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Mechanisms of the influence of magnolol on eicosanoid metabolism in neutrophils

Mei-Feng Hsu[a], Min-Chi Lu[b], Lo-Ti Tsao[c], Yu-Hsing Kuan[d], Chien-Chih Chen[e], Jih-Pyang Wang[c,*]

[a]*Department of Biochemistry, China Medical University, Taichung, Taiwan, ROC*
[b]*Department of Physiology, China Medical University, Taichung, Taiwan, ROC*
[c]*Department of Education and Research, Taichung Veterans General Hospital, Taichung, Taiwan, ROC*
[d]*Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan, ROC*
[e]*National Research Institute of Chinese Medicine, Taipei, Taiwan, ROC*

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Abstract

We have demonstrated that magnolol suppressed thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4) formation in A23187-stimulated rat neutrophils. Maximum inhibition was obtained with about 10 μM magnolol. Magnolol was more effective in the inhibition of cyclooxygenase (COX) activity than in the inhibition of 5-lipoxygenase (5-LO) activity as assessed by means of enzyme activity determination *in vitro* and COX and 5-LO metabolic capacity analyses *in vivo*. Magnolol alone stimulated cytosolic phospholipase A_2 ($cPLA_2$) phosphorylation and the translocation of 5-LO and $cPLA_2$ to the membrane, and evoked arachidonic acid (AA) release. Recruitment of both 5-LO and $cPLA_2$ to the membranes was suppressed by EGTA. Arachidonyl trifluoromethyl ketone ($AACOCF_3$), a PLA_2 inhibitor, bromoenol lactone (BEL), a Ca^{2+}-independent PLA_2 ($iPLA_2$) inhibitor, and EGTA suppressed the magnolol-induced AA release. However, none of the follows affected magnolol-induced AA-release: 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB203580), a p38 mitogen-activated protein kinase (MAPK) inhibitor, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), a MAPK kinase (MEK) inhibitor, or 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF109203X), a protein kinase C (PKC) inhibitor. In addition, magnolol at 30 μM did not stimulate the p38 MAPK and extracellular signal-regulated kinase 2 (ERK2) enzyme activities. These results indicated that magnolol inhibits the formation of prostaglandins and leukotrienes in A23187-stimulated rat neutrophils, probably through a direct blockade of COX and 5-LO activities. The stimulatory effects of magnolol at high concentration on the membrane association of 5-LO and $cPLA_2$ are attributable to the elevation of $[Ca^{2+}]_i$, and on the AA release is likely via activation of $cPLA_2$ and $iPLA_2$.

Keywords: Neutrophils; Magnolol; Cyclooxygenase; 5-Lipoxygenase; Cytosolic phospholipase A_2; Arachidonic acid release

1. Introduction

Many different cell types release AA upon stimulation with diverse stimuli. Depending on the cell type, the released AA is then metabolized to generate various biologically important eicosanoids, which are potent regulators of inflammation, allergy, reproduction and neurotoxicity [1,2]. The rate-limiting step in eicosanoid biosynthesis is the liberation of AA, a process that involves phospholipase A_2 (PLA_2). PLA_2 comprises a superfamily of enzymes that hydrolyze the ester bond of phospholipids at the *sn*-2 position, including secretory PLA_2 ($sPLA_2$),

* Corresponding author. Tel.: +886-4-2359-2705x4023; fax: +886-4-2359-2705.
E-mail address: w1994@vghtc.gov.tw (J.-P. Wang).

Abbreviations: AA, arachidonic acid; $AACOCF_3$, arachidonyl trifluoromethyl ketone; BEL, bromoenol lactone; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; ERK, extracellular signal-regulated kinase; fMLP, formyl-methionyl-leucyl-phenylalanine; GF109203X, 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide; HBSS, Hanks' balanced salt solution; $iPLA_2$, Ca^{2+}-independent PLA_2; 5-LO, 5-lipoxygenase; LTB_4, leukotriene B_4; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PKC, protein kinase C; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole; TXB_2, thromboxane B_2; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene.

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iPLA$_2$ and cPLA$_2$, all of which have been shown to mediate AA release in some systems [3]. Among the members of this superfamily, 85-kDa cPLA$_2$ has attracted attention because it preferentially hydrolyses arachidonyl phospholipids [4]. Increases in the cytosolic concentration of Ca^{2+} and phosphorylation by MAPKs and PKC have been shown to play important roles in cPLA$_2$ activation [5,6]. Activation of cPLA$_2$ involves its translocation from the cytosol to the nuclear membrane and perinuclear region to provide access to phospholipid [7]. Activated neutrophils release AA, which also acts as a multifunctional agonist for the mobilization of Ca^{2+}, degranulation and superoxide anion generation [8,9].

