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Myrtucommulone from *Myrtus communis* Exhibits Potent Anti-Inflammatory Effectiveness in Vivo

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ABSTRACT

Myrtucommulone (MC), a nonprenylated acylphloroglucinol contained in the leaves of myrtle (*Myrtus communis*), has been reported to suppress the biosynthesis of eicosanoids by inhibition of 5-lipoxygenase and cyclooxygenase-1 in vitro and to inhibit the release of elastase and the formation of reactive oxygen species in activated polymorphonuclear leukocytes. Here, in view of the ability of MC to suppress typical proinflammatory cellular responses in vitro, we have investigated the effects of MC in in vivo models of inflammation. MC was administered to mice intraperitoneally, and paw edema and pleurisy were induced by the subplantar and intrapleural injection of carrageenan, respectively. MC (0.5, 1.5, and 4.5 mg/kg i.p.) reduced the development of mouse carrageenan-induced paw edema in a dose-dependent manner. Moreover, MC (4.5 mg/kg i.p. 30 min before and after carrageenan) exerted anti-inflammatory effects in the pleurisy model. In particular, 4 h after carrageenan injection in the pleurisy model, MC reduced: 1) the exudate volume and leukocyte numbers; 2) lung injury (histological analysis) and neutrophil infiltration (myeloperoxidase activity); 3) the lung intercellular adhesion molecule-1 and P-selectin immunohistochemical localization; 4) the cytokine levels (tumor necrosis factor-α and interleukin-1β) in the pleural exudate and their immunohistochemical localization in the lung; 5) the leukotriene B_4, but not prostaglandin E_2, levels in the pleural exudates; and 6) lung peroxidation (thiobarbituric acid-reactant substance) and nitrotyrosine and poly (ADP-ribose) immunostaining. In conclusion, our results demonstrate that MC exerts potent anti-inflammatory effects in vivo and offer a novel therapeutic approach for the management of acute inflammation.

Inflammatory diseases are accompanied by neutrophil infiltration, release of proinflammatory cytokines [such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β] and bioactive mediators [e.g., leukotrienes (LTs) and prostaglandins], and expression of adhesion molecules [intercellular adhesion molecule (ICAM)-1 and P-selectin]. Several experimental findings have shown that these mediators contribute to tissue damage characteristic of the inflammatory process (Fröde et al., 2001; Ueno and Oh-ishi, 2002; Cuzzocrea et al., 2003; Fialkow et al., 2007; Mazzon and Cuzzocrea, 2007). In addition, the elevation of intracellular Ca^{2+} concentration causes release of proteases (e.g., leukocyte elastase or cathepsin G) and formation of reactive oxygen species (ROS) (Krause et al., 1990; Conner and Grisham, 1996; Parekh and Penner, 1997), both of which destroy invading particles but also damage cells and tissues of the host. Moreover, ROS generation induced by intracellular Ca^{2+} elevation causes lipid peroxidation and DNA single-strand damage (Dix et al., 1996; Pérez-De La Cruz et al., 2008). Interference with the generation or action of these proinflammatory mediators exerts beneficial effects in a variety of inflammation models, including the carrageenan-induced paw edema and pleurisy model (Moore, 2003; Morris, 2003).

Myrtle (*Myrtus communis* L., Myrtaceae) is widely distributed in the Mediterranean area and is used as a culinary spice and an antiseptic and anti-inflammatory agent in folk

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ABBREVIATIONS: TNF, tumor necrosis factor; IL, interleukin; LT, leukotriene; ICAM, intercellular adhesion molecule; ROS, reactive oxygen species; MC, myrtucommulone; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; MPO, myeloperoxidase; PMN, polymorphonuclear leukocyte; PAR, poly (ADP-ribose); PGE_2, prostaglandin E_2; COX, cyclooxygenase; CAR, carrageenan.

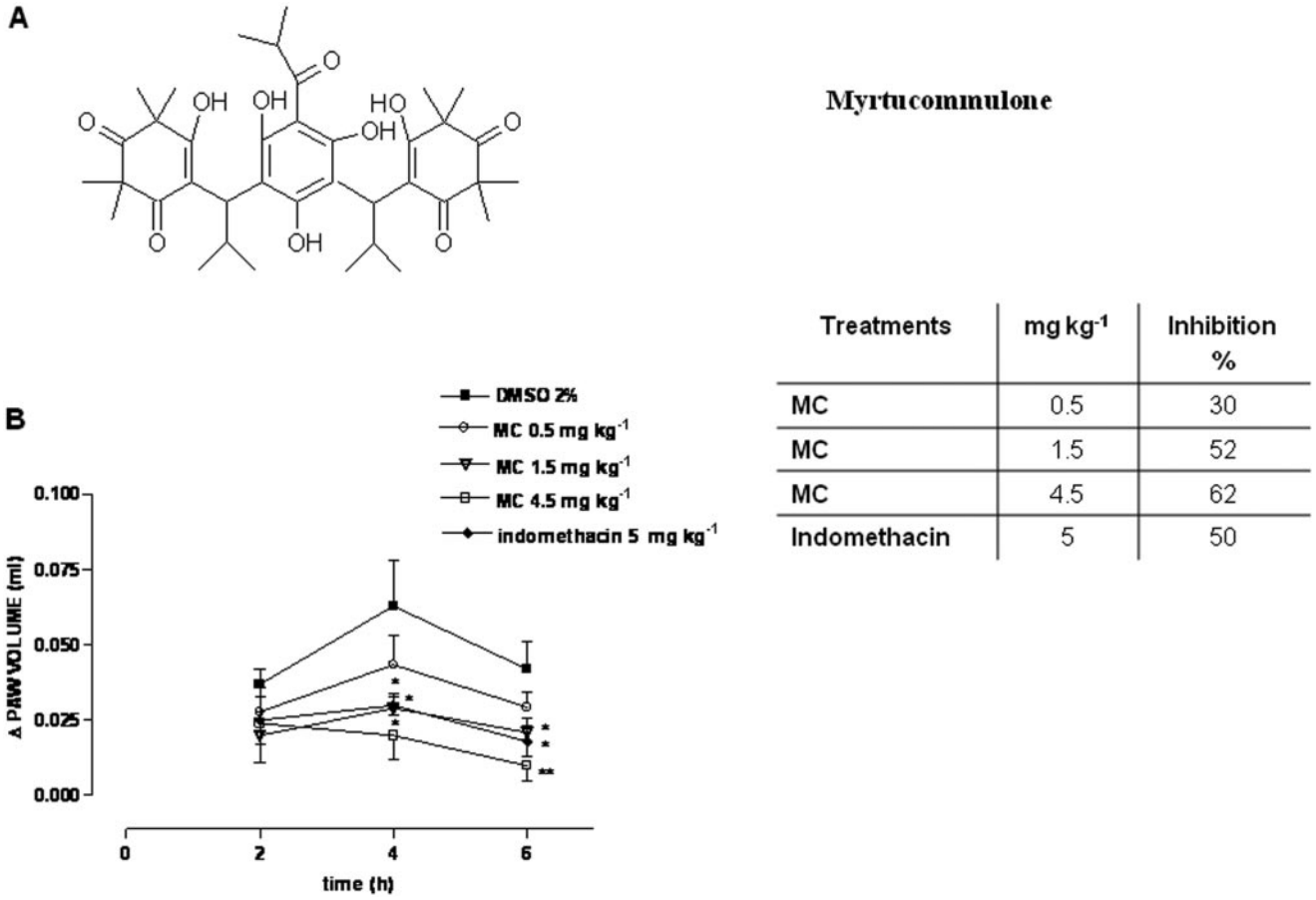
medicine (Appendino et al., 2002). Nevertheless, only a small number of studies have investigated the pharmacological effects of the plant or its specific ingredients. For example, myrtle extracts have been reported to be efficient as antibacterial (Al-Saimary et al., 2002), antihyperglycemic (Elfellah et al., 1984; Onal et al., 2005), and analgesic (Lévesque and Lafont, 2000) treatment, but there is yet no clinical use of the plant or its characteristic ingredients for treating any disease. Myrtucommulone (MC) and semimyrtucommulone are unique oligomeric, nonprenylated acylphloroglucinols contained in the leaves of myrtle. In particular, they are considered to be responsible for the antioxidative (Rosa et al., 2003) and the antibacterial (Rotstein et al., 1974; Appendino et al., 2002) activities of myrtle preparations. Moreover, MC potently suppresses the biosynthesis of eicosanoids by inhibition of 5-lipoxygenase and cyclooxygenase-1, and it inhibits the release of elastase and the formation of ROS, apparently by blocking receptor-coupled Ca^{2+} mobilization (Feisst et al., 2005). It was shown recently that MC induces apoptosis of cancer cells via the intrinsic mitochondrial pathway and only marginally affects normal nontransformed cells (Tretiakova et al., 2008).

