Inducible nitric oxide synthase and cyclooxygenase-2 participate in anti-inflammatory and analgesic effects of the natural marine compound lemnalol from Formosan soft coral *Lemnalia cervicorni*

Yen-Hsuan Jean a,1, Wu-Fu Chen b,1, Chang-Yi Duh c,d, Shi-Ying Huang c, Chi-Hsin Hsu c,d, Chan-Shing Lin c,d, Chun-Sung Sung e, I-Ming Chen c,d, Zhi-Hong Wen c,d,*

**Abstract**

Lemnalol (8-isopropyl-5-methyl-4-methylene-decahydro-1,5-cycloc-naphthalen-3-ol) is a natural compound isolated from the marine soft coral *Lemnalia cervicorni*. In the present study, the anti-inflammatory and anti-nociceptive properties of lemnalol were investigated in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells and carrageenan-injected rats, respectively. Our results demonstrate that lemnalol significantly inhibited the expression of the pro-inflammatory proteins, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), in LPS-stimulated RAW 264.7 cells. An **in vivo** inflammation model was induced by intraplantar injection of carrageenan into rat hind paws. An intramuscular injection of lemnalol (15 mg/kg) 10 min before carrageenan injection resulted in significant inhibition of carrageenan-induced rat paw edema and thermal hyperalgesia behavior. Western blot experiments revealed that the carrageenan-induced expression of iNOS and COX-2 in paw tissue was significantly down-regulated by lemnalol. Moreover, post-intrathecal injection of lemnalol produced a dose-dependent antinociceptive effect in carrageenan-injected rats (1 and 5 μg). The present results indicate that the marine-derived compound lemnalol had anti-inflammatory and analgesic effects in LPS-stimulated RAW 264.7 cells and carrageenan-injected rats, respectively. In addition, inhibition of elevated iNOS and COX-2 protein expression as well as neurophil infiltration of carrageenan-injected paws may be involved in the beneficial effects of lemnalol.

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**Keywords:** Lemnalol; iNOS; COX-2; Intrathecal; Carrageenan; Inflammation; Analgesia

1. **Introduction**

Marine organisms have proven to be rich sources of structurally novel and biologically active natural compounds. These compounds have served as important chemical prototypes for the discovery of new drugs for use in the treatment of various human diseases. Many studies on plant- or marine-derived anti-inflammatory compounds have investigated the potential inhibitory effects of natural products in an **in vitro** system, lipopolysaccharide (LPS)-stimulated macrophage. Using this system, bacterial LPS has become one of the best-characterized stimuli used to induce the up-regulation of pro-inflammatory proteins such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Inducible COX-2 could be responsible for the high prostaglandins observed in many inflammatory pathologies (Simmons et al., 2004). Similar to COX-2, iNOS produces large amounts of nitric oxide (NO) and has been suggested to play a central role in inflammatory disease (Suschek et al., 2004). Numerous studies have reported that NO and prostaglandins participate in inflammatory and...
nociceptive events (Ferreira et al., 1973; Holthusen and Arndt, 1994). However, only a few studies have directly examined the ability of marine-derived natural products to inhibit inflammatory responses and elucidate their possible mechanisms in an in vivo model. The carrageenan-induced rat paw inflammatory response (including, paw edema and hyperalgesia) is a widely used animal model used to determine anti-inflammatory activity, and has been fully characterized (Di and Willoughby, 1971; Di, 1972; Garcia et al., 1973).

Lemnalol (8-isopropyl-5-methyl-4-methylene-decahydro-1,5-cyclo-naphthalen-3-ol) was originally isolated from the soft coral *Lemnalia cervicorni* by Kikuchi et al. (1983). Duh et al. (2004) also isolated lemnalol from the same genus but different species of soft coral, *Lemnalia cervicorni*. In the present study, we initially examined the effect of lemnalol on the up-regulation of iNOS and COX-2 protein expression in LPS-treated RAW 264.7 macrophages, a well-characterized cell inflammation model. From preliminary screening, we had found that lemnalol is a potential anti-inflammatory compound that significantly inhibits the expression of the pro-inflammatory proteins iNOS and COX-2 in LPS-stimulated RAW 264.7 macrophage cells. Importantly, it has been demonstrated that iNOS and COX-2 play key roles in the development of carrageenan-induced inflammatory responses such as paw edema and nociception (Seibert et al., 1994; Salvenini et al., 1996). Thus, in the present study, we investigated the possible anti-inflammatory, anti-hyperalgesia, and anti-nociceptive effects of lemnalol in a carrageenan-injected rat model. Moreover, using this model, we also examined whether lemnalol affects the time course of the inflammatory response and the up-regulation of iNOS and COX-2 protein expression. We demonstrate that the marine-derived natural compound lemnalol promotes a significant inhibition of the carrageenan-induced inflammatory effect.