In neutrophils, the principal lipoxygenase is 5-LO, the non-heme iron-containing enzyme, which catalyses the biosynthesis of leukotriene A_4 (LTA_4) from AA. LTA_4 can be subsequently transformed into LTB_4 or leukotriene C_4 (LTC_4) by LTA_4 hydrolase or LTC_4 synthase, respectively [10]. 5-LO is localized in the cytosol in resting neutrophils and after cell stimulation undergoes a Ca^{2+}-dependent translocation to the nuclear membrane, where it interacts with 5-LO-activating protein for LTs biosynthesis [11]. LTB_4, the main product of LTs, is the crucial eicosanoid for recruiting neutrophils to the inflammatory site. COX, a heme-containing oxygenase, catalyzes the biosynthesis of endoperoxides (PGG_2, PGH_2) from AA, which can be subsequently transformed into prostaglandins, thromboxane and prostacyclin. Unlike 5-LO, COX is a glycoprotein associated with membrane of the endoplasmic reticulum and nuclei in resting cells [12].

Magnolol, a hydroxylated biphenyl compound isolated from the Chinese herb Hou p'u, the cortex of *Magnolia officinalis* [13], has been found to relax rat vascular smooth muscle [14], scavenge hydroxyl radicals [15], inhibit neutrophil aggregation and superoxide anion generation [16,17], suppress the expression of vascular cell adhesion molecule-1 in endothelial cells [18] and the proliferation of cultured human colon and liver cancer cells [19] *in vitro*, and to exert anti-inflammatory and analgesic effects [20,21] and protect rat heart against injury during ischaemia-reperfusion [22] *in vivo*. Our previous studies revealed that the inhibition of eicosanoid formation could be a mechanism by which magnolol exerts its anti-inflammatory action [20,21]. The aim of the present study was to evaluate how magnolol exerts its pharmacological effects on eicosanoid formation in neutrophils.

2. Materials and methods

2.1. Materials

Magnolol was isolated and purified from the cortex of *M. officinalis* as previously described [13]. Dextran T-500, enhanced chemiluminescence reagent, TXB_2 and LTB_4 enzyme immunoassay (EIA) kits were purchased from Amersham Pharmacia Biotech. HBSS was obtained from Gibco Life Technologies. C_{18} Sep-Pak cartridge and 5 μm μBondapak C_{18} column were purchased from Waters Co. CR Acrodisc 13 PTFEP was purchased from Gelman Sciences. Ram seminal vesicles COX and BEL were obtained from Biomol Research Laboratories. COX inhibitor screening assay kit and human recombinant 5-LO were obtained from Cayman Chemical. Myelin basic protein (MBP) and anti-phospho-MBP antibody were purchased from Upstate Biotechnology. GF109203X and SB203580 were purchased from Calbiochem-Novabiochem Co. U0126 was obtained from Promega Co. [5,6,8,9,11,12,14,15-^{3}H]AA was obtained from NEN Life Science Products. Rabbit polyclonal antibodies against ERK2 and p38 MAPK, and mouse monoclonal antibodies against cPLA$_2$ and p38 MAPK were obtained from Santa Cruz Biotechnology. Mouse monoclonal antibodies against 5-LO and pan-ERK were purchased from BD Transduction Laboratories. Polyvinylidene difluoride membrane was obtained from Millipore Co. 3-Amino-1-(3-trifluoromethylphenyl)-2-pyrazoline HCl (BW755C) was supplied by Wellcome Research Laboratories. Other chemicals were purchased from Sigma Chemical Co. The final percentage of DMSO in the reaction mixture was $\leq$0.5% (v/v).

2.2. Isolation of neutrophils

Blood was collected from the abdominal aorta of pentobarbital-anesthetized rats (Sprague–Dawley). Neutrophils were purified by dextran sedimentation, centrifugation through Ficoll-Hypaque, and hypotonic lysis of erythrocytes [23]. Purified neutrophils containing >95% viable cells were resuspended in HBSS containing 10 mM HEPES, pH 7.4, and 4 mM $NaHCO_3$, and kept in an ice bath before use.

2.3. Measurement of TXB_2 and LTB_4 formation

Neutrophils (2×10^6/mL) were incubated with test drugs in the presence of 1 mM $CaCl_2$ for 3 min at 37° before stimulation with 3 μM A23187 for 45 min. The amounts of TXB_2 and LTB_4 in the medium were determined by enzyme immunoassay.