Despite the strong effectiveness of MC as an anti-inflammatory agent in various experimental cell-based models, so far, no data concerning the in vivo action of MC in inflammation have been published. In this article, in view of the ability of MC to suppress typical proinflammatory cellular responses in vitro, we have investigated the effects of MC in in vivo models of inflammation, such as mouse carrageenan-induced paw edema and pleurisy, widely used for the in vivo screening of anti-inflammatory compounds. Our data show that MC is a highly efficient anti-inflammatory agent with a promising pharmacological profile and potential therapeutical application.

Materials and Methods

Materials. MC (Fig. 1A) was isolated from myrtle leaves as described previously (Appendino et al., 2002). The compound was dissolved in dimethyl sulfoxide (DMSO) (13.4 mg/ml) before being diluted with saline and kept in the dark at −20°C, and freezing/thawing cycles were kept to a minimum. The final concentration of DMSO in the MC solution was 2%. λ-Carrageenan type IV isolated from *Gigartina aciculaire* and *Gigartina pistillata* was purchased from Sigma-Aldrich (St. Louis, MO). Biotin and avidin were obtained from DBA (Milan, Italy). All other reagents and compounds used were obtained from Sigma-Aldrich.

Animals. Male adult CD1 mice (25–35 g; Harlan, San Pietro al Natisone, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care complied with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116192) and with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986).



Treatments	mg kg^{-1}	Inhibition %
MC	0.5	30
MC	1.5	52
MC	4.5	62
Indomethacin	5	50

Fig. 1. Chemical structure of myrtucommulone (A) and effect of MC on mouse paw edema (B). Animals (n = 10 for each experimental group) were treated i.p. with 0.5, 1.5, and 4.5 mg/kg test compound (MC) or 5 mg/kg indomethacin or 2% DMSO 30 min before carrageenan subplantar injection. Insert, percentages of inhibition caused by the treatments calculated from the relative areas under the curves. Data are expressed as mean ± S.E.M. *, $p < 0.05$; **, $p < 0.01$ versus 2% DMSO.

Carrageenan-Induced Paw Edema. Mice were divided into groups ($n = 10$ for each group) and lightly anesthetized with enflurane 4% mixed with 0.5 l/min O_2 and 0.5 l/min N_2O. Each group of animals received subplantar administration of saline (0.05 ml) or 1% λ-carrageenan type IV (w/v) (0.05 ml) in saline. The paw was marked to immerse it always at the same extent in the measurement chamber. The volume was measured by using a hydroplethismometer, specially modified for small volumes (Ugo Basile, Comerio, Italy) immediately before subplantar injection and 2, 4, and 6 h thereafter (Posadas et al., 2004). The assessment of paw volume was always performed double blind and by the same operator. The increase in paw volume was calculated by subtracting the initial paw volume (basal) from the paw volume measured at each time point.

Carrageenan-Induced Pleurisy. Mice were anesthetized with 4% enflurane mixed with 0.5 l/min O_2 and 0.5 l/min N_2O and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected, and saline (0.1 ml) or λ-carrageenan type IV 2% (w/v) (0.1 ml) was injected into the pleural cavity. The skin incision was closed with a suture, and the animals were allowed to recover. At 4 h after the injection of carrageenan, the animals were killed by inhalation of CO_2. The chest was carefully opened, and the pleural cavity was rinsed with 1 ml of saline solution containing heparin (5 U/ml) and indomethacin (10 μg/ml). The exudate and washing solution were removed by aspiration, and the total volume was measured. Any exudate that was contaminated with blood was discarded. The amount of exudate was calculated by subtracting the volume injected (1 ml) from the total volume recovered. Leukocytes in the exudate were resuspended in phosphate-buffered saline (PBS) and counted with an optical light microscope in a Burker's chamber after vital trypan blue staining.

Experimental Design. For the carrageenan-induced paw edema model in the treated group of animals, MC (0.5, 1.5, and 4.5 mg/kg) (MC-treated groups) or indomethacin (5 mg/kg) (INDO-treated group) was given i.p. 30 min before carrageenan. The vehicle-treated group of mice received vehicle (2% DMSO i.p.) instead of MC 30 min before carrageenan.

For carrageenan-induced pleurisy model in the treated group of animals, MC (4.5 mg/kg) was given i.p. 30 min before and after carrageenan (MC-treated group). The vehicle-treated group of mice received vehicle (2% DMSO i.p.) instead of MC 30 min before and after carrageenan. A group of animals underwent surgery and received saline instead of carrageenan (sham group) and received injections of vehicle (2% DMSO i.p.) or MC (4.5 mg/kg).

Determination of Myeloperoxidase Activity. Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as described previously (Laight et al., 1994). At 4 h after intrapleural injection of carrageenan, lung tissues were obtained and weighed. Each piece of tissue was homogenized in a solution containing 0.5% hexadecyltrimethylammonium bromide dissolved in 10 mM PBS, pH 7, and centrifuged for 30 min at 20,000*g* at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol peroxide/min at 37°C and was expressed in units per gram weight of wet tissue.

Histological Examination. Lung biopsies were taken 4 h after injection of carrageenan. Lung biopsies were fixed for 1 week in 10% (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol, and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Tissue sections (thickness) were then deparaffinized with xylene and stained with hematoxylin and eosin. All sections were studied using light microscopy (Dialux 22 Leitz).

Immunohistochemical Localization of TNF-α, IL-1β, ICAM-1, P-Selectin, Nitrotyrosine, and Poly(ADP-Ribose). At 4 h after carrageenan administration, the lungs were fixed in 10% buffered formaldehyde, and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 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 section in 2% normal goat serum in PBS for 20 min. Endogenous biotin or avidin-binding sites were blocked by sequential incubation for 15 min with avidin and biotin. The sections were then incubated overnight with primary anti-ICAM-1 (1:500), anti-P-selectin (1:500), anti-TNF-α (1:500), anti-IL-1β (1:500), anti-nitrotyrosine (1:1000), and anti-poly(ADP-ribose) (PAR) (1:500) antibodies or with control solutions. Sections were washed with PBS and incubated with secondary antibody for 2 h at room temperature. To confirm that the immunoreaction for the nitrotyrosine was specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess of nitrotyrosine (10 mM) to verify the binding specificity. To verify the binding specificity for ICAM-1, P-selectin, TNF-α, IL-1β, and PAR, sections were also incubated with the primary antibody (no secondary) or with the secondary antibody (no primary). Under these conditions, no positive staining was found in the sections, indicating that every immunoreaction was specific. Immunocytochemistry photographs were assessed by densitometry and scored by two independent blind situations. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266).

