ELSEVIER

European Journal of Pharmacology 343 (1998) 79–86

ejp

Magnolol inhibits Mac-1 (CD11b/CD18)-dependent neutrophil adhesion: Relationship with its antioxidant effect

Yuh-Chiang Shen [a], Yen-Jen Sung [b], Chieh-Fu Chen [a,c,*]

[a] *Institute of Pharmacology, School of Life Science, National Yang-Ming University, Taipei, Taiwan*
[b] *Department of Anatomy, School of Medicine, National Yang-Ming University, Taipei, Taiwan*
[c] *National Research Institute of Chinese Medicine, Section 2, 155-1 Li-Nung Street, Shih-Pai, Taipei 11221, Taiwan*

Received 28 July 1997; revised 5 November 1997; accepted 11 November 1997

Abstract

Magnolol, a phenolic compound isolated from a Chinese herbal drug, *Magnolia officinalis*, has been shown to protect rat heart from ischemia/reperfusion injury. Neutrophil adhesion plays a crucial process during this inflammatory response. To evaluate whether magnolol prevents ischemia/reperfusion injury by inhibiting neutrophil adhesion, we determined whether magnolol can inhibit adhesion of phorbol-12-myristate-13-acetate (PMA)-activated human neutrophils to a fibrinogen-coated surface in a dose-dependent manner. Using flow cytometric analysis, we observed that magnolol pretreatment (10 min at 37°C) diminished PMA (100 ng/ml)-induced Mac-1 upregulation. PMA also induced rapid intracellular accumulation of superoxide ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2) in neutrophils; magnolol pretreatment attenuated the accumulation of these two substances. Inhibition of reactive oxygen species by superoxide dismutase and/or catalase, which decompose $O_2^{-\cdot}$ and H_2O_2, respectively, also abolished Mac-1 upregulation and neutrophil adhesion. We conclude that magnolol inhibits neutrophil adhesion and that this can account for its anti-ischemia/reperfusion injury effect. We propose that the inhibitory effect of magnolol on neutrophil adhesion to the extracellular matrix is mediated, at least in part, by inhibition of the accumulation of reactive oxygen species, which in turn suppresses the upregulation of Mac-1 that is essential for neutrophil adhesion.

Keywords: Magnolol; Neutrophil; Mac-1 (CD11b/CD18); Superoxide; Hydrogen peroxide; Phorbol-12-myristate-13-acetate (PMA)

1. Introduction

Magnolol, an active principle isolated from the Chinese herb 'Houp,u' (*Magnolia officinalis*), has been shown to be an anti-platelet aggregation (Teng et al., 1988), vessel dilation (Teng et al., 1990) and anti-inflammation (Wang et al., 1992) agent. The anti-inflammatory effects of magnolol can be accounted for by its multiple pharmacological activities including inhibition of prostaglandin D_2 formation (Wang et al., 1992), suppression of non-selective vascular hyporeactivity to mediators (Wang et al., 1993), reduction of the formation of eicosanoids mediators (Wang et al., 1995) as well as its antioxidant potential (Lo et al., 1994; Chang et al., 1994; Chan et al., 1996) and its ability to prevent ischemic-reperfusion injury (Hong et al., 1996). Despite the numerous studies that have been performed, the mechanisms involved in these anti-inflammatory effects, especially the ischemic-reperfusion injury prevention effect, of magnolol remain unclear.

Recruitment of neutrophils into areas of inflammation is a key event during inflammatory responses. This begins with the binding of neutrophils to the endothelium, followed by their transmigration into tissues (Albelda et al., 1994). Neutrophil binding to the endothelium is characterized by distinct phases, including rolling, activation and firm adhesion (Ley, 1996). The molecular basis for these phases is the upregulation of various cell adhesion molecules that belong to three major families: (1) the selectins (e.g. P-selectin, L-selectin and E-selectin) (Bevilacqua and Nelson, 1993; Lefer et al., 1994), (2) the $\beta 2$ integrins (e.g. CD11/CD18) (Ruoslahti, 1991) and (3) the immunoglobulin superfamily (e.g. intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and platelet-endothelial cell adhesion molecule-1) (Springer, 1990). While the selectins are important for rolling, firm adhesion of neutrophils is essentially $\beta 2$ integrin dependent (Springer and Anderson, 1986; Arfors et al., 1987). The $\beta 2$ integrins comprise a group of heterodimeric glycoproteins, including CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1) and CD11c/CD18 (p150,95), with

* Corresponding author. Tel.: +886-2-8225592; fax: +886-2-8230488.

LFA-1 and Mac-1 being the major integrins on neutrophils. Although all three of these $\beta 2$ integrins may participate in the intercellular adhesive interaction, Mac-1 appears to be the principal form accountable for the firm adhesion of neutrophils (Lefer and Lefer, 1996). Increased neutrophil adhesion has been demonstrated to be an important factor in the pathogenesis of vascular injury during inflammatory processes, such as ischemia/reperfusion injury of the heart (Lefer, 1995), liver (Monden et al., 1995) and brain (Matsuo et al., 1994). Hence, therapeutic interventions targeting these phases of neutrophil adhesion may prove to be effective. This can be illustrated by the observation that administration of a monoclonal antibody directed against CD11b (Mac-1) could prevent intestine ischemia/reperfusion-induced lung injury, regardless of the activation state of neutrophils (Koike et al., 1995). However, it has been demonstrated that both superoxide and hydrogen peroxide can modulate leukocyte Mac-1 expression and leukocyte endothelial adhesion, an effect which can be diminished by superoxide dismutase and/or catalase pretreatment (Fraticelli et al., 1996; Serrano et al., 1996). Given the anti-oxidant potential of magnolol (Chang et al., 1994; Lo et al., 1994; Chan et al., 1996), whether magnolol can inhibit neutrophil adhesion as a mechanism to prevent ischemia/reperfusion injury, therefore, warrants further investigation.