2.4. Release of free [^{3}H]AA and HPLC analysis of AA metabolites

For the determination of [^{3}H]AA release, neutrophils (1.5×10^6/mL) were loaded with 0.05 μCi [^{3}H]AA (180–240 Ci/mmol) for 90 min at 37°. Cells were then washed three times and resuspended in HBSS containing 0.15% BSA, which trapped free AA and prevents further reuptake or metabolism of AA. Cells were incubated with test drugs in the presence of 1 mM $CaCl_2$ for 3 min at 37° before stimulation with A23187 or magnolol. The amount of [^{3}H]AA released into the medium was measured by scintillation

counting, and the results are expressed as percent release of the total radioactivity incorporated. Background release (<2.5%) from unstimulated cells treated with vehicle (DMSO) is subtracted from each experimental point.

In the AA metabolites analyses, neutrophils (1×10^7/mL) were loaded with 1 μCi [^{3}H]AA for 90 min at 37°, then washed three times with HBSS. Cells were incubated with test drugs in the presence of 1 mM $CaCl_2$ for 3 min before stimulation with 3 μM A23187 for 30 min. The reaction mixtures were centrifuged at 4000 *g* for 5 min at 4°. The supernatants were diluted with 2 volumes of methanol and applied to a C_{18} Sep-Pak cartridge prewashed with CH_3OH followed by water. The cartridge was washed with 20% CH_3OH in 0.1 M phosphate buffer, pH 7.4, followed by water, then eluted with 80% CH_3OH in water. The eluates containing AA and eicosanoids were evaporated under N_2 gas and stored at −70° until analysis. The dried lipid extract was dissolved in CH_3OH followed by 2 volumes of water, then filtered through a 0.45 μm CR Acrodisc 13 PTFE. Filtrate (200 μL) was injected into the HPLC (Waters 600), and the eicosanoids were separated by a 5 μm μBondapak C_{18} column (30 cm × 0.4 cm). For a total 105-min, COX metabolites were eluted during an initial 25-min with the mobile phase of acetonitrile:water:trifluoroacetic acid (33:67:0.1, v/v), then 5-LO metabolites and free AA were eluted with a stepwise gradient increase of acetonitrile to 100:0:0.1 (v/v) [24], pumped at a flow rate of 1 mL/min. Eicosanoids were monitored continuously with a flow scintillation analyzer (A Canberra 150 TR). Eicosanoid standards were utilized to determine the elution time of individual eicosanoids by an UV detector (Waters 486) at 210 nm for COX metabolites and free AA, 280 nm for LTs and 235 nm for HETE. The metabolic capacities of COX and 5-LO were assessed by summation of the peak areas of COX metabolites and 5-LO metabolites, respectively, and expressed as a percent of total radioactivity released.

2.5. COX and LO activities

For COX-1 assay, the reaction mixture (0.1 M Tris–HCl, pH 8.0, 5 mM tryptophan, 8 mM hematin, test drugs and 10 μg/mL of ram seminal vesicles COX) was incubated for 3 min at 30°. The reaction was initiated by adding 50 μM AA. For human recombinant 5-LO assay, the reaction mixture (50 mM Tris–HCl, pH 7.5, test drugs, 2 mM $CaCl_2$, 1 mM ATP, 20 μg/mL of lecithin, and 4 U/mL of 5-LO) was incubated for 3 min at 30°. The reaction was initiated by adding 100 μM AA. The velocity of oxygen consumption in the reaction mixture was monitored continuously with a Clark-type oxygen electrode using a YSI biological oxygen monitor (Model 5300). COX-2 assay was carried out using COX inhibitor screening assay kit according to manufacturer's guidelines to directly measure $PGF_{2\alpha}$ production by $SnCl_2$ reduction of COX-derived PGH_2 at 415 nm.

2.6. Immunoblot analysis of cPLA$_2$ and 5-LO

Reactions (5×10^7 cells/mL) were terminated by the addition of ice-cold HBSS, then washed and resuspended in lysis buffer (0.34 M sucrose, 10 mM Tris, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 10 mM NaF, 1 mM Na_3VO_4, 2 mM *p*-nitrophenyl phosphate, 10 μg/mL each of leupeptin, antipain and pepstatin). Lysates were centrifuged at 800 *g* for 5 min to remove unbroken cells and nuclei. In some experiments, the lysates were then further centrifuged at 180,000 *g* for 30 min at 4° to separate the supernatants (as cytosol fraction) and pellets (as membrane fraction). After determination of the protein concentration, Laemmli sample buffer was added to the lysates, cytosol or membrane fraction, then SDS–PAGE and immunoblotting was performed. Proteins were resolved by 7.5% SDS–PAGE for analysis of cPLA$_2$ and 5-LO translocation and cPLA$_2$ gel shift (16 cm long gel). Proteins were then transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% (w/v) non-fat dried milk in TBST buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20) and probed with anti-cPLA$_2$ or anti-5-LO antibody, and revealed using enhanced chemiluminescence reagent.