2. Materials and methods

2.1. Chemicals

Lemnalol (8-isopropyl-5-methyl-4-methylene-decahydro-1,5-cyclo-naphthalen-3-ol, Fig. 1) was provide by Professor Duh Chang-Yi, Department of Marine Biotechnology and Resources, National Sun Yat-sen University. It was isolated from the soft coral *L. cervicorni* collected from Green Island, off Taiwan, as described previously (Duh et al., 2004). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, antibiotic–antimycotic solution, and trypsin-EDTA were purchased from Invitrogen Co. (Grand Island, NY, USA). Lipopolysaccharide (LPS; *Escherichia coli*), carrageenan lambda, and dimethyl sulfoxide (DMSO) were purchased from Sigma Co., Ltd. (St Louis, MO, USA). Artificial cerebrospinal fluid (aCSF) comprised 151.1 mM Na+, 2.6 mM K+, 122.7 mM Cl−, 21.0 mM HCO3−, 0.9 mM Mg2+, 1.3 mM Ca2+, 2.5 mM HPO42−, and 3.5 mM dextrose; this solution was bubbled with 5% CO2:95% O2 in order to adjust the final pH to 7.3.

2.2. Cell culture and in vitro anti-inflammatory assay

The anti-inflammatory activity method used was modified from that of Ho et al. (2004) and Park et al. (2005), and our previous study (Huang et al., 2006). Murine RAW 264.7 macrophages were obtained from the American Type Culture Collection (ATCC, No TIB-71) and cultured in DMEM containing 10% heat-inactivated FBS, 2 mM glutamine, 1 mM pyruvate, 4.5 g/l glucose, 50 U/ml penicillin and 50 μg/ml streptomycin at 37 °C in a humidified 5% CO2:95% air incubator under standard conditions. Inflammation in macrophages was induced by incubating them for 16 h in a medium containing only LPS (0.01 μg/ml) without compounds. For anti-inflammatory activity assay, lemnalol (0.1, 1, 10 or 100 μM) were added to the cells 5 min before LPS challenge. The cells were then washed with ice-cold phosphate-buffered saline (PBS), lysed in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin), and then centrifuged at 20,000 ×g for 30 min at 4 °C. The supernatant was decanted from the pellet and retained for western blot analysis of iNOS and COX-2. Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA, USA) modified from the method of Lowry et al. (1951).

2.3. Western blot analysis for iNOS, nNOS, COX-2, and COX-1

Western blotting was performed according to the method described in our previous study (Wen et al., 2005). An equal volume of sample buffer (2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromophenol blue, 2% 2-mercaptoethanol, and 50 mM Tris–HCl, pH 7.2) was added to the sample, which was then loaded onto a tricine SDS-polyacrylamide gel and electrophoresed at 150 V for 90 min. The proteins were transferred to a polyvinylidene difluoride membrane (PVDF; Immobilon-P, Millipore, 0.45-μM pore size) at 125 mA overnight at 4 °C in transfer buffer (50 mM Tris–HCl, 380 mM glycine, 1% SDS, and 20% methanol). The membrane was blocked for 50 min at room temperature with 5% non-fat dry milk in Tris-buffered saline (TTBS; 0.1% Tween 20, 20 mM Tris–HCl, 137 mM NaCl, pH 7.4), and then incubated for 180 min at room temperature with antibodies against iNOS (1:1000 dilution; BD Pharmingen, San Diego, CA, USA; catalog no. 610322; polyclonal antibody), neuronal nitric oxide synthase (nNOS, 1:1000 dilution; BD Pharmingen, San Diego, CA, USA; catalog no. 610308; monoclonal antibody), and COX-2 (1:1000 dilution; BD Pharmingen, San Diego, CA, USA; catalog no. 610332; monoclonal antibody).
2.6. Carrageenan-induced paw edema and thermal hyperalgesia

Rats were excluded from the study if they exhibited evidence of suffering. Those that survived then were returned to their home cages for a 4-day recovery period. The rats were then anesthetized by the National Sun Yat-sen University and Use Committee. Animals of the American Physiology Society and was approved by the National Sun Yat-sen University and Use Committee. Every effort was made to minimize the number of animals used and their suffering.