We hypothesize that inhibition of ischemia/reperfusion injury by magnolol may be mediated by interference with the upregulation of adhesion molecules, leading to inhibition of neutrophil adhesion. As mentioned above, firm adhesion of neutrophils to the endothelium and to a large array of extracellular matrix proteins is primarily Mac-1 dependent (Beatty et al., 1983; Lefer and Lefer, 1996). Adhesion of phorbol-12-myristate-13-acetate (PMA)-activated neutrophils to a fibrinogen-coated surface was measured to investigate the effects of magnolol. We also examined the expression of Mac-1 on the surface of neutrophils after magnolol treatment, since both the expression and activation of Mac-1, through conformational change, have been shown to be important in the firm adhesion of neutrophils (Albelda et al., 1994; Ley, 1996). Moreover, because it has been shown that reactive oxygen species (such as superoxide, $O_2^{-\cdot}$, and hydrogen peroxide, H_2O_2) can stimulate the translocation of Mac-1 from intracellular stores to the cell surface (Simms and D'amico, 1995), we examined the role of reactive oxygen species inhibition in the anti-adhesive effect of magnolol.

2. Materials and methods

2.1. Preparation of neutrophils

Venous blood samples were collected with syringes containing heparin (final concentration 20 unit/ml) from healthy volunteers of both sexes between 20 and 40 years old. Neutrophils were isolated by the Ficoll gradient centrifugation method, followed by hypotonic lysis of contaminating erythrocytes (Boyum, 1974). Briefly, blood samples were mixed with an equal volume of 3% dextran solution in a 50 ml centrifuge tube and incubated in an upright position for 20 min at room temperature to allow sedimentation of erythrocytes. The upper, leukocyte-rich layer was then collected and subjected to centrifugation at $250 \times g$ for 15 min at 4°C. After centrifugation, the pellet was resuspended immediately in a volume of phosphate-buffered saline (PBS) equal to the starting volume of blood. The cell suspension was then apportioned, 25 ml per tube, into 50 ml centrifuge tubes, followed by layering of 10 ml of 1.077 g/ml Ficoll solution (Histopaque 1077; Sigma Chemicals Co., St. Louis, MO) beneath the cell suspension, using a pipette. After centrifugation at $400 \times g$ for 40 min at 20°C without brake, the upper (PBS) and lower (Ficoll) layers were carefully removed, leaving the granulocyte/erythrocyte pellet. To remove residual erythrocytes, the pellet was resuspended in 20 ml cold 0.2% NaCl for 30 s, followed by addition of 20 ml cold 1.6% NaCl to restore tonicity. The remaining neutrophils were then pelleted, washed twice with ice-cold PBS and resuspended in an adequate volume of ice-cold Hank's buffered saline solution (HBSS) until further manipulation. The preparation contained more than 95% neutrophils, as estimated by counting 200 cells under a microscope after Giemsa staining (Sigma). In all cases where neutrophils were pretreated with magnolol, the cells were mixed with the drug at concentrations from 0.1 to 10 μM in HBSS for 10 min at 37°C.

2.2. Measurement of neutrophil adhesion

Adhesion of neutrophils to extracellular matrix was determined in 24-well tissue culture plates (Costar Mesa, CA) coated with fibrinogen. Prior to the addition of neutrophils, the plates were incubated with 250 μl per well of human fibrinogen (50 μg/ml in PBS; Chemicon International, CA) for 2 h at 37°C. The wells were washed once with HBSS, blocked with 1% BSA (Sigma, USA) in HBSS for 1 h at 37°C, and washed twice with HBSS containing 0.1% Tween-20 (Sigma, USA) and once with HBSS. 500 μl per well of magnolol-pretreated neutrophils (5×10^5 cells/ml in HBSS) was then added to individual wells. After stimulation with PMA (100 ng/ml) for 15 min at 37°C, non-adherent cells were removed by aspiration and the wells were gently washed twice with warm PBS containing 1 mM Ca^{2+}. Adherent neutrophils were then stained with 250 μl of 0.25% rose bengal solution (Sigma, USA) for 10 min at room temperature. The staining solution was aspirated off and each well was washed twice with PBS and then 250 μl of ethanol:PBS (1:1) solution was added. After incubation at 37°C for 30 min to

allow cell-retained stain to be completely dissolved, the optical density at 570 nm (OD_{570}) of each well was determined with a microplate reader (EL311sx, Biotek Instruments, Winoski, VT), using wells containing medium alone as blanks.

2.3. Measurement of Mac-1 upregulation by flow cytometry

Expression of Mac-1 (CD11b/CD18) was analyzed as described by Endemann et al. (Endemann et al., 1996) with some modifications. Magnolol-pretreated neutrophils were stimulated with PMA (100 ng/ml) for 15 min. The cells were then pelleted and resuspended in 1 ml ice-cold PBS containing 10% heat-inactivated fetal bovine serum and 10 mM sodium azide. For staining of Mac-1, all subsequent steps were carried out in an ice bath. Cells were incubated for 60 min with 20 μg/ml of an anti-Mac-1 primary antibody (mouse anti-human CD11b, class IgG_1; Pharmingen, San Diego, CA) or a non-specific mouse antibody (class IgG_1, Sigma) as a negative control. After two washes with ice-cold PBS–FBS-azide, the cells were further incubated in the dark with 20 μg/ml of a fluorescein isothiocyanate (FITC)-labeled secondary antibody (goat anti-mouse IgG; Serotec, USA) for 30 min and then washed twice with PBS containing 5% FBS. Finally, stained cells were resuspended in flow cytometer sheath fluid (Becton Dickinson) containing 1% paraformaldehyde and analyzed on a flow cytometer (FACSort; Becton Dickinson) for Mac-1 expression. Data are expressed as peak channel fluorescence (PCF) for each sample as calculated by the CellQuest® software (Becton Dickinson) on a Power Macintosh 6100/66 computer.