2.7. Immunoprecipitation of MAPK activity

For the immunoprecipitation of ERK activity, neutrophils (2×10^7 cells) were lysed on ice in 0.2 mL of lysis buffer, and then clarified by centrifugation at 4° for 10 min at 12,000 *g* as our previously described [25]. Briefly, ERK2 were immunoprecipitated by the addition of rabbit polyclonal anti-ERK2 and 50% slurry of protein A-Sepharose beads in 0.2 mL of lysis buffer, and the samples were rotated at 4° for 2 hr. The beads were washed twice in lysis buffer and twice in kinase assay buffer. For the immunoprecipitation of p38 MAPK activity, neutrophils (2×10^7 cells) were lysed on ice in lysis buffer (20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 10% glycerol, 1% NP-40, 0.5 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 μg/mL each of aprotinin, pepstatin and leupeptin). The p38 MAPK in lysate was immunoprecipitated by the addition of rabbit polyclonal anti-p38 MAPK and protein A-Sepharose beads, and the samples were rotated at 4° for overnight. The beads were washed twice in lysis buffer and twice in kinase assay buffer (40 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM Na_3VO_4, and 2 mM dithiothreitol). The kinase activities of ERK2 and p38 MAPK were assayed using 10 μg of MBP as substrate and 200 μM ATP in an assay volume of 20 μL, which was incubated at 30° for 20 min. The reaction was stopped by the addition of Laemmli sample buffer and boiled for 5 min. Samples were separated using 12.5% SDS–PAGE, transferred to polyvinylidene difluoride membranes, and probed with anti-phospho-MBP, ERK2 or p38 MAPK monoclonal antibody.

2.8. Statistical analysis

Statistical analyses were performed using the Bonferroni *t*-test method after analysis of variance. $P < 0.05$ was considered significant for all tests. Analysis of the regression line test was used to calculate IC_{50} values. Data are expressed as means ± SD.

3. Results

3.1. Effect of magnolol on eicosanoid formation

In the presence of extracellular Ca^{2+}, rat neutrophils produced significant amounts of TXB_2 and LTB_4 in response to Ca^{2+}-ionophore A23187. As shown in Fig. 1A, TXB_2 formation induced by A23187 was concentration-dependently suppressed by indomethacin, a COX inhibitor, magnolol or BW755C, a dual COX/LO inhibitor, with IC_{50} values about 0.02, 0.6 and 1.9 μM, respectively. The A23187-induced LTB_4 formation was inhibited by BW755C (IC_{50} value about 7 μM) but enhanced by indomethacin. Magnolol showed stimulation of A23187-induced LTB_4 formation at low concentrations (<3 μM), but exhibited an inhibitory effect at concentrations ≥3 μM (Fig. 1B).

We next examined whether magnolol affected formation of other eicosanoids in response to A23187 by using HPLC with an on-line radiodetector. Figure 2A shows the representative AA metabolic profiles of rat neutrophils stimulated with A23187. The mean COX and 5-LO metabolic

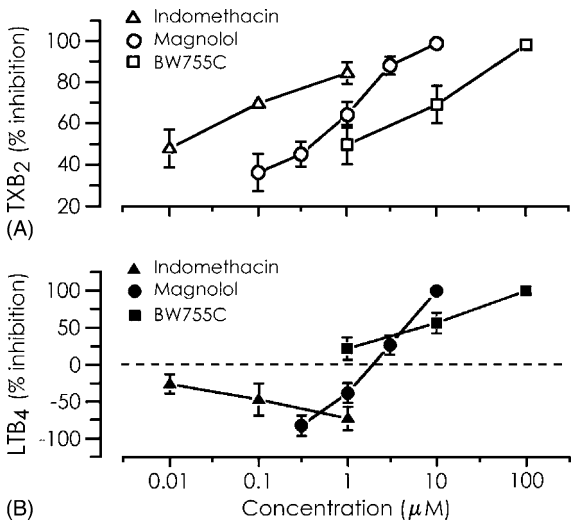

Fig. 1. Concentration dependence of the inhibition of magnolol on TXB_2 and LTB_4 formation in rat neutrophils. Cells were preincubated with DMSO (as control), indomethacin, magnolol or BW755C at the indicated concentration for 3 min at 37° before stimulation with 3 μM A23187 for 45 min. The supernatants were collected for (A) TXB_2 and (B) LTB_4 determination. Results were calculated as the percentage inhibition of control values (8.2 ± 0.6 ng and 22.8 ± 2.8 ng per 2×10^6 cells for TXB_2 and LTB_4 formation, respectively). Values are means ± SD of 4–6 independent experiments.