Measurement of Prostaglandin E_2 and LTB_4 Levels. The amount of prostaglandin E_2 (PGE_2) and LTB_4 was evaluated in the exudate at 4 h after the induction of pleurisy by carrageenan injection. The assay was carried out by using a colorimetric, commercial kit (Calbiochem, San Diego, CA).

Total Protein Extraction and Western Blot Analysis for COX-2. At 4 h after carrageenan administration, the lungs were homogenized in a buffer containing 20 mM HEPES, pH 7.6, 1.5 mM $MgCl_2$, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 15 μg/ml trypsin inhibitor, 3 μg/ml pepstatin, 2 μg/ml leupeptin, 40 μM benzamidine, 1% Nonidet P-40, 20% glycerol, and 50 mM NaF. The homogenates were centrifuged at 10,000*g* for 15 min and at 4°C, the supernatant was collected, and protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein (50 μg) were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, and 2 mg/ml bromphenol) in a ratio of 1:1, boiled for 3 min, and centrifuged at 10,000*g* for 10 min. Each sample was loaded and electrophoresed on a 10% SDS-polyacrylamide gel. The proteins were transferred on to nitrocellulose membranes. The membranes were blocked with 0.1% PBS-Tween containing 5% nonfat dry milk. After the blocking, the membranes were incubated with the primary antibody overnight at 4°C. Rabbit monoclonal antibody anti-COX-2 was diluted 1:1000 in 0.1% PBS-Tween. After the incubation, the membranes were washed six times with 0.1% PBS-Tween and were incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated anti-mouse secondary antibodies (Dako North America, Inc., Carpinteria, CA) diluted 1:1000 in 0.1% PBS-Tween containing 5% nonfat dry milk. The membranes were washed, and protein bands were detected by an enhanced chemiluminescence system (GE Healthcare, Chalfont St. Giles, UK). Densitometric analysis was performed with a Fluor S quantitative imaging system (Bio-Rad).

Measurement of TNF-α and IL-1β Levels. TNF-α and IL-1β levels were evaluated in the exudates at 4 h after the induction of pleurisy by carrageenan injection. The assay was carried out by using a colorimetric, commercial enzyme-linked immunosorbent assay kit (Calbiochem).

Thiobarbituric Acid-Reactant Substance Measurement. Thiobarbituric acid-reactant substance measurement, which is considered a good indicator of lipid peroxidation in the lung tissues, was carried out. Tissues, collected 4 h after carrageenan administration, were homogenized in 1.15% KCl solution. An aliquot (0.1 ml) of the homogenate was added to a reaction mixture containing 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid, pH 3.5, 1.5 ml of 0.8% thiobarbituric acid, and 0.7 ml of distilled water. Samples were then boiled

for 1 h at 95°C and centrifuged at 3000*g* for 10 min. The optical density at 650 nm (OD_{650}) was measured using an enzyme-linked immunosorbent assay microplate reader (SLT-Labinstruments, Salzburg, Austria). Thiobarbituric acid-reactant substances were calculated by comparison with OD_{650} of standard solutions of 99% 1,1,3,3-tetramethoxypropan and 99% malondialdehyde bis(dimethyl acetal).

Statistical Procedures. All values in the figures and text are expressed as means ± S.E. of *n* observations, where *n* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three independent experiments performed on different experimental days. The data were analyzed by one-way analysis of variance followed by a Bonferroni post hoc test for multiple comparisons. A p value less than 0.05 was considered significant.

Results

Carrageenan-Induced Mouse Paw Edema

Injection of carrageenan into the mouse paw produced a marked increase of paw volume, reaching its maximal effect after 4 h (Fig. 1B). Pretreatment of mice with MC (0.5, 1.5, and 4.5 mg/kg), injected 30 min before carrageenan, dose-dependently attenuated the inflammatory response at 4 and 6 h (Fig. 1B). In mice treated with 0.5, 1.5, and 4.5 mg/kg MC, the peak of the response to carrageenan at 4 h was reduced by 31, 54, and 68%, respectively, whereas indomethacin (5 mg/kg), a nonsteroidal anti-inflammatory drug used as a reference, caused 57% inhibition. Comparison of the areas under the curves of each group, between 2 and 6 h after carrageenan injection, yielded similar degrees of inhibition (Fig. 1B, insert).

Carrageenan-Induced Pleurisy

Inflammatory Response To further assess the effectiveness of MC as an anti-inflammatory agent in vivo, the compound was tested in carrageenan-induced pleurisy in mice. Injection of carrageenan into the pleural cavity of mice (2% DMSO group) elicited an acute inflammatory response characterized by the accumulation of fluid (Fig.