2.4. Flow cytometric analysis of intracellular $O_2^{-\cdot}$ and H_2O_2 production

Intracellular production of $O_2^{-\cdot}$ and H_2O_2 was analyzed on a flow cytometer (FACSort; Becton Dickinson) according to Robinson et al. (1994). Briefly, neutrophils were incubated at 37°C for 5 min with 20 mM 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Eugene, OR) and for an additional 15 min with 10 mM of hydroethidine (Molecular Probes). The acetate moieties of DCFH-DA are cleaved off intracellularly by esterases, liberating the membrane impermeable 2′,7′-dichlorofluorescin, which fluoresces when oxidized to 2′,7′-dichlorofluorescein (DCF) by H_2O_2; hydroethidium, on the other hand, can be directly oxidized by $O_2^{-\cdot}$ to ethidium bromide (EB), which fluoresces after intercalating with nucleic acids. After labeling, cells were treated with magnolol and stimulated with PMA (100 ng/ml). Production of $O_2^{-\cdot}$ and H_2O_2 was then monitored every 10 min on FACSort by measuring emission at 525 nm (FL1) for DCF and 590 nm (FL2) for EB. Data are expressed as peak channel fluorescence for each sample as described above.

2.5. Magnolol and other chemicals

Magnolol was purchased from Nacalai Tesque (Japan). It was first dissolved in DMSO (dimethyl sulfoxide) at 10 mM and then serially diluted in PBS immediately prior to experiments. Magnolol stock solution was used within 1 week after preparation. For examination of the effect of magnolol, 5 μl of magnolol solution was added to 500 μl neutrophil suspension and incubated at 37°C for 10 min prior to the addition of 100 ng/ml PMA (Sigma).

2.6. Statistical analysis

Data were analyzed by one-way or two-way analysis of variance (ANOVA) depending on the number of experimental variables. In cases where ANOVA showed a significant difference, a protected Fisher's LSD (least significant difference) test was used to compare group means at an alpha level equal to 0.05. Concentration dependency was analyzed by performing simple linear regression across doses of magnolol and testing the slope of the regression line against 0 by Student's *t*-test at an α level equal to 0.05. All values in figures represent means ± S.E.M.

3. Results

3.1. Magnolol inhibits neutrophil adhesion to fibrinogen

To examine whether magnolol could inhibit neutrophil adhesion in-vitro, we established an assay system in which PMA (100 ng/ml) was used to induce neutrophil adhesion to a plastic surface coated with an extracellular matrix protein, fibrinogen. Whereas untreated neutrophils displayed spontaneous adhesion, with a rose bengal staining value of 23.0 ± 2.0 (Fig. 1; values are expressed as $OD_{570} \times 1000$), PMA caused a markedly enhanced adhesion to the fibrinogen-coated surface ($OD_{570} \times 1000 = 100.0 \pm 6.0$). Pretreatment of neutrophils with magnolol (0.1 to 10 μM) dose dependently inhibited the adhesion of neutrophils to the fibrinogen-coated surfaces induced by PMA (100 ng/ml) (Fig. 1; $P < 0.05$, $n = 6$). Magnolol alone did not influence spontaneous neutrophil adhesion (Fig. 1). The concentrations of magnolol used in this study were not cytotoxic to neutrophils (viability after magnolol treatment > 95% by Trypan blue exclusion assay).

3.2. Magnolol inhibits Mac-1 upregulation

Because upregulation of Mac-1 (CD11b/CD18), a cell surface adhesion molecule of the integrin family, has been shown to be crucial to adhesion of neutrophils to the extracellular matrix (Walzog et al., 1995; Everitt et al., 1996), we examined whether magnolol inhibits neutrophil adhesion by virtue of down regulation of Mac-1. The requirement for Mac-1 for PMA-induced neutrophil adhe-

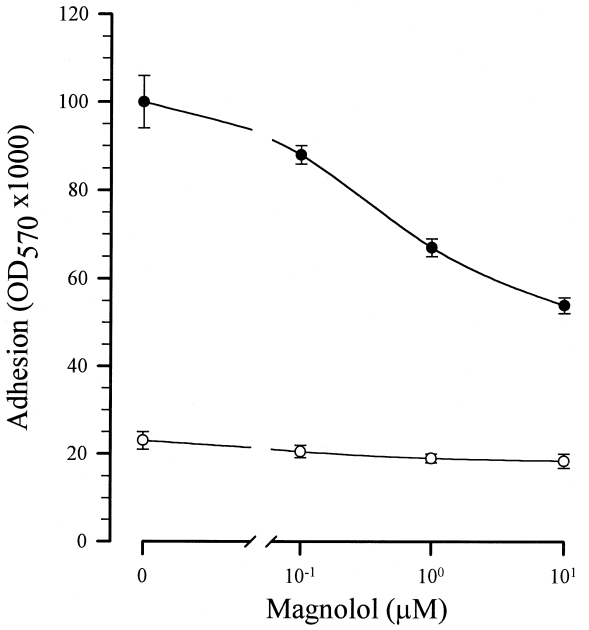

Fig. 1. Mean concentration–response curves for magnolol for the inhibition of PMA-induced neutrophil adhesion to extracellular matrix. Human neutrophils were pretreated with magnolol (0.1–10 μM) for 10 min at 37°C and then plated on a fibrinogen-coated surface in the presence (●) or absence (○) of PMA (100 ng/ml) for 15 min at 37°C. After removal of non-adherent cells, adherent cells were stained with 0.25% rose bengal and the optical density at 570 nm (OD_{570}) of each sample was measured. Control levels represent spontaneous adhesion of neutrophils without PMA stimulation. Values are means and vertical lines S.E.M. of 6 experiments.