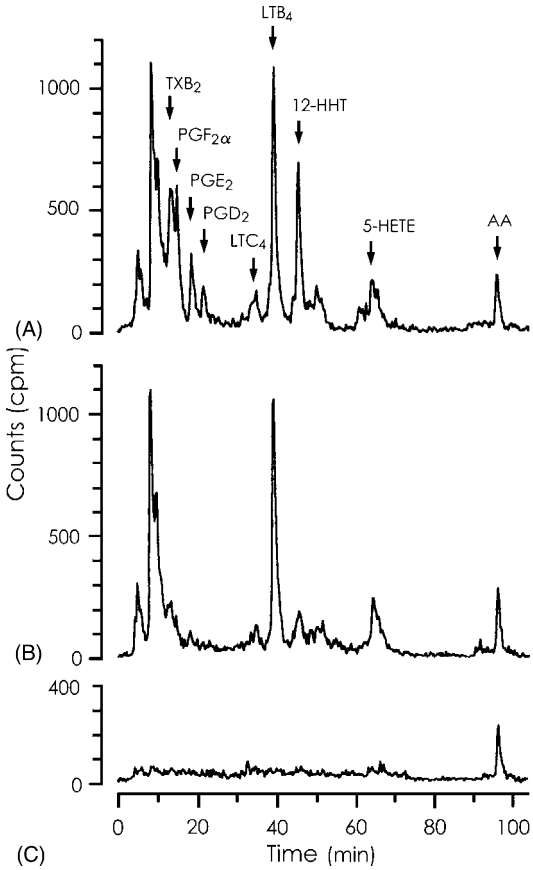

Fig. 2. Effect of magnolol on AA metabolite formation. [^{3}H]AA-loaded cells were preincubated with (A) DMSO (as control), (B) 3 μM or (C) 10 μM magnolol for 3 min at 37° before stimulation with 3 μM A23187 for 30 min. The released eicosanoids were extracted from medium and separated by HPLC. Radioactivity was detected by an on-line radiodetector. Similar results were obtained from 3 to 4 independent experiments. Retention times of authentic standards, including prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), TXB_2, and 12-hydroxyheptadecatrienoic acid (12-HHT), LTB_4, leukotriene C_4 (LTC_4), 5-hydroxyeicosatetraenoic acid (5-HETE), are shown in the chromatogram.

capacities, which are assessed by the release of COX and 5-LO products were about 17.5 and 39.6%, respectively, of the total radioactivity released. Magnolol at 3 μM abolished the formation of COX products but had little effect on the 5-LO metabolic capacity (Fig. 2B). Both COX and 5-LO metabolic capacities were lost in cells pretreated with 10 μM magnolol (Fig. 2C). We were unable to detect the formation of any specific AA metabolite among the profiles, which was selectively suppressed by magnolol. The viability was >93% when cells were incubated with 30 μM magnolol for 20 min at 37° (as assessed by trypan blue exclusion).

3.2. Effect of magnolol on COX and 5-LO activity

Ram seminal vesicles COX has long been used for the study of COX-1 activity. In order to determine the effect of magnolol on COX and 5-LO activity, the oxygen uptake was monitored to study the dioxygenation reaction of ram

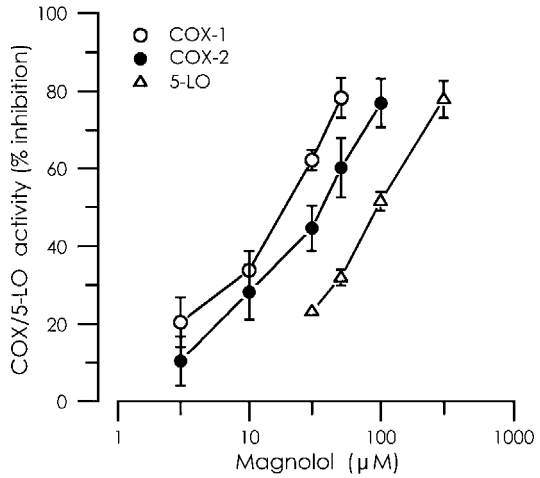

Fig. 3. Effect of magnolol on COX and 5-LO activities. Ram seminal vesicles COX (COX-1), or human recombinant 5-LO was incubated with DMSO (as control) or magnolol at the indicated concentrations for 3 min at 30° before addition of AA. Oxygen consumption in the reaction mixture was monitored continuously. Human recombinant COX-2 assay was carried out using COX inhibitor screening assay kit according to manufacturer's guidelines. The plate was read at 415 nm. Results were calculated as the percentage inhibition of control values (10.7 ± 0.3 nmol O_2/min and 4.2 ± 0.2 nmol O_2/min for COX-1 and human recombinant 5-LO, respectively; 0.45 ± 0.02 μg $PGF_{2\alpha}$/mL for COX-2). Values are means ± SD of 4–7 independent experiments.