2.4. Animals

Male Wistar rats weighing 250–285 g were used throughout the experiments. The rats were maintained in Plexiglas cages within a temperature-controlled (22 ± 1 °C) room, on a 12-h light/dark cycle, and given free access to food and water. Each rat was used only once during the study. All surgery and drug injection were performed under isoflurane anesthesia. The use of animals conformed to the Guiding Principles in the Care and Use of Animals of the American Physiology Society and was approved by the National Sun Yat-sen University and Use Committee. Every effort was made to minimize the number of animals used and their suffering.

2.5. Intrathecal catheter implantation

Using the method described by Yaksh and Rudy (1976) and in our previous study (Wen et al., 2005), an intrathecal (i.t.) catheter (0.008 inch inner diameter, 0.014 inch outer diameter) was inserted via the atlantooccipital membrane into the i.t. space at the level of the lumbar enlargement of the spinal cord, and then externalized and fixed to the cranial aspect of the head. The rats were returned to their home cages for a 4-day recovery period. Rats were excluded from the study if they exhibited evidence of gross neurological injury or the presence of fresh blood in the CSF.

2.6. Carrageenan-induced paw edema and thermal hyperalgesia

Paw edema was induced by intraplantar injection of 100 μl of 1.5% sterile carrageenan lambda in saline into the right hind paw (Winter et al., 1962). Carrageenan caused visible redness and pronounced swelling that was well developed by 4 h and persisted for more than 48 h. The paws were marked in order to facilitate replicate positioning in the measurement chamber. The volume of paw was measured by using a paw volume meter (plethysmometer, Singa Inc.). The increase in paw volume was calculated by subtracting the initial paw volume (basal) from the paw volume measured at each time point. Thermal hyperalgesia was assessed by placing the hind paw on a radiant heat source and measuring the paw withdrawal latency at a low intensity heat set to a cut-off time of 30 s using an IITC analgesiometer (IITC Inc., Woodland Hills, CA, USA). The paw withdrawal latency was measured as described previously by Hargreaves et al. (1988) as the average of two measurements per paw. The hind paw volume and thermal hyperalgesia were measured before and after the administration of carrageenan at 1 to 2 h intervals for 12 h.

2.7. The effect of systemic lemnalol on the carrageenan-induced inflammatory response

The peripheral effect of lemnalol was assessed by intramuscular (i.m.) injection (0.1 ml) 10 min before carrageenan administration. Naïve rats were allocated randomly to groups of 3:

(i) Control group (C); rats received no treatment.
(ii) Carrageenan group; rats received 100 μl of 1.5% carrageenan (intraplantar).
(iii) Carrageenan+lemnalol group; rats received 100 μl of 1.5% carrageenan (intraplantar) plus 15 mg/kg lemnalol (i.m.).

The lemnalol was dissolved in 20% DMSO and delivered in a volume of 100 μl. The edema component of inflammation was quantified by measuring the difference in hind footpad edema before carrageenan injection and at 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, and 12 h after carrageenan injection. For statistical analysis, the area under the curve (AUC) for the plot of paw edema versus time was calculated by the trapezoidal method (Rowland and Tozer, 1995) from 0 to 12 h after carrageenan or carrageenan plus lemnalol injection.

2.8. The effect of i.t. lemnalol injection on carrageenan-induced thermal hyperalgesia

A further group of rats (n=18) were implanted with an i.t. catheter for lemnalol or DMSO (20%) administration. On the fourth day after i.t. catheter implantation, the rats were assigned to 1 of 4 groups:

(i) Control group (C); rats received 10 μl of 20% DMSO (i.t.), followed by 10 μl of aCSF to flush the catheter.
(ii) Carrageenan only group; rats received 100 μl of 1.5% carrageenan (intraplantar) at 4 h, following an injection of 10 μl of 20% DMSO (i.t.) and 10 μl of aCSF to flush the catheter.
(iii) Carrageenan+lemnalol (1 μg) group; rats received 100 μl of 1.5% carrageenan (intraplantar) at 4 h, following an injection of 1 μg lemnalol (i.t.) and 10 μl of aCSF to flush the catheter.
(iv) Carrageenan+lemnalol (5 μg) group; rats received 100 μl of 1.5% carrageenan (intraplantar) at 4 h, following an injection of 5 μg lemnalol (i.t.) and 10 μl of aCSF to flush the catheter.