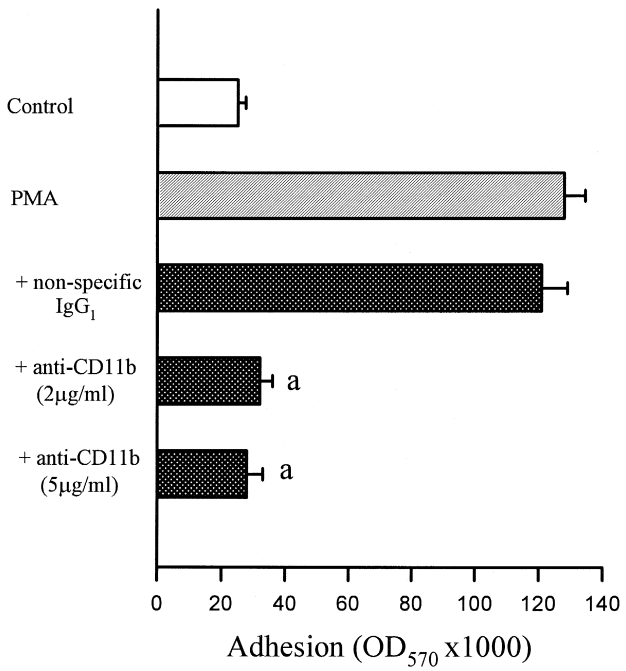

Fig. 2. Dependency of neutrophil adhesion on Mac-1 (CD11b/CD18). PMA (100 ng/ml)-stimulated neutrophils were plated in the presence of anti-CD11b (2 and 5 μg/ml) or a non-specific IgG_1 and adhesion was measured as in Fig. 1. Values are means ± S.E.M. of 3 experiments. [a] $P < 0.05$ as compared with samples incubated with PMA alone.

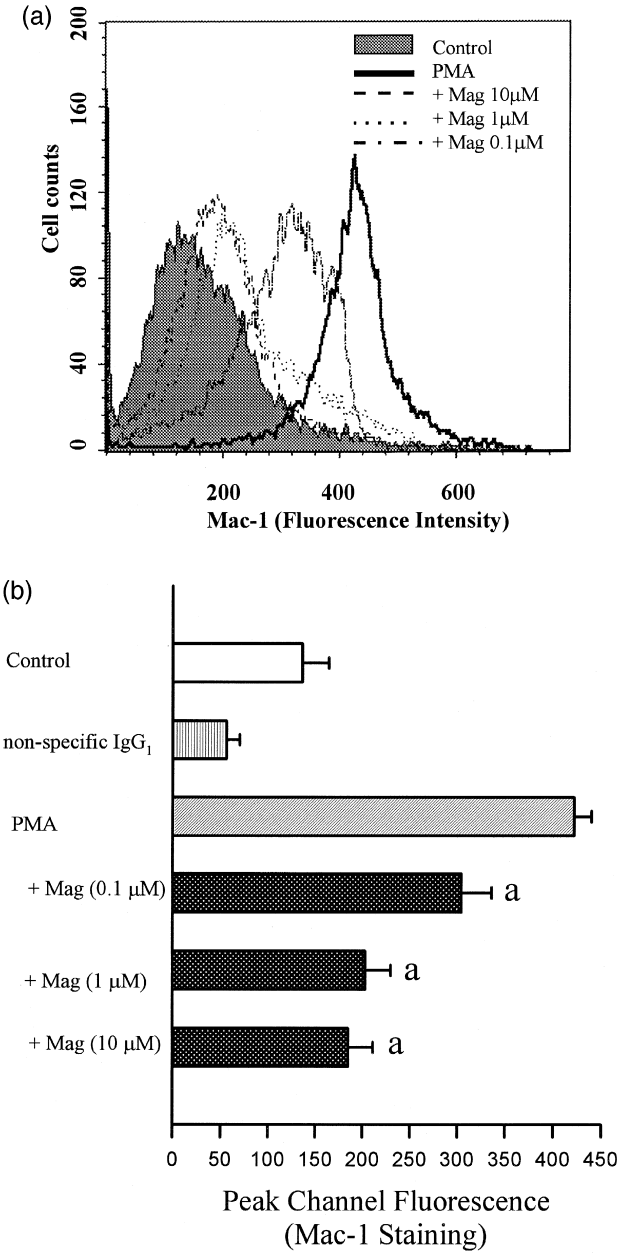

Fig. 3. Effect of magnolol on PMA (100 ng/ml)-induced Mac-1 upregulation. (a) Flow cytometric analysis of a representative experiment. PMA-stimulated neutrophils, with or without magnolol pretreatment, were stained, in an ice-bath, with anti-CD11b (Mac-1) antibody and total Mac-1 level on the cell surface was quantitated by FACSort. Control level represents neutrophils that were neither treated with magnolol nor stimulated with PMA. All the magnolol-pretreated groups, designated + Mag, were stimulated with PMA. (b) Statistical summary of PMA-upregulated Mac-1 expression in the presence of magnolol (Mag; 0–10 μM). Non-specific IgG_1 was included to indicate the specificity of anti-CD11b staining. Values represent the means ± S.E.M. of 3 experiments. [a] $P < 0.05$ as compared to samples treated with PMA alone.

sion was demonstrated by using an antibody against the α subunit, CD11b, of heterodimeric Mac-1. As shown in Fig. 2, anti-CD11b almost completely blocked the PMA-induced adhesion of neutrophils. To assess the effect of magnolol on Mac-1 expression, we measured surface lev-

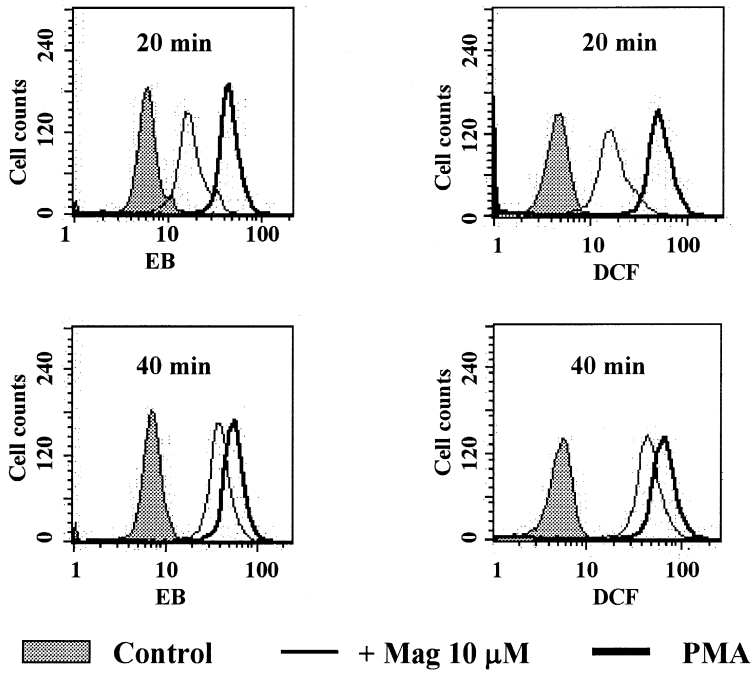

Fig. 4. Effects of magnolol on intracellular accumulation of peroxides in neutrophils upon PMA (100 ng/ml) stimulation. Flow cytometric analysis of the production of $O_2^{-\cdot}$ (EB fluorescence) and H_2O_2 (DCF fluorescence) from a representative experiment.