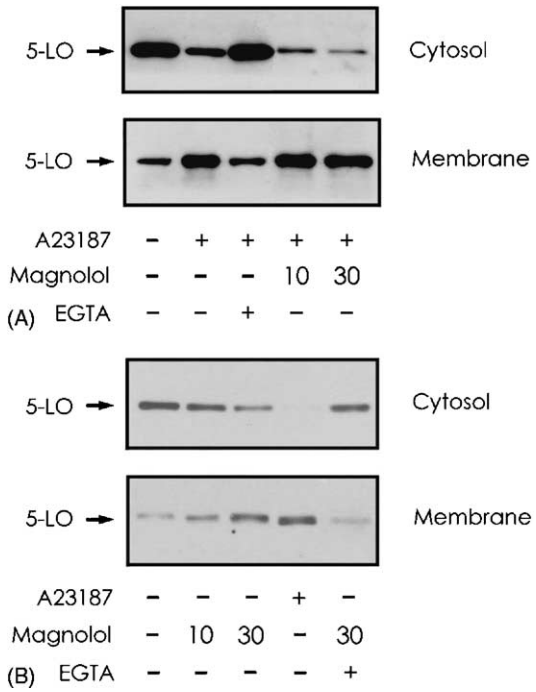

Fig. 4. Effect of magnolol on 5-LO translocation. (A) Cells were incubated with DMSO, 1 mM EGTA or 10–30 μM magnolol for 3 min at 37° before addition of 3 μM A23187 for 10 min. (B) Cells were incubated with DMSO or 10–30 μM magnolol or 3 μM A23187 for 10 min in the presence or absence of 1 mM EGTA. Cells were then disrupted and the cytosol and membrane fractions were prepared. Proteins were resolved by 7.5% SDS–PAGE, and 5-LO was detected by immunoblot analysis using anti-5-LO antibody. Results presented are representative of 3–4 independent experiments with similar results.

seminal vesicles COX and human recombinant 5-LO. The incorporation of oxygen into AA was determined by the difference in measurements of oxygen tension in solution. In addition, the effect of magnolol on COX-2 activity was also assessed using COX inhibitor screening assay kit, and $PGF_{2\alpha}$ production by $SnCl_2$ reduction of COX-derived PGH_2 was determined at 415 nm. As shown in Fig. 3, magnolol inhibited COX and 5-LO activities in a concentration-dependent manner. Magnolol inhibited COX-1 and COX-2 to a similar extent (IC_{50} values 26.0 ± 1.9 μM and 31.2 ± 2.8 μM, respectively), and was less active on 5-LO with the IC_{50} value about 3.5-fold higher than that for the inhibition of COX-1.

3.3. Effect of magnolol on membrane translocation of 5-LO

5-LO is localized in the cytosol in resting neutrophils and undergoes translocation to the membrane after cell stimulation. The membrane-associated enzyme is preferentially utilized for LTs synthesis. The effect of magnolol on the distribution of 5-LO protein between the cytosol and membrane fractions in rat neutrophils was determined by immunoblot analysis using mouse monoclonal 5-LO antibody. Figure 4A shows that the incubation of neutrophils with A23187 resulted in the loss of a substantial portion of the cytosolic 5-LO protein and a proportional increase in 5-LO in the membrane fraction, in agreement with the previous report in rat alveolar macrophages [26]. A23187-induced 5-LO translocation was reversed by EGTA. Magnolol had no inhibitory effect on A23187-induced response. By contrast, incubation of cells with magnolol alone resulted in a concentration-dependent change in the distribution of 5-LO protein in cytosol and membrane fractions. EGTA reversed the magnolol-stimulated 5-LO translocation (Fig. 4B).

3.4. Effect of magnolol on PLA_2 activity

To determine whether magnolol affected the total AA release *in vivo*, [^{3}H]AA-loaded neutrophils were stimulated in the presence of 0.15% BSA. Under these conditions, released AA is trapped in the medium, bound to BSA. Magnolol (3–30 μM) had no inhibitory effect on A23187-induced AA release. In contrast, removal of the extracellular Ca^{2+} from medium by EGTA or pretreatment of cells with $AACOCF_3$, a PLA_2 inhibitor [27], both significantly attenuated A23187-induced response (Fig. 5A). In the absence of A23187, magnolol alone evoked [^{3}H]AA release in a concentration-dependent manner. Treatment of cells with EGTA, $AACOCF_3$, and BEL, an $iPLA_2$ inhibitor [28], also attenuated this response ($P < 0.05$ for EGTA, and $P < 0.01$ for $AACOCF_3$ and BEL) (Fig. 5B).