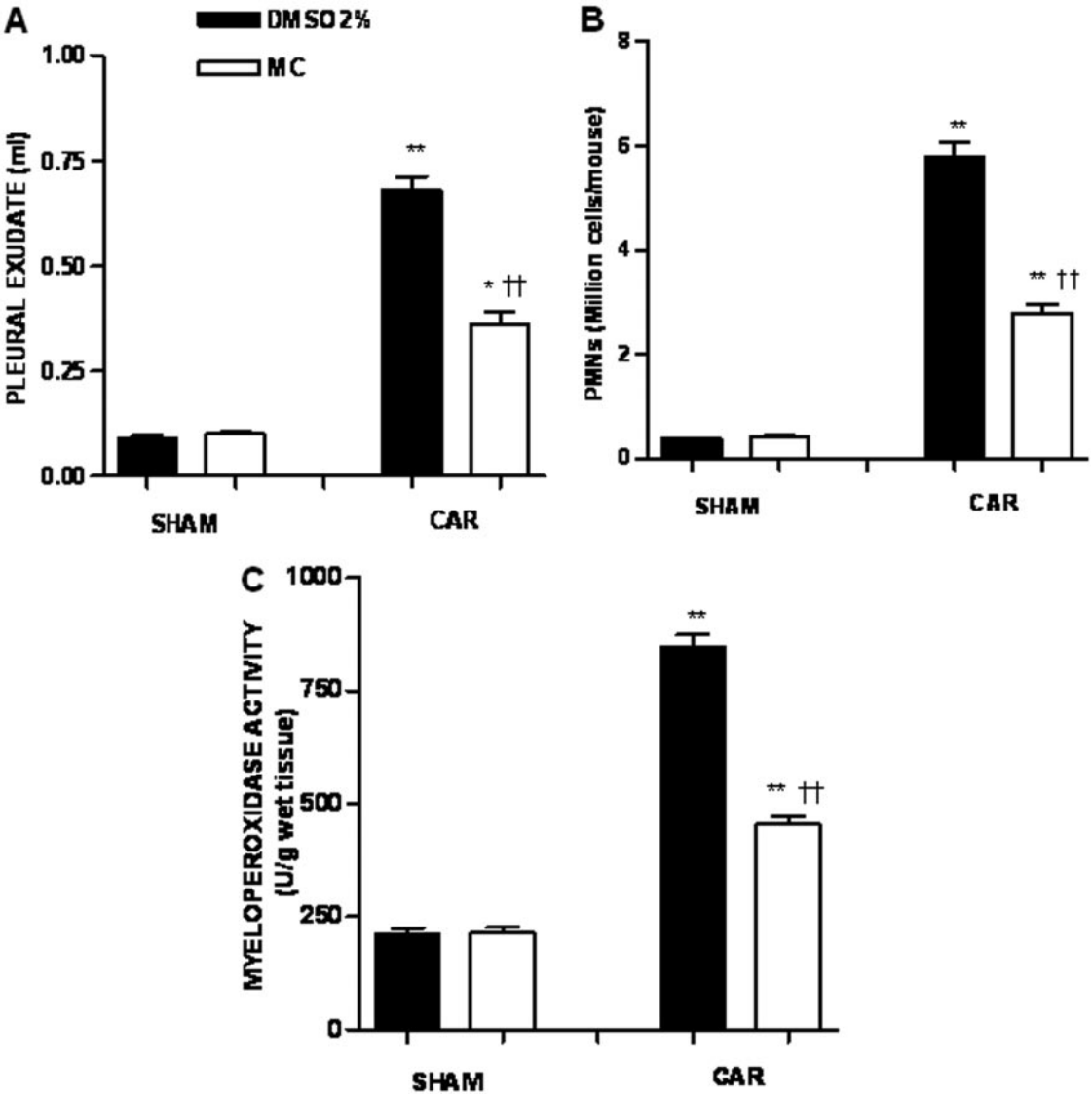


Fig. 2. Effect of MC on carrageenan-induced pleurisy. Thirty min before and after carrageenan (CAR) or saline (SHAM) intrapleural injection, mice (n = 10 for each experimental group) were treated i.p. with 4.5 mg/kg MC or 2% DMSO. The exudate volume (A) and PMN accumulation (B) in pleural cavity and lung MPO activity (C) were assessed 4 h after carrageenan or saline injection. Data are expressed as mean ± S.E.M. *, $p < 0.05$; **, $p < 0.01$ versus SHAM; ††, $p < 0.01$ versus 2% DMSO.

2A) that contained large numbers of PMNs (Fig. 2B) compared with the corresponding sham animals. As observed in carrageenan-induced paw edema, MC (4.5 mg/kg i.p., 30 min before and after the carrageenan) significantly ($p < 0.01$) inhibited the inflammatory response induced by intrapleural injection of carrageenan, as demonstrated by the significant attenuation of exudate formation (Fig. 2A) and PMN infiltration (Fig. 2B). The action of MC on cell migration to the inflammatory site was further supported by the measurement of lung MPO activity, an index of neutrophil infiltration characteristic of the inflammatory process (Fröde and Medeiros, 2001). In fact, MC (4.5 mg/kg i.p., 30 min before and after the carrageenan) significantly ($p < 0.01$) reduced the increase of lung MPO activity induced by carrageenan administration (Fig. 2C).

Histological examination of lung sections from sham-treated (Fig. 3A) and carrageenan-treated (Fig. 3B) mice showed that injection of carrageenan induced edema, lung injury, and infiltration of inflammatory cells within 4 h (Fig. 3B, see arrows). Treatment with MC (4.5 mg/kg i.p., 30 min before and after carrageenan) (Fig. 3C) significantly attenuated the edema and the infiltration of PMNs into the lung, leading to a clear reduction of tissue injury.

Expression of ICAM-1 and P-Selectin. Carrageenan-induced neutrophil infiltration in lung tissues was associated with expression of adhesion molecules. In fact, positive staining for ICAM-1 (Fig. 4, A and A1, see arrows; Table 1) and P-selectin (Fig. 4, C and C1, see arrows; Table 1) was increased along the bronchial epithelium and in vessels from carrageenan-treated (4 h) mice. Less positive staining for ICAM-1 and P-selectin was obvious in the lungs of carrageenan-treated mice that received MC (4.5 mg/kg i.p., 30 min before and after the carrageenan) (Fig. 4, B and D; Table 1).

LTB_4 and PGE_2 Levels. Because MC is an inhibitor of 5-lipoxygenase and COX-1, the metabolites of these enzymes, i.e., LTB_4 and PGE_2, have been determined in pleural exudates (Fig. 5, A and B). In comparison with the corresponding sham animals, the levels of LTB_4 and PGE_2 were significantly ($p < 0.01$) elevated in pleural exudates from 2% DMSO-treated mice at 4 h after carrageenan administration. Treatment with MC (4.5 mg/kg i.p., 30 min before and after the carrageenan) significantly decreased ($p < 0.01$) the levels of LTB_4, whereas it did not affect PGE_2 production (Fig. 5, A and B) and lung COX-2 expression (data not shown).

TNF-α and IL-1β Levels. Production of inflammatory mediators (e.g., TNF-α and IL-1β) by neutrophils represents a pivotal biochemical event to maintain and propagate the inflammatory response within injured tissues (Fröde et al., 2001). In comparison with the corresponding sham animals,