els of Mac-1 on PMA-activated neutrophils with or without magnolol pretreatment, using flow cytometric analysis. As shown in Fig. 3a, the expression of Mac-1 (expressed as Mac-1 fluorescence intensity) on PMA-activated neutrophils was significantly elevated relative to the control level (Mac-1 fluorescence of untreated neutrophils). An apparent shift-to-the-left of Mac-1 fluorescence was observed in samples pretreated with magnolol. A statistical summary of 3 flow cytometric experiments is shown in Fig. 3b; magnolol significantly inhibited PMA-induced upregulation of Mac-1 in a concentration-dependent manner ($P < 0.05$).

3.3. Magnolol inhibits the production of intracellular $O_2^{-\cdot}$ and H_2O_2

It has recently been shown that reactive oxygen species (e.g. $O_2^{-\cdot}$ and H_2O_2) can upregulate Mac-1 expression and enhance neutrophil adhesion (Fraticelli et al., 1996; Serrano et al., 1996), possibly by triggering the translocation of Mac-1 from subcellular locations to the cell surface (Simms and D'amico, 1995). Since magnolol has been claimed to be an antioxidant (Chang et al., 1994; Lo et al., 1994; Chan et al., 1996), we hypothesized that the de novo production of reactive oxygen species by neutrophils may up regulate Mac-1 expression, which can be diminished by magnolol. We used a flow cytometric method that enabled us to measure intracellular reactive oxygen species changes in PMA-stimulated neutrophils with or without magnolol pretreatment. Fig. 4 illustrates the fluorescence intensity measured in a representative experiment and Table 1 summarizes the results from 6 experiments of PMA-stimulated accumulation of intracellular $O_2^{-\cdot}$ (measured as EB fluorescence) and H_2O_2 (measured as DCF fluorescence), respectively. At 40 min, PMA enhanced the fluorescence of EB and DCF from the control levels of 8.0 ± 2.2 and 6.2 ± 1.0 to 47.2 ± 4.1 and 64.4 ± 3.0, respectively. In magnolol-pretreated samples, a significant decrease in the fluorescence intensity of both EB and DCF was observed at 20 and 40 min as compared with that of the PMA alone group ($P < 0.05$, $n = 6$). Introduction of antioxidants, such as ascorbic acid, superoxide dismutase or catalase, to the sample buffer completely inhibited the PMA-induced accumulation of $O_2^{-\cdot}$ and H_2O_2 (data not shown). Induction of intracellular accumulation of reactive oxygen species oc-

Table 1
Statistical summary of the PMA-induced production of $O_2^{-\cdot}$. (EB) and H_2O_2 (DCF) at 20 and 40 min

	Mean channel fluorescence			
	EB		DCF	
	20 min	40 min	20 min	40 min
PMA (100 ng/ml) + Magnolol (μM)				
0	44.2 ± 3.0	47.2 ± 4.1	54.2 ± 2.0	64.4 ± 3.0
1	20.5 ± 6.0[a]	44.3 ± 4.0	18.4 ± 8.0[a]	49.1 ± 5.2[a]
10	18.3 ± 3.2[a]	31.0 ± 7.0[a]	17.0 ± 5.3[a]	49.0 ± 6.4[a]
Control (buffer only)	7.2 ± 3.4	8.0 ± 2.2	5.4 ± 2.0	6.2 ± 1.0

PMA-induced production of $O_2^{-\cdot}$. (EB) and H_2O_2 (DCF) was monitored by flow cytometer at 20 and 40 min after addition of PMA.
[a] $P < 0.05$ as compared with samples treated with PMA alone (magnolol 0 μM) at the same time point. Values are means ± S.E.M. from 6 experiments.

curred immediately after addition of PMA, indicating that changes in reactive oxygen species production may precede other functional changes in neutrophils after proinflammatory challenge.

3.4. Inhibition of reactive oxygen species abolishes neutrophil Mac-1 upregulation and adhesion

To further confirm that production of reactive oxygen species could constitute a mechanism for Mac-1-mediated adhesion of neutrophils, we measured cell surface Mac-1 levels of PMA-stimulated neutrophils in the presence of superoxide dismutase or catalase. As shown in Fig. 5, both superoxide dismutase and catalase markedly inhibited Mac-1 upregulation. Combination of the two reagents further diminished Mac-1 upregulation.

The effect of superoxide dismutase and catalase on neutrophil adhesion is summarized in Fig. 6. In the presence of superoxide dismutase and/or catalase, neutrophil adhesion to the fibrinogen-coated surface was significantly inhibited ($P < 0.05$, $n = 4$), from 120.4 ± 15.2 (PMA) to 45.5 ± 8.2, 36.8 ± 5.4 and 28.2 ± 4.2 for superoxide dismutase, catalase and superoxide dismutase plus catalase, respectively. However, superoxide dismutase and/or catalase did not completely inhibit adhesion, since the $OD_{570} \times 1000$ value for the control samples (spontaneous adhesion) was significantly lower than that of samples treated with superoxide dismutase and/or catalase ($P < 0.05$, $n = 4$).