3.5. Effect of magnolol on $cPLA_2$ activation

Activation of $cPLA_2$ involves its translocation from cytosol to membrane to provide access to phospholipid

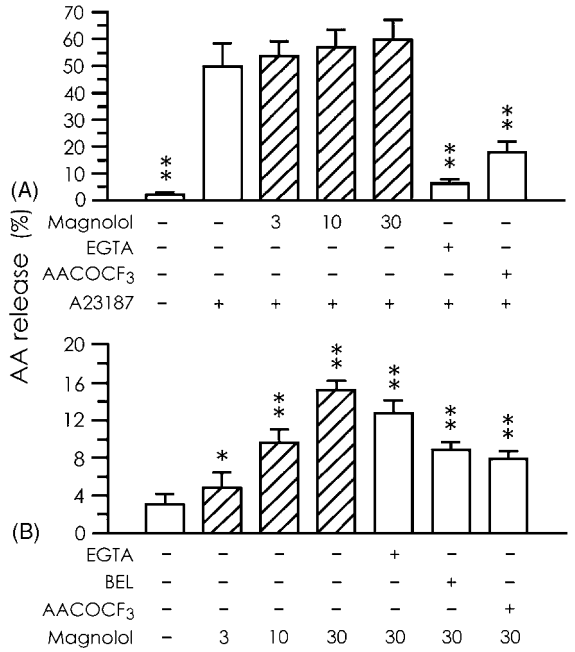

Fig. 5. Effect of magnolol on [^{3}A]AA release. The radioactivity in the medium was detected by liquid scintillator. Results were calculated as percent of the total radioactivity incorporated (14,460 ± 2009 dpm). (A) [^{3}H]AA-loaded cells were incubated with DMSO, 3–30 μM magnolol, 1 mM EGTA or 30 μM $AACOCF_3$ for 3 min at 37° before addition of DMSO or 3 μM A23187 for 20 min at 37°. Values are means ± SD of 4–7 independent experiments. $^{**}P < 0.01$, as compared with the control value (second column). (B) [^{3}H]AA-loaded cells were incubated with DMSO or 3–30 μM magnolol for 20 min, or with 1 mM EGTA, 30 μM BEL or 30 μM $AACOCF_3$ for 3 min before addition of magnolol for 20 min at 37°. Values are means ± SD of 4–6 independent experiments. $^{*}P < 0.01$, $^{**}P < 0.01$, as compared with the resting value (first column).

[4]. A23187 greatly induced the membrane association of $cPLA_2$. Figure 6 shows magnolol alone also evoked $cPLA_2$ membrane translocation in a concentration-dependent manner. Detectable membrane-associated $cPLA_2$ was seen as early as 1 min after stimulation of cells with magnolol, and was maintained for 10 min. Both A23187- and magnolol-induced responses were abolished by EGTA pretreatment.

It has been reported that the phosphorylation of $cPLA_2$ results in an increase in catalytic activity [5,6]. The phosphorylation of $cPLA_2$ is detected by the retarded mobility of the phosphorylated enzyme. A23187 induced a partial $cPLA_2$ gel shift in rat neutrophils. Pretreatment of cells with magnolol had no inhibitory effect on A23187-induced response (Fig. 6C). Magnolol alone produced a time- and concentration-dependent increase in the gel shift of $cPLA_2$ (Fig. 6D and E). The phosphorylation of $cPLA_2$ is detectable within 1 min of incubation with magnolol, and phosphorylation levels continued to rise for at least 10 min.

Pretreatment of cells with U0126, a MEK inhibitor [29], SB203580, a p38 MAPK inhibitor [30], or GF109203X, a protein kinase C inhibitor [31] had no significant effect on A23187- and magnolol-induced AA release (Fig. 7). Only U0126 exhibited an inhibitory effect on AA-release in response to fMLP (about 38% inhibition).

3.6. Effect of magnolol on MAPK activation

The immunoprecipitated MAPK, prepared from the cells stimulated with fMLP by using polyclonal antibody against ERK2 or p38 MAPK, greatly increased the phosphorylation of MBP. These effects were attenuated by the pretreatment of cells with U0126 or SB203580, respectively. However, the immunoprecipitated MAPK from the magnolol (30 μM)-stimulated cells did not increase the intensity of phosphorylation of MBP as compared to the resting levels (Fig. 8A and B).