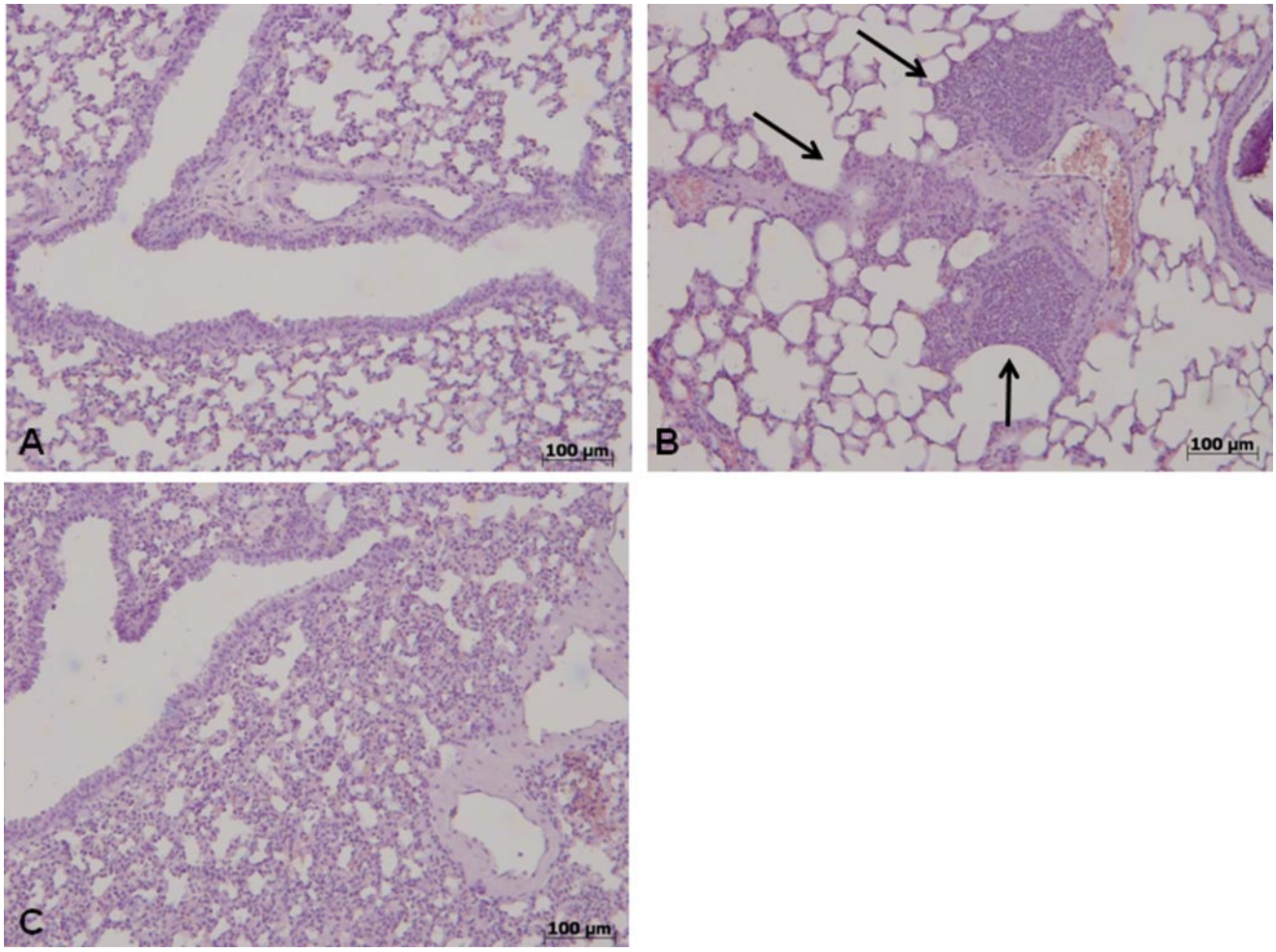


Fig. 3. Effect of MC on lung injury. Compared with lung sections taken from sham-treated animals (A), lung sections from carrageenan-treated mice (B) demonstrated edema and PMN infiltration (see arrows). Lung sections from carrageenan-treated mice that received MC exhibited reduced tissue injury and inflammatory cell infiltration (C). Figure is representative of all the animals in each group.

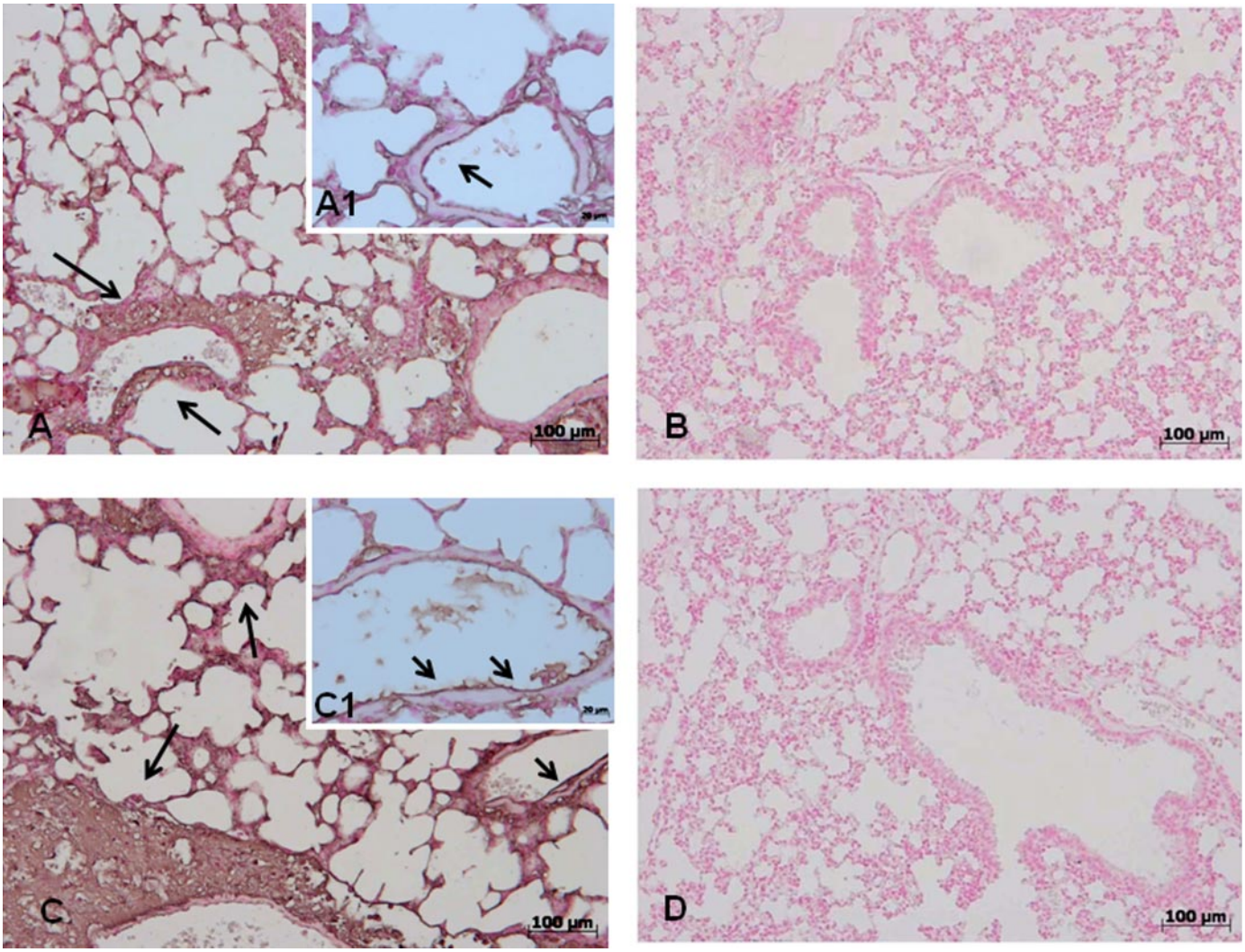


Fig. 4. Effect of MC on the immunohistochemical localization of ICAM-1 and P-selectin in the lung. Immunohistochemical analyses of sections from carrageenan-treated mice showed positive staining for ICAM-1 (A and A1, see arrows) and P-selectin (C and C1, see arrows). The intensity of staining for ICAM-1 (B) and P-selectin (D) was markedly reduced in tissue sections obtained from MC-treated mice. The figures are representatives of all the animals in each group.

TABLE 1

Typical densitometry evaluation: analysis of ICAM-1, P-selectin, TNF-α , IL-1β , nitrotyrosine, and PAR

The assay was carried out by using Optilab Graftek software. Data are expressed as means ± S.E. of percentage of total tissue area. In particular, the densitometry analysis was carried out in the section in which the lung was orientated to observe all the histological portions.

	Percentage of Total Lung Tissue Area					
	ICAM-1	P-Selectin	TNF-α	IL-1β	Nitrotyrosine	PAR
CAR + 2% DMSO	5.1 ± 0.5	6.0 ± 0.5	5.2 ± 0.5	5.1 ± 0.5	6.4 ± 0.5	6.1 ± 0.5
CAR + MC	1.0 ± 0.08**	0.9 ± 0.08**	1.0 ± 0.5**	1.1 ± 0.08**	1.0 ± 0.08**	1.1 ± 0.08**

** $p < 0.01$ vs. CAR + 2% DMSO.

the levels of TNF-α and IL-1β were significantly ($p < 0.01$) elevated in pleural exudates from 2% DMSO-treated mice at 4 h after carrageenan administration (Fig. 5, C and D). Treatment with MC (4.5 mg/kg i.p., 30 min before and after the carrageenan) significantly decreased ($p < 0.01$) the levels of these proinflammatory cytokines (Fig. 5, C and D). In addition, tissue sections obtained from carrageenan-treated mice (2% DMSO group) showed positive staining for TNF-α (Fig. 6, A and A1; Table 1) and IL-1β (Fig. 6, C and C1; Table 1). In carrageenan-treated mice, which received MC, the staining for TNF-α and IL-1β was significantly ($p < 0.01$) and visibly reduced (Fig. 6, B and D; Table 1) in comparison with 2% DMSO-treated animals.

