-inflammatory effect in animal models. This result suggests that PL has a potential to be developed into an anti-inflammatory agent for treating inflammatory diseases.

References


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