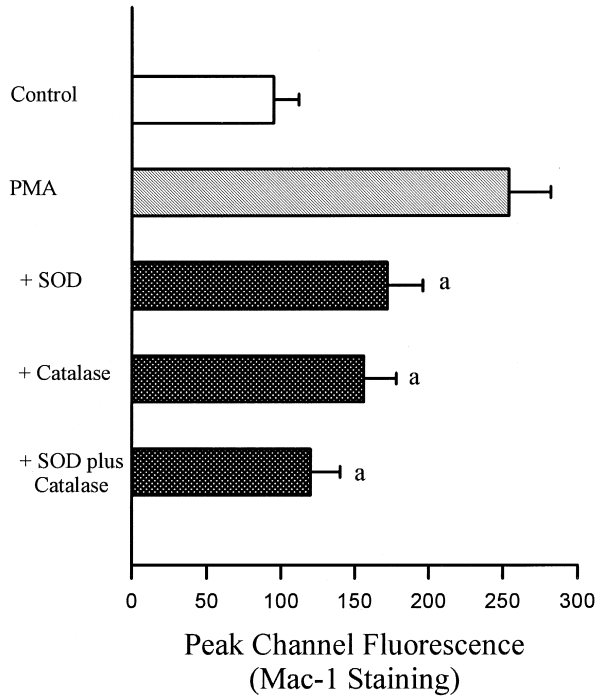

Fig. 5. Effect of peroxide scavengers on PMA (100 ng/ml)-upregulated Mac-1 expression of neutrophils. Neutrophils were processed as previously and Mac-1 expression was measured by flow cytometry in the presence or absence of superoxide dismutase (SOD, a scavenger of $O_2^{-\cdot}$; 200 units/ml) and/or catalase (a scavenger of H_2O_2; 500 units/ml). $^aP < 0.05$ as compared with samples treated with PMA alone. Values are means ± S.E.M. from 4 experiments.

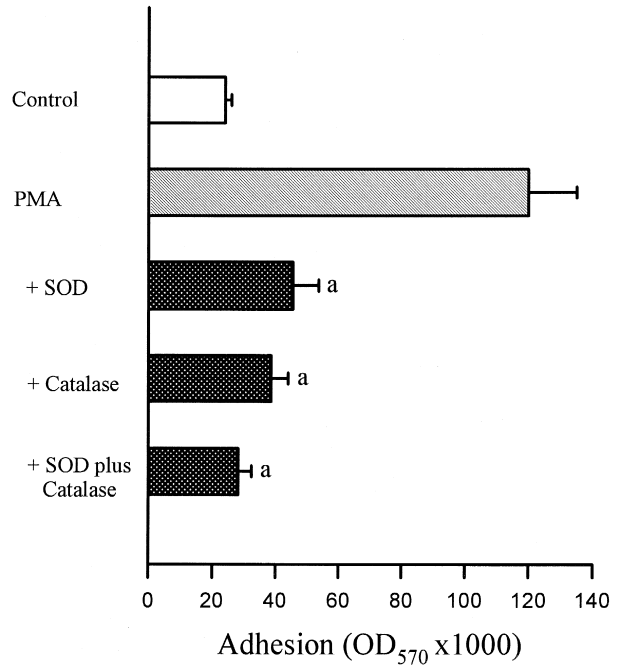

Fig. 6. Effect of peroxide scavengers on PMA (100 ng/ml)-induced neutrophil adhesion. Superoxide dismutase (SOD; 200 units/ml) and/or catalase (500 units/ml) were used to scavange $O_2^{-\cdot}$ and H_2O_2, respectively. $^aP < 0.05$ as compared with samples treated with PMA alone. Values are means ± S.E.M. from 4 experiments.

4. Discussion

Neutrophil migration from blood to sites of infection or injury is an important process during inflammation (Springer and Anderson, 1986). A crucial mechanism behind this process is the adhesion of neutrophils to either endothelial cells or extracellular matrix (Corbi et al., 1988; Tonnesen et al., 1989; Bohnsack et al., 1990). Therefore, drugs that block neutrophil adhesion should be effective as antiinflammatory agents. In the present report, neutrophils pretreated with magnolol at pharmacologically applicable concentrations (0.1–10 μM) for 10 min had an impaired adhesion to fibrinogen. The anti-adhesive effect of magnolol was not due to cytotoxic effects since under these conditions there was no difference in viability between treated neutrophils and control cells (viability $> 95\%$ at the end of experiments). In order to further clarify the mechanism by which magnolol suppressed neutrophil adhesion, in the light of the importance of Mac-1 in neutrophil adhesion (Jaeschke et al., 1996; Albelda et al., 1994), we examined the effect of magnolol on cell surface levels of Mac-1. We established that the anti-adhesive

effect of magnolol was, at least in part, mediated by inhibition of the Mac-1 upregulation on the membrane of neutrophils elicited by exposure to PMA.

Recently, it has been demonstrated that both superoxide and hydrogen peroxide induce Mac-1 upregulation and Mac-1-mediated neutrophil adhesion (Serrano et al., 1996; Fraticelli et al., 1996) through increased translocation of Mac-1 from secretory vesicles and specific granules to the plasma membrane (Simms and D'amico, 1995). In this report, the effect of magnolol, as a reactive oxygen species scavenger, on Mac-1 upregulation and neutrophil adhesion was compared with that of superoxide dismutase and catalase, two well-known scavengers of $O_2^{-\cdot}$ and H_2O_2, respectively. In agreement with prior studies, our data confirmed that magnolol could effectively inhibit the generation of both $O_2^{-\cdot}$ and H_2O_2 by neutrophils (Table 1) and that both superoxide dismutase and catalase significantly down regulated Mac-1 expression (Fig. 5) as well as neutrophil adhesion to fibrinogen (Fig. 6). The flow cytometric method used in this study for the measurement of reactive oxygen species production enabled on-line monitoring of the intracellular accumulation of reactive oxygen species in neutrophils. Accumulation of reactive oxygen species began almost immediately after PMA stimulation. Thus, the prompt accumulation of reactive oxygen species in response to PMA stimulation and our observation that Mac-1 upregulation could be inhibited by reactive oxygen species scavengers suggest that reactive oxygen species are early signaling molecules participating in the regulation of neutrophil function. This argument is further strengthened by the previous observation that reactive oxygen species could upregulate Fcγ receptor IIa during ligation of Fcγ receptor IIIb (Salmon et al., 1995) as well as the activity of p58 (c-fgr) and p53/56 (c-lyn) tyrosine kinases during neutrophil adhesion (Yan and Berton, 1996). Thus, it was suggested that reactive oxygen species could regulate neutrophils functions through an autocrine-like mechanism.