The lemnalol was dissolved in 20% DMSO and delivered in a volume of 10 μl. The paw withdrawal latency was also transformed into the percentage of maximum possible effect (MPE) using the following formula: % MPE = (post-drug latency − baseline)/(cut-off time of 30 s)
off—baseline) × 100%, where the post-drug latency is the response measured 2, 4, 6, and 8 h after injection of lemnalol or saline, the baseline is the response measured immediately prior to test injection at 4 h, and the cut-off time is 30 s.

2.9. Preparation of paw tissues for western blot analysis, histopathology, and immunohistochemistry

After the last determination of paw edema (12 h after carrageenan injection), the animals were sacrificed and paw samples were collected for western blotting and histopathological observation. For western blotting analysis, samples from the paws of control, carrageenan-injected, carrageenan plus lemnalol-injected, and lemnalol-injected rats collected at different time points were washed with ice-cold PBS and homogenized in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin) using a Polytron homogenizer (5 cycles of 10 s at 3000 rpm). After centrifugation at 20,000 × g for 60 min at 4 °C, the protein content of the supernatant was measured using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Protein samples were already for western blotting analysis of COX-2, COX-1, iNOS, nNOS and β-actin of paw tissue. For histopathological examination, biopsies of paws were taken 24 h after the induction of inflammation with carrageenan. Rats under deep anesthesia induced with sodium pentobarbital (100 mg/kg) were already perfused transcardially with 500 ml of cold PBS containing 1% sodium nitrite and heparin (0.2 U/ml) and then with 4% paraformaldehyde in 500 ml of 0.1 M PBS (pH 7.4). The paw samples were then harvested and fixed in 10% neutral buffered formalin, placed in a decalcifying solution for 30 h, and then stored in 10% formalin prior to processing. The paws were bisected longitudinally, placed in embedding cassettes, embedded in paraffin, and then cut into 4-µm sections. The sections were stained with hematoxylin and eosin (H & E) for histopathological observations. For immunohistochemistry, following deparaffinization, endogenous peroxidase was quenched with 0.3% H2O2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the sections in 5% normal goat serum in PBS for 30 min. The sections were incubated overnight at 4 °C with anti-iNOS (1:300 dilution) or anti-COX-2 (1:300 dilution) antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and an avidin–biotin peroxidase complex (DBA; Vector, Milan, Italy). Finally, the sections were reacted with 3,3′-diaminobenzidine tetrahydrochloride (DAB) for 1–2 min. All slides for histopathology and immunohistochemistry were analyzed under a Leica DM1000 microscope (Leica Instruments Inc.). A microscope digital camera system (SPOT, RT slider; Diagnostic Instruments Inc.) was used to photograph all specimens.

2.10. Data and statistical analysis

All data are presented as the mean ± S.E.M. For the immunoreactivity data, the intensity of each test band is expressed as the integrated optical density (IOD), calculated with respect to the average optical density of the corresponding control (LPS-only treatment) band. For statistical analysis, all the data were analyzed by a one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls post hoc test for multiple comparisons. A significant difference was defined as a P value of <0.05. In order to simplify the data analysis, values derived from the temporal determination of paw withdrawal latency and paw edema were transformed to AUC. The AUC for the time–response curves for paw withdrawal latency was calculated for individual animals using Sigmaplot software that takes into account time on the X-axis and response on the Y-axis.