Lipid Peroxidation, Nitrotyrosine, and PAR Formation. Infiltration of neutrophils into tissues and the subsequent production of ROS induce lipid peroxidation, nitrosative stress, and DNA damage (Azad et al., 2008). To evaluate the effects of MC on lipid peroxidation in the lung, the levels of thiobarbituric acid-reactant substance were measured. As shown in Fig. 7A, thiobarbituric acid-reactant substance levels were significantly ($p < 0.01$) increased in the lung of carrageenan-treated mice (2% DMSO group) compared with

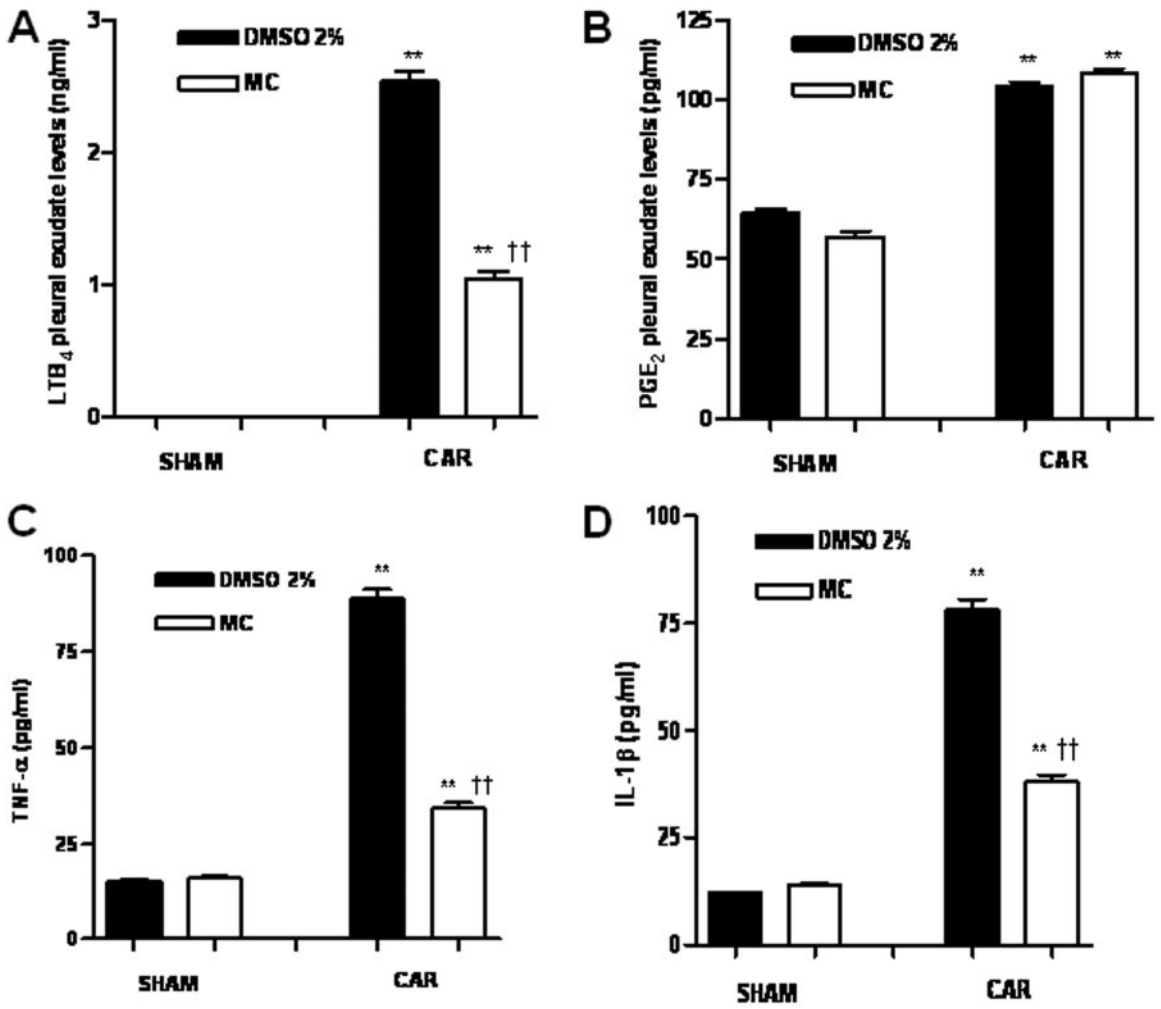


Fig. 5. Effect of MC on LTB_4, PGE_2, TNF-α, and IL-1β levels in the pleural exudates. Thirty minutes before and after CAR or saline (SHAM) intrapleural injection, mice ($n = 10$ for each experimental group) were treated i.p. with 4.5 mg/kg MC or 2% DMSO. The LTB_4 (A), PGE_2 (B), TNF-α (C), and IL-1β (B) levels in the pleural were assessed 4 h after carrageenan or saline injection. Data are expressed as mean ± S.E.M. **, $p < 0.01$ versus SHAM; ††, $p < 0.01$ versus 2% DMSO.

the corresponding sham animals. In mice treated with MC (4.5 mg/kg i.p., 30 min before and after carrageenan), lung thiobarbituric acid-reactant substance levels were significantly ($p < 0.01$) reduced in comparison with those of 2% DMSO-treated mice (Fig. 7A). Nitrosative stress and DNA damage were evaluated by immunohistological staining for nitrotyrosine (Fig. 7, B and B1, see arrows) and PAR (Fig. 7, D and D1, see arrows) performed on lung sections taken 4 h after carrageenan injection, respectively. MC (4.5 mg/kg i.p., 30 min before and after carrageenan) significantly ($p < 0.01$) attenuated the staining for nitrotyrosine (Fig. 7C, see arrows) and PAR (Fig. 7E, see arrows) (Table 1) in comparison with 2% DMSO-treated animals.

Discussion

MC, an oligomeric, nonprenylated acylphloroglucinol contained in the leaves of myrtle, exhibits inhibitory properties in vitro visualized by the suppression of various functional cellular leukocyte responses, including the biosynthesis of prostaglandins and LT, the release of leukocyte elastase, and the generation of ROS (Feisst et al., 2005). In view of these findings, MC and myrtle preparations have been suggested as a potential therapeutic for the treatment of inflammatory and allergic diseases. To explore the anti-inflammatory potential of MC to allow the judgment of its therapeutic benefit for the treatment of inflammation in humans, data from appropriate in vivo studies using animal models are required.

To this aim, we have investigated the effect of MC in in vivo models of acute inflammation in mice, such as carrageenan-induced paw edema and pleurisy. In fact, these inflammatory models are widely used to determine the anti-inflammatory activity of compounds in vivo (Moore, 2003; Morris, 2003). In particular, the mouse paw edema has been used to screen for new anti-inflammatory drugs, whereas carrageenan-induced pleurisy is more suitable to study the mechanisms involved in the anti-inflammatory action of test compounds.