In addition to inhibition of reactive oxygen species production, magnolol may also inhibit other biochemical pathways that regulate Mac-1 expression. Since protein kinase C is also involved in the regulation of Mac-1 expression (Monk and Banks, 1991; Roubey et al., 1991), it is likely that magnolol can suppress Mac-1 upregulation by inhibiting protein kinase C activity in neutrophils treated with PMA, a well-known protein kinase C activator. Mac-1 expression may also be regulated by phospholipase A_2, which catalyzes the synthesis of arachidonates, because inhibitors of phospholipase A_2 can inhibit the surface expression of Mac-1 (Jacobson and Schrier, 1993). As magnolol has been shown to decrease the production of prostaglandin D_2 and thromboxane B_2 (Wang et al., 1992; Wang et al., 1995), two downstream metabolites of arachidonate, it is likely that magnolol also influences this biochemical pathway and in turn regulates Mac-1 expression. Whether these biochemical pathways are a target of magnolol in the regulation of Mac-1 awaits further research and is currently under investigation in this laboratory.

In conclusion, we have demonstrated that the ability of magnolol to prevent ischemia-reperfusion injury can be explained by its ability to inhibit neutrophil adhesion through inhibition of Mac-1 upregulation. The inhibitory effect of magnolol on Mac-1 expression could be mediated by down regulation of reactive oxygen species generation. As an effective anti-adhesive agent at pharmacological concentrations, magnolol may be clinically useful for the prevention of inflammatory injury by inhibiting the early phases of neutrophil recruitment.

Acknowledgements

This study was supported, in part, by grants from National Science Council (NSC-87-2314-B-077-015) and National Research Institute of Chinese Medicine, Taiwan, Republic of China, to C.F.C. and Y.J.S., respectively.

References

Albelda, S.M., Smith, C.W., Ward, P.A., 1994. Adhesion molecules and inflammatory injury. FASEB J. 8, 504–512.

Arfors, K.E., Lundberg, C., Lindbom, L., Lundberg, K., Beatty, P.G., Harlan, J.M., 1987. A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo. Blood 69, 338–340.

Beatty, P.G., Ledbetter, J.A., Martin, P.J., Price, T.H., Hansen, J.A., 1983. Definition of a common leukocyte cell-surface antigen (Lp95-150) associated with diverse cell-mediated immune functions. J. Immunol. 131, 2913–2918.

Bevilacqua, M., Nelson, R.M., 1993. Selectins. J. Clin. Invest. 91, 379–387.

Bohnsack, J.F., Akiyama, S.K., Damsky, C.H., Knape, W.A., Zimmerman, G.A., 1990. Human neutrophil adherence to laminin in vitro. Evidence for a distinct neutrophil integrin receptor for laminin. J. Exp. Med. 171, 1221–1237.

Boyum, A., 1974. Separation of blood leukocytes, granulocytes and lymphocytes. Tissue Antigens 4, 269–274.

Chan, P., Cheng, J.T., Tsao, C.W., Niu, C.S., Hong, C.Y., 1996. The in vitro antioxidant activity of trilinolein and other lipid-related natural substance as measured by enhanced chemiluminescence. Life Sci. 59, 2067–2073.

Chang, W.S., Chang, Y.H., Lu, F.T., Chiang, H.C., 1994. Inhibitory effect of phenolics on xathine oxidase. Anticancer Res. 14, 501–506.

Corbi, A.L., Kishimoto, T.K., Miller, L.J., Springer, T.A., 1988. The human leukocyte adhesion glycoprotein Mac-1 (complement receptor type 3, CD11b) alpha subunit. Cloning, primary structure and relation to the integrins, von Willebrand factor and factor B. J. Biol. Chem. 263, 12403–12411.

Endemann, G., Feng, Y., Bryant, C.M., Hamilton, G.S., Perumattam, J., Mewshaw, R.E., Liu, D.Y., 1996. Novel anti-inflammatory compounds prevent CD11b/CD18, $\alpha_m \beta_2$ (Mac-1)-dependent neutrophil adhesion without blocking activation-induced changes in Mac-1. J. Pharmacol. Exp. Ther. 276, 5–12.

Everitt, E.A., Malik, A.B., Hendey, B., 1996. Fibronectin enhances the migration rate of human neutrophils in vitro. J. Leukoc. Biol. 60, 199–206.

Fraticelli, A., Serrano, C.V.J., Bochner, B.S., Capogrossi, M.C., Zweier,

J.L., 1996. Hydrogen peroxide and superoxide modulate leukocyte adhesion molecule expression and leukocyte endothelial adhesion. Biochim. Biophys. Acta 1310, 251–259.

Hong, C.Y., Hung, S.S., Tsai, S.K., 1996. Magnolol reduces infarct size and suppresses ventricular arrhythmia in rats subjected to coronary ligation. Clin. Exp. Pharmacol. Physiol. 23, 660–664.

Jacobson, P.B., Schrier, D.J., 1993. Regulation of CD11b/CD18 expression in human neutrophils by phospholipase A2. J. Immunol. 151, 5639–5652.

Jaeschke, H., Smith, C.W., Clemens, M.G., Ganey, P.E., Roth, R.A., 1996. Mechanisms of inflammatory liver injury: adhesion molecules and cytotoxicity of neutrophils. Toxicol. Appl. Pharmacol. 139, 213–226.

Koike, K., Moore, E.E., Moore, F.A., Franciose, R.J., Fontes, B., Kim, F.J., 1995. CD11b blockade prevents lung injury despite neutrophil priming after gut ischemia/reperfusion. J. Trauma. 39, 23–27, discussion.

Lefer, A.M., 1995. Role of selectins in myocardial ischemia-reperfusion injury. Ann. Thorac. Surg. 60, 773–777.