3. Results

3.1. The effect of lemnalol on LPS-induced iNOS and COX-2 protein expression in macrophage

The up-regulation of pro-inflammatory iNOS and COX-2 in the LPS-stimulated RAW 264.7 macrophage cells was evaluated by western blot analysis (Fig. 2). The analysis was carried out on whole cell lysates using antibodies against mouse macrophage iNOS and COX-2. The dose responses for inhibition of the LPS-induced 130-kDa iNOS and 71-kDa COX-2 protein expression by lemnalol are shown in Fig. 2. At 0.1, 1, 10, and 100 µM doses of lemnalol, the levels of iNOS protein were significantly reduced to 62.5±7.3%, 40.7±5.4%, 0.21±0.1%, and 0.1±0.1% of the control level, respectively. At concentrations of 10 and 100 µM, the level of COX-2 protein were also reduced significantly to 17.5±6.5% and 13.3±5.3% of the control level, respectively. Therefore, both iNOS and COX-2 were significantly inhibited by lemnalol at 10 µM, and 100 µM, with protein expression in all cases being almost completely abolished. Only vehicle (DMSO) or lemnalol (0.1, 1, 10 and 100 µM) did not induce any iNOS and COX-2 protein expression in RAW264.7 cells. Lennalol (from 0.1 to 50 µM) did not induce cytotoxicity in RAW264.7 cell by trypan blue staining.

3.2. The effect of systemic injection of lemnalol on the carrageenan-induced inflammatory response

Intraplantar injection of 100 µl 1.5% carrageenan into the rat hind paw induced a significant decrease in paw withdrawal latency (Fig. 3) and an increase in paw edema (Fig. 4) as compared to the baseline (before carrageenan injection). These observations indicated the development of an inflammatory pain response, thermal hyperalgesia, and paw swelling (edema). However, in the present experiment, contralateral inflammatory responses were not observed. The thermal hyperalgesia behavior increased progressively reaching a maximum value from 4 to 8 h after carrageenan injection (Fig. 3). A 15 mg/kg dose of lemnalol (i.m.) 10 min before carrageenan injection significantly reduced the carrageenan-induced thermal hyperalgesia. At 12 h after carrageenan injection, there was a 96.8±6.7% increase in paw volume in the carrageenan-injected group compared with the pre-
carrageenan control value (Fig. 4). The results are calculated as AUC (from baseline to 12 h after carrageenan injection) for simplified comparison of data between the carrageenan and carrageenan plus lemnalol groups. The AUC of carrageenan and carrageenan plus lemnalol are 1052.1±57.2 and 676.5±47.52, respectively. Lemnalol (15 mg/kg) injected i.m. 10 min before intraplantar injection of carrageenan, significantly inhibited the edema formation. In the present study, there was no macroscopic evidence of either paw erythema or edema in the saline or lemnalol only groups (data not shown).

3.3. The effect of intrathecal lemnalol on carrageenan-induced thermal hyperalgesia

The baselines of the paw withdrawal latency for all experimental groups before carrageenan or carrageenan plus

Fig. 2. The effect of lemnalol on pro-inflammatory iNOS and COX-2 protein expression in RAW 264.7 cells induced by LPS. (A) Western blots for iNOS, COX-2, and β-actin proteins from RAW 264.7 cells; (B) relative density of immunoblot of iNOS; (C) relative density of immunoblot of COX-2. Relative intensity of LPS-stimulated group was taken to be 100%. Band intensities were quantified by densitometry and are indicated as a percentage change relative to that of the LPS-stimulated group. Western blotting with β-actin was performed to verify that equivalent amounts of protein were loaded in each lane. This experiment was repeated 5 times. *Significantly different from the LPS-stimulated group (P<0.05).

Fig. 4. The effect of lemnalol at a dose of 15 mg/kg on the development of paw edema after intraplantar carrageenan injection. (A) Lemnalol was injected i.m. 10 min before carrageenan injection. The basal volume of each rat paw was taken to be 100% and variations from this value were given as a percentage change from the control (pre-drug) values. Each point represents the mean±S.E.M. of 6 rats per group. *Indicates significant difference compared with the saline group (P<0.05). Each point or bar represents the mean±S.E.M. of 6 rats.
drug injection were the same. The average baseline paw withdrawal latency in the thermal nociceptive test for naïve rats was 29.2±1.2 s (n=34). The paw withdrawal latency was decreased progressively, reaching a maximum response (10.5±2.1 s) at 4th h after intraplantar carrageenan injection. Intrathecal lemnalol (1 or 5 μg) injected 4th h after carrageenan injection significantly inhibited the thermal hyperalgesia behavior (Fig. 5A). The same results are presented in Fig. 5B as % MPE from 4 h to 12 h after carrageenan injection, which simplified the comparison of data between the groups.

Four hours after intraplantar carrageenan injection, both 1 and 5 μg (i.t.) lemnalol produced a significant dose-dependent anti-nociceptive effect.