Injection of carrageenan in the mouse paw induced an acute inflammatory response (0–6 h) characterized by an increase in vascular permeability and PMN infiltration related to edema formation at the site of inflammation (Posadas et al., 2004). The i.p. treatment of mice with MC reduced the development of edema induced by carrageenan at 4 and 6 h in a dose-dependent manner. In particular, MC seemed to

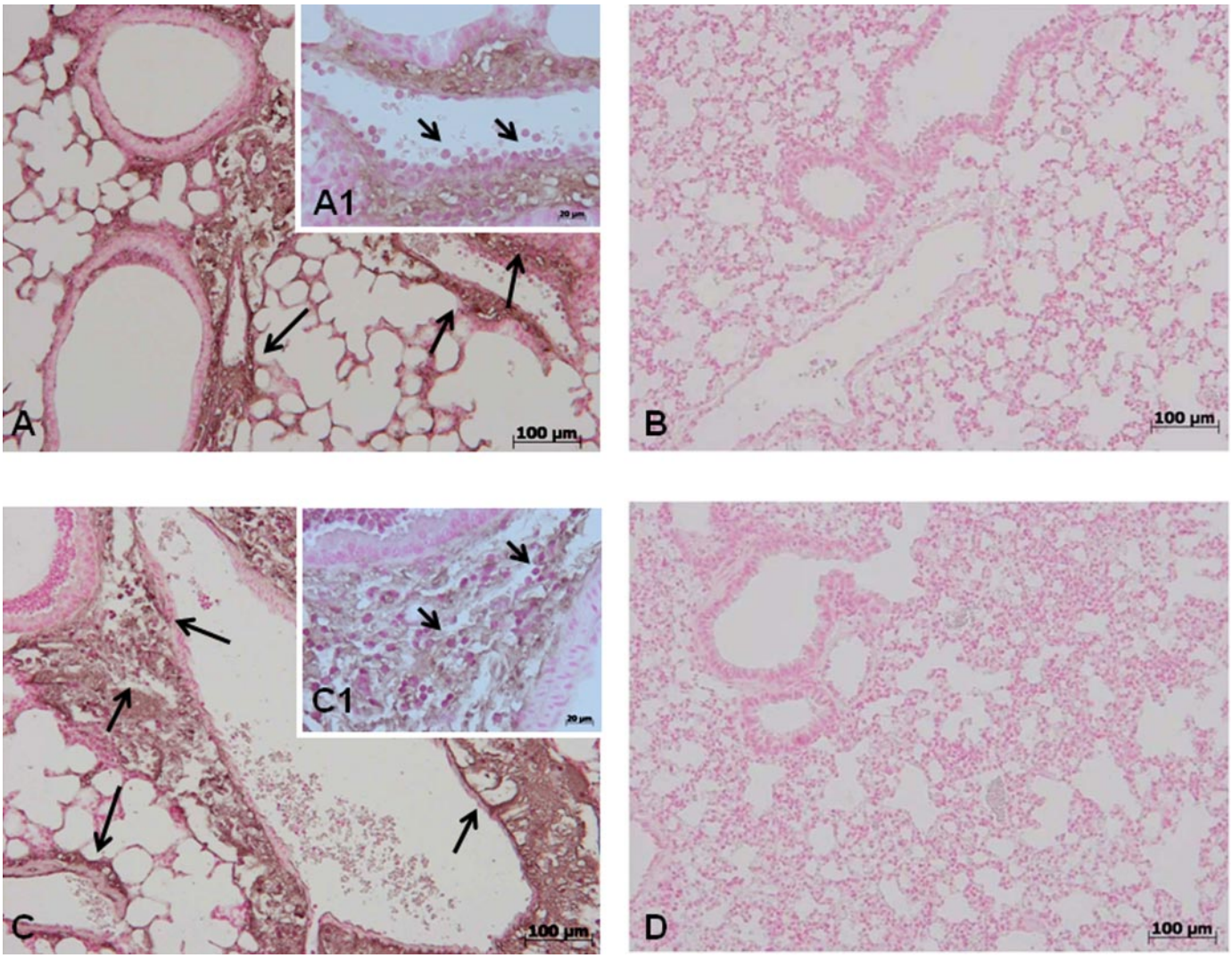


Fig. 6. Effect of MC on the TNF-α and IL-1β immunohistochemical localization in the lung. Immunohistochemical analyses of sections from carrageenan-treated mice showed positive staining for TNF-α (A and A1, see arrows) and IL-1β (C and C1, see arrows). The intensity of staining for TNF-α (B) and IL-1β (D) was markedly reduced in tissue sections obtained from MC-treated mice. The figures are representatives of all the animals in each group.

be more potent than indomethacin, confirming a remarkable anti-inflammatory effectiveness of the compound in vivo.

The onset of local inflammation after carrageenan is linked to generation of ROS, release of leukocyte elastase, and formation of LT from neutrophils. Because MC blocked these neutrophil responses in vitro, we attribute the effectiveness of MC in carrageenan-induced paw edema to such interrelations. This hypothesis is supported by the results obtained in the in vivo model of acute inflammation, the carrageenan-induced pleurisy, which enables the analysis of the cellular and molecular mechanisms responsible for the anti-inflammatory action of the test compound. In fact, one possible mechanism by which MC attenuated carrageenan-induced pleurisy (exudate volume and cell number) might be the reduction of PMN infiltration by down-regulating the adhesion molecules ICAM-1 and P-selectin. In particular, we observed that the appearance of P-selectin on the endothelial vascular wall and the up-regulation of expression of ICAM-1 on endothelial cells induced by acute lung inflammation (4 h after carrageenan administration) was abolished by MC treatment. As a consequence, MC might interrupt the interactions between neutrophils and endothelial cells, both at the early rolling phase mediated by P-selectin and at the late firm adhesion phase mediated by ICAM. The reversal of increased expression of the adhesion molecules in the lung tissue of carrageenan-treated mice treated with MC correlated with the reduction of leukocyte infiltration, as assessed by measuring the granulocyte-specific MPO activity, and with the attenuation of lung tissue damage as evaluated by histological examination. Moreover, our results demonstrating that MC inhibits LTB_4 generation in the pleural exudates of carrageenan-treated mice are in agreement with the report of Feisst et al. (2005), who have showed that MC potently inhibits the formation of LT (IC_{50} = 1.8 μM) by interference with 5-lipoxygenase (Feisst et al., 2005). Because 5-lipoxygenase regulates the expression of adhesion molecules (Cuzzocrea et al., 2003), it is reasonable to speculate that MC might reduce their expression via inhibition of LT synthesis. Leukotriene B_4 is a potent chemotactic and chemokinetic factor that recruits neutrophils (Ford-Hutchinson et al., 1980), and MC clearly reduced the number of PMN in the exudates of inflamed mouse pleurisy. However, more de-