Lefer, A.M., Lefer, D.J., 1996. The role of nitric oxide and cell adhesion molecules on the microcirculation in ischemia-reperfusion. Cardiovascular Res. 32, 743–751.

Lefer, A.M., Weyrich, R.M., Buerke, M., 1994. Role of selectins, a new family of adhesion molecules, in ischemia-reperfusion injury. Cardiovasc. Res. 28, 289–294.

Ley, K., 1996. Molecular mechanisms of leukocyte recruitment in the inflammatory process. Cardiovasc. Res. 32, 733–742.

Lo, Y.C., Teng, C.M., Chen, C.F., Chen, C.C., Hong, C.Y., 1994. Magnolol and honokiol isolated from *Magnolia officinalis* protect rat heart mitochondria against lipid peroxidation. Biochem. Pharmacol. 47, 549–553.

Matsuo, Y., Onodera, H., Shiga, Y., Shozuhara, H., Ninomiya, M., Kihara, T., Tamatani, T., Miyasaka, M., Kogure, K., 1994. Role of cell adhesion molecules in brain injury after transient middle cerebral artery occlusion in the rat. Brain Res. 656, 344–352.

Monden, K., Arii, S., Ishiguro, S., Nakamura, T., Fujita, S., Mise, M., Niwano, M., Sasaoki, T., Imamura, M., 1995. Involvement of ICAM-1 expression on sinusoidal endothelial cell and neutrophil adherence in the reperfusion injury of cold-preserved livers. Transplant. Proc. 27, 759–761.

Monk, P.N., Banks, P., 1991. The role of protein kinase C activation and inositol phosphate production in the regulation of cell-surface expression of Mac-1 by complement fragment c5a. Biochim. Biophys. Acta 1092, 251–255.

Robinson, P.J., Carter, W.O., Narayana, P.D., 1994. Oxidative product formation analysis by flow cytometry. In: Darzynkiewicz, Z., Robison, J.P., Crissman, H.A. (Eds.), Methods in Cell Biology, Flow Cytometry, Part A, 2nd ed. Academic Press, San Diego, CA, pp. 437–442.

Roubey, R.A., Ross, G.D., Merrill, J.T., Walton, F., Reed, W., Winchester, R.J., Buyon, J.P., 1991. Staurosporine inhibits neutrophil phagocytosis but not ic3b binding mediated by CR3 (CD11b/CD18). J. Immunol. 146, 3557–3562.

Ruoslahti, E., 1991. Integrins. J. Clin. Invest. 87, 1–5.

Salmon, J.E., Millard, S.S., Brogle, N.L., KimberlY, R.P., 1995. Fc gamma receptor iiib enhances Fc gamma receptor iia function in an oxidant-dependent and allele-sensitive manner. J. Clin. Invest. 95, 2877–2885.

Serrano, C.V.J., Mikhail, E.A., Wang, P., Noble, B., Kuppusamy, P., Zweier, J.L., 1996. Superoxide and hydrogen peroxide induce CD18-mediated adhesion in the postischemic heart. Biochim. Biophys. Acta 1316, 191–202.

Simms, H.H., D'amico, R., 1995. Subcellular location of neutrophil opsonic receptors is altered by exogenous reactive oxygen species. Cell Immunol. 166, 71–82.

Springer, T.A., 1990. Adhesion receptors of the immune system. Nature 346, 425–434.

Springer, T.A., Anderson, D.C., 1986. The importance of the Mac-1, LFA-1 glycoprotein family in monocyte and granulocyte adherence, chemotaxis, and migration into inflammatory sites: Insights from an experiment of nature. Ciba. Found. Symp. 118, 102–126.

Teng, C.M., Chen, C.C., Ko, F.N., Lee, L.G., Hung, T.F., Chen, Y.P., Hsu, H.Y., 1988. Two antiplatelet agents from *Magnolia officinalis*. Thromb. Res. 50, 757–765.

Teng, C.M., Yu, S.M., Chen, C.C., Hung, Y.L., Hung, T.F., 1990. EDRF-release and Ca^{2+}-channel blockade by magnolol, an antiplatelet agent isolated from Chinese herb *Magnolia officinalis*, in rat thoracic aorta. Life Sci. 47, 1153–1161.

Tonnesen, M.G., Anderson, D.C., Springer, T.A., Knedler, A., Avdi, N., Henson, P.M., 1989. Adherence of neutrophils to cultured human microvascular endothelial cells. Stimulation by chemotactic peptides and lipid mediators and dependence upon the Mac-1, LFA-1, p150,95 glycoprotein family. J. Clin. Invest. 83, 637–646.

Walzog, B., Schuppan, D., Heimpel, C., Hafezi-Moghadam, A., Gaehtgens, P., Ley, K., 1995. The leukocyte integrin Mac-1 (CD11b/CD18) contributes to binding of human granulocytes to collagen. Exp. Cell Res. 218, 28–38.

Wang, J.P., Hsu, M.F., Raung, S.L., Chen, C.C., Kou, J.S., Teng, C.M., 1992. Anti-inflammatory and analgesic effects of magnolol. Naunyn-Schmiedebergs Arch. Pharmacol. 346, 707–712.

Wang, J.P., Raung, S.L., Chen, C.C., Kou, J.S., Teng, C.M., 1993. The inhibitory effect of magnolol on cutaneous permeability in mice is probably mediated by a nonselective vascular hyporeactivity to mediators. Naunyn-Schmiedebergs Arch. Pharmacol. 348, 663–669.

Wang, J.P., Ho, T.F., Chang, L.C., Chen, C.C., 1995. Anti-inflammatory effect of magnolol, isolated from *Magnolia officinalis*, on A23187-induced pleurisy in mice. J. Pharm. Pharmacol. 47, 857–860.

Yan, S.R., Berton, G., 1996. Regulation of Src family tyrosine kinase activities in adherent human neutrophils. Evidence that reactive oxygen intermediates produced by adherent neutrophils increase the activity of the p58c-fgr and p53/56lyn tyrosine kinases. J. Biol. Chem. 271, 23464–23471.