3.4. Effect of lemnalol on carrageenan-induced iNOS and COX-2 expression in paws

As expected, neither iNOS nor COX-2 protein expression was detectable in the paws of naïve rats and rats injected with lemnalol alone. Intraplantar injection of carrageenan evoked significantly

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Fig. 5. A time course showing the effects of i.t. lemnalol on the carrageenan-induced thermal hyperalgesia behavior. (A) Lemnalol (1 or 5 μg) was injected i.t. at 4 h after carrageenan injection. (B) Anti-nociceptive effect of i.t. lemnalol in carrageenan-induced thermal hyperalgesia. Anti-nociception data are expressed as a percentage of the MPE after lemnalol injection. Each point or bar represents the mean±S.E.M. of 6 rats. The results for the different doses of lemnalol (1 and 5 μg) groups were significantly different from those for the carrageenan group (*, a P<0.05 compared to the carrageenan-injected alone group; b P<0.05 compared to the lemnalol 1 μg group) and were dose-dependent.

Fig. 6. The inhibitive effect of systemic lemnalol on iNOS and COX-2 protein expression in homogenized rat paws from carrageenan-induced inflammatory rats. Western blot analyses of the expression of the iNOS, COX-2, nNOS, COX-1, and β-actin in paw tissues after various treatments (A). Intraplantar carrageenan injection significantly induced iNOS and COX-2 protein expression in paws at 24 h. Lemnalol (15 mg/kg) administered i.m. 10 min before carrageenan injection significantly inhibited carrageenan-induced up-regulation of iNOS (B) and COX-2 (C) protein expression. The expression of nNOS, COX-1, and β-actin proteins remained unchanged in all groups. Quantification data used are the mean±S.E.M. of 3 different experiments (*P<0.05 compared to the carrageenan-injected group).
up-regulated expression of iNOS and COX-2 proteins at 24 h. Lemnalol administered at a dose of 15 mg/kg (i.m.) significantly reduced the levels of the iNOS and COX-2 to 34.5%±7.5% and 59.4%±6.8%, respectively, in comparison with the carrageenan-injected group (100% for both iNOS and COX-2). In the present study, there was no change in nNOS and COX-1 protein expression in paw tissue after any of the treatments (Fig. 6).

3.5. Paw histopathology and immunohistochemistry

Photomicrographs of sections stained with H & E and immunohistochemistry illustrate the carrageenan-induced inflammatory severity and the anti-inflammatory effect of lemnalol on paw histology at 24 h after carrageenan or carrageenan plus lemnalol (15 mg/kg, i.m.) injection. No inflammation, tissue destruction, or iNOS and COX-2 immunoreactive cells were observed in the paws of normal rats (Fig. 7A, D, G). In contrast, carrageenan-induced enlarged cavities resulting from tissue erosion were observed; these cavities were populated by infiltrating cells (neutrophils) (Fig. 7B). Treatment with lemnalol clearly inhibited the leukocyte infiltration (Fig. 7C). However, the administration of the lemnalol alone had no effect on the inflammatory response. Twenty-four hours after intraplantar carrageenan injection, numerous iNOS and COX-2 immunoreactive cells were observed in the paw tissue (Fig. 7E, H). Administration of lemnalol (15 mg/kg, i.m.) 10 min prior to the injection of carrageenan markedly reduced the increase in iNOS and COX-2 immunoreactive cells in paws (Fig. 7F, I). Intraperitoneal-administered lemnalol alone produced no inflammatory histology nor any iNOS and COX-2 immunoreactive cells in paws.

4. Discussion

Intraplantar carrageenan injection is a suitable technique for the evaluation of the effects of anti-inflammatory compounds (Di et al., 1971). The results of the present study indicated that in addition to the anti-inflammatory effect in an LPS-stimulated macrophage in vitro assay, the marine-derived natural compound lemnalol was able to down-regulate the in vivo expression of carrageenan-induced pro-inflammatory proteins iNOS and COX-2 in rat paws. We also demonstrated that carrageenan-evoked thermal hyperalgesia was significantly inhibited by the systemic or central administration of lemnalol. Moreover, systemically administered lemnalol also inhibited carrageenan-induced edema.