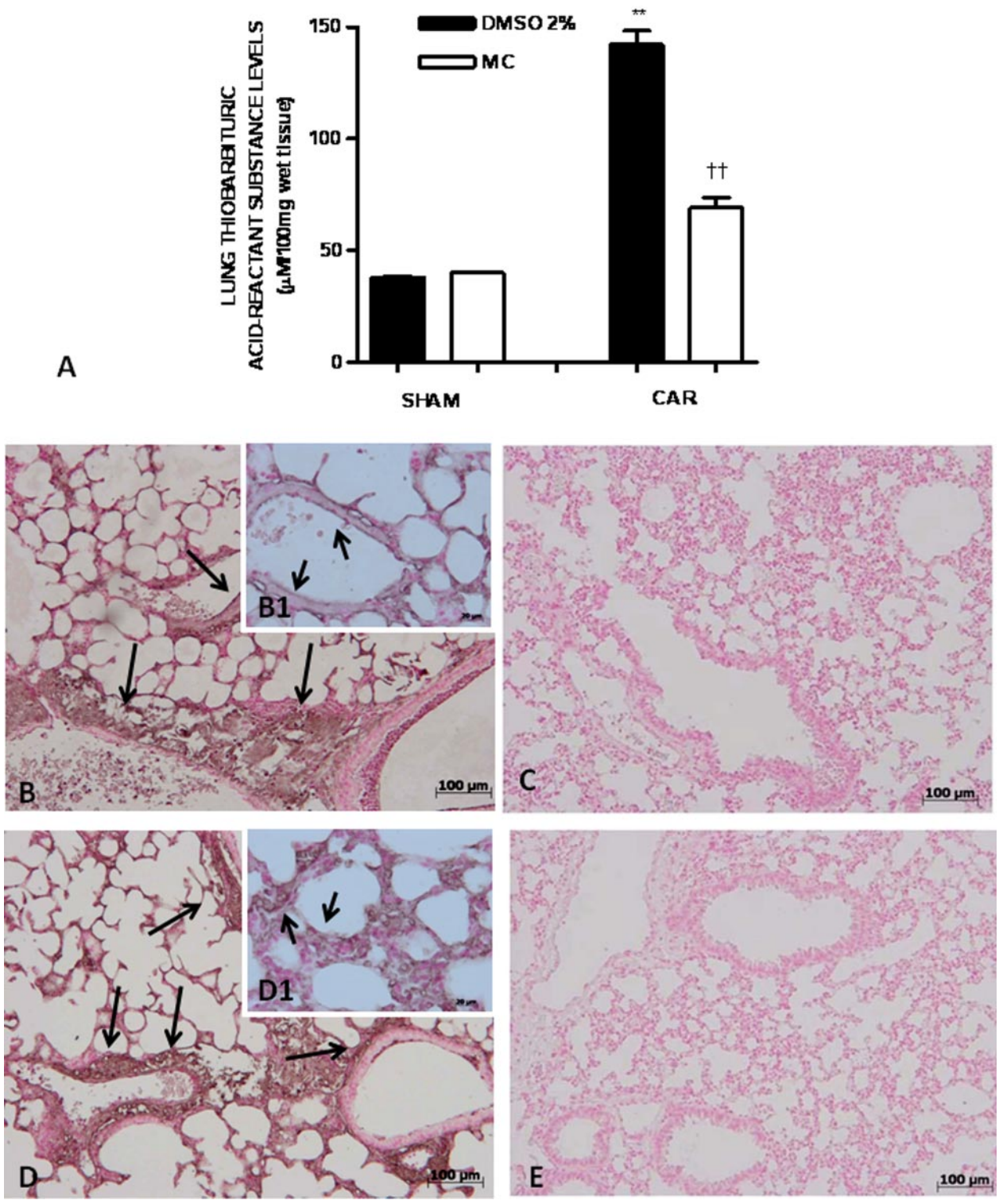


Fig. 7. Effect of MC on lung lipid peroxidation (A) and on the immunohistochemical localization of nitrotyrosine (B and B1) and PAR (D and D1) in the lung. Thirty minutes before and after CAR or saline (SHAM) intrapleural injection, animals ($n = 10$ for each experimental group) were treated i.p. with 4.5 mg/kg MC or 2% DMSO. Thiobarbituric acid-reactant substance levels (A) were determined in the lung 4 h after carrageenan injection. Data are expressed as mean ± S.E.M. **, $p < 0.01$ versus SHAM; ††, $p < 0.01$ versus 2% DMSO. Immunohistochemical analyses of sections from carrageenan-treated mice showed positive staining for nitrotyrosine (B and B1, see arrows) and PAR (D and D1, see arrows). The intensity of staining for nitrotyrosine (C) and PAR (E) was markedly reduced in tissue sections obtained from MC-treated mice. The figures are representatives of all the animals in each group.

tailed studies are needed to reveal a clear interrelation between these processes.

It is interesting that in our experimental conditions, MC did not inhibit PGE_2 generation. Thus, although there is good evidence in this and in other models of inflammation that an enhanced formation of prostanoids contributes to the pathophysiology of local inflammation (Futaki et al., 1993; Mitchell et al., 1993; Salvemini et al., 1995; Harada et al., 1996;

Sautebin et al., 1998), the anti-inflammatory action of MC does not involve PGE_2 generation.

It has been suggested that neutrophil activation in lung injury represents an important source of other inflammatory molecules, such as TNF-α, IL-1β, and ROS (Conner and Grisham, 1996). Moreover, various studies have shown clearly that inhibition of TNF-α and IL-1β formation significantly prevents the development of the inflammatory process (Mageed et al., 1998; Fröde et al., 2001; Mukherjee et al., 2005). Our results demonstrate that MC attenuates the production of TNF-α and IL-1β in pleural exudates and lungs of carrageenan-treated mice, which might contribute to the mechanism underlying the anti-inflammatory effectiveness of MC in carrageenan-induced pleurisy. Alternatively, the inhibition of TNF-α and IL-1β formation by MC might reflect its inhibitory effects on neutrophil infiltration (Fröde et al., 2001; Mazzon and Cuzzocrea, 2007). With regard to ROS, it is well known that their generation by neutrophils is responsible for lipid peroxidation, nitrosative stress, and DNA single-strand damage (Dix et al., 1996), which then cause cellular dysfunction, damage, and eventually cell death (Carmody and Cotter, 2001). Lung damage because of ROS production is associated with high concentrations of thiobarbituric acid-reactant substances, which are considered a good indicator of lipid peroxidation, whereas nitration of tyrosine and PARS activation can be considered as global biomarker of nitrosative stress (Beckman, 1996) and DNA damage, respectively. The reduced lipid peroxidation, nitrosative stress, and DNA damage observed in the present study suggest that MC decreases ROS production in the carrageenan-treated mice. Because MC is able to suppress ROS generation in isolated PMN stimulated with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine by its ability to block receptor-coupled Ca^{2+} mobilization at submicromolar concentrations (Feisst et al., 2005), these observed in vivo actions could be related to this effect. In addition, a mechanism has been identified where G protein-coupled receptor-mediated Ca^{2+} signals are translated into mitochondrion-derived superoxide, which up-regulates the expression of ICAM-1 and elicits leukocyte/endothelial cell firm adhesion (Hawkins et al., 2007). Thus, the inhibitory effect of MC on ROS formation and on the expression of adhesion molecules might eventually depend on interference with intracellular Ca^{2+} signaling. In addition, MC may also have a direct effect on oxidative enzymes, in accordance with the antioxidant activity observed by others (Rosa et al., 2003).

Taken together, these findings indicate that MC exerts potent anti-inflammatory effects in vivo, as evidenced by its inhibitory action in two well known models of acute experimental inflammation. Therefore, we successfully expanded our initial observations that MC acts as an anti-inflammatory agent based on its inhibitory action on 5-lipoxygenase (Feisst et al., 2005) and other proinflammatory leukocyte functions.

In conclusion, our data suggest that the suppression of adhesion molecules and inhibition of LTB_4 generation and PMN infiltration are part of the significant mechanisms of lung injury protection by MC. Our findings further support a future potential use of MC as a therapeutic agent in the therapy of conditions associated with acute inflammation.

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