Many studies have demonstrated that the massive production of NO and PGE2 via pro-inflammatory proteins iNOS and COX-2, respectively, plays an important pathophysiological role in the development of carrageenan-induced thermal hyperalgesia and paw edema (Ialenti et al., 1992; Meller et al., 1994; Dirig et al., 1988; Nantel et al., 1999). Omote et al. (2001) and Salvemini et al. (1996) demonstrated the peripheral release of NO by both iNOS and nNOS in a carrageenan-induced model. Both iNOS and nNOS are selective inhibitors that are able to inhibit carrageenan-induced nociceptive behavior (Salvemini et al., 1996; Handy and Moore, 1998). However, in the present results, the intraplantar injection of carrageenan did not produce nNOS up-regulation in paw homogenates. We suggest that the lemnalol induced anti-inflammatory effects might occur via iNOS but not nNOS.

In the present study, both iNOS and COX-2 protein expression at the site of inflammation were significantly inhibited by the i.m.
injection of lemnalol. However, in both LPS-stimulated macrophage and the carrageenan-injection experiment, we found that the ability of lemnalol to down-regulate iNOS expression is greater than that of COX-2. Several studies have suggested an interaction between NO and COX-2 (Salvemini et al., 1993, 1995, 1996). The administration of the NOS non-selective inhibitor L-NMMA suppressed COX-2 expression in carrageenan-induced inflammation (Toriyabe et al., 2004). Moreover, Chun et al. (2004) demonstrated NO-induced COX-2 up-regulation through the eukaryotic transcription factor NF-κB, which is a critical regulator of COX-2 and iNOS expression. The iNOS protein induces massive NO production at the inflammatory site and contributes to COX-2 up-regulation (Toriyabe et al., 2004). Based on the foregoing evidence, we propose that lemnalol inhibits carrageenan-induced COX-2 protein expression through the down-regulation of iNOS.

Of the anti-inflammatory drugs currently in use, the steroid-related agents (such as corticosteroids) are the most potent suppressors of inflammation. There are, however, many problems and side effects associated with the long-term use of steroids. In the present study, lemnalol a chemical lacking a steroid backbone was observed to exhibit anti-inflammatory activity. Based on the present results, we suggest that lemnalol can be considered as a member of the group of drugs referred to as non-steroid anti-inflammatory agents (such as corticosteroids) are the most potent suppressors of inflammation. There are, however, many problems and side effects associated with the long-term use of steroids. In the present study, lemnalol a chemical lacking a steroid backbone was observed to exhibit anti-inflammatory activity. Based on the present results, we suggest that lemnalol can be considered as a member of the group of drugs referred to as non-steroid anti-inflammatory drugs (NSAID) that have significant therapeutic advantages in terms of steroid-induced side effects.

It is interesting to note that lemnalol has been previously reported to exhibit anti-tumor activity in several cancer cell lines (Kikuchi et al., 1983; Duh et al., 2004). The massive production of NO and prostaglandin E₂ via iNOS and COX-2, respectively, is associated with many disorders including cancer. The up-regulation of iNOS and COX-2 protein expression has been found in several human and animal cancer cell types (Xu et al., 2002; Wu, 2006). Numerous studies have provided direct evidence for the role of iNOS and COX-2 in tumorigenesis by using selective iNOS or COX-2 inhibitors (Reddy et al., 1996; Fukutake et al., 1998; Rao et al., 2002; Chen et al., 2004), which inhibit the development of tumors. Coussens and Werb (2002) have indicated that the inflammatory process is a critical component of tumor progression, and that anti-inflammatory drugs are an effective anticancer therapy. In the present study we demonstrated that lemnalol significantly inhibited the expression of pro-inflammatory protein iNOS and COX-2. Although the anticancer effects of lemnalol have been observed, the mechanism of action is not fully understood. The molecular mechanisms by which lemnalol inhibits iNOS and COX-2 protein expression remain to be investigated.

In summary, our results have demonstrated that lemnalol significantly inhibits the expression of the pro-inflammatory proteins iNOS and COX-2 in LPS-stimulated macrophage. In an in vivo study, we also found that lemnalol could produce significant anti-inflammatory and analgesic effects in intraplantar carrageenan-induced thermal hyperalgesia and paw edema in rats. Furthermore, we propose that the possible mechanism by which lemnalol exerts its anti-inflammatory and analgesic effects involves the inhibition of neutrophil infiltration and the up-regulation of iNOS and COX-2 protein expression. Our results suggest that the marine-derived natural compound lemnalol could be a potent therapeutic agent for inflammatory pain.

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