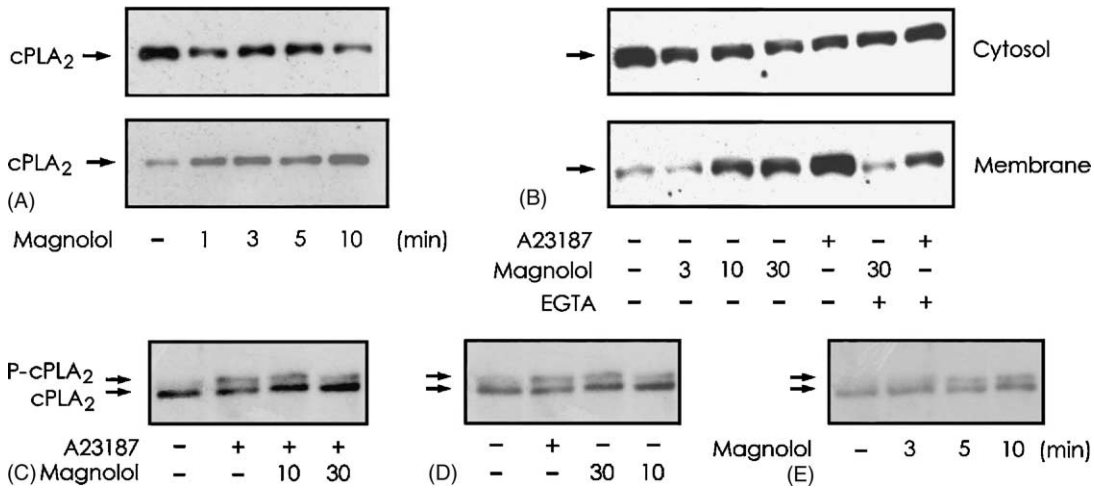

Fig. 6. Effects of magnolol on $cPLA_2$ translocation and phosphorylation. For $cPLA_2$ translocation, cells were incubated with (A) DMSO for 10 min or 30 μM magnolol for 1 to 10 min, (B) DMSO, 3–30 μM magnolol or 3 μM A23187 for 10 min at 37° in the presence or absence of 1 mM EGTA. Cells were then disrupted and the cytosol and membrane fractions were prepared. For $cPLA_2$ phosphorylation, cells were incubated with (C, D) DMSO or 10–30 μM magnolol for 3 min before the addition of 3 μM A23187 for 10 min, or with (E) DMSO for 10 min or 30 μM magnolol for 3–10 min at 37°. Cell lysates were then prepared. Proteins were resolved by 7.5% SDS–PAGE, and $cPLA_2$ was detected by immunoblot analysis using anti-$cPLA_2$ antibody. Results presented are representative of 3–4 independent experiments with similar results.

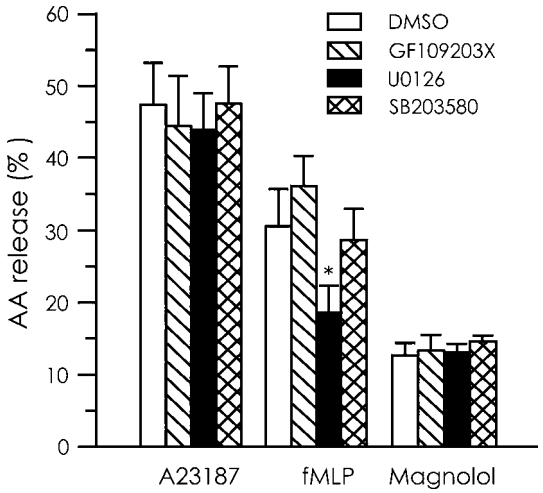

Fig. 7. Effects of GF109203X, U0126, and SB203580 on [^{3}A]AA release. The radioactivity in the medium was detected by liquid scintillator. Results were calculated as percent of the total radioactivity incorporated (17,511 ± 2784 dpm). [^{3}H]AA-loaded cells were incubated with DMSO, 10 μM GF109203X, 1 μM U0126 or 1 μM SB203580 for 3 min at 37° before addition of 3 μM A23187, 1 μM fMLP in the presence of 5 μg/mL of cytochalasin B, or 30 μM magnolol for 20 min at 37°. Values are means ± SD of 4–7 independent experiments. $^*P < 0.01$, as compared with the corresponding control value (the first column of each group).

4. Discussion

Our previous report demonstrated that magnolol ameliorates the A23187-induced pleurisy in mice, which is dependent on the reduction of the formation of eicosanoid mediators in the inflammatory site [21]. In the present study, magnolol concentration-dependently attenuated the formation of TXB_2 and LTB_4 in A23187-stimulated rat neutrophils. LTB_4, a potent chemoattractant, mediated plasma exudation indirectly via the circulating neutrophils. Inhibition of LTB_4 formation might account for the suppression of neutrophil recruitment to the pleural cavity [21]. Using HPLC with an on-line radiodetector showed LTB_4 is the major metabolite of AA in rat neutrophils, in agreement with a previous report [32]. Magnolol did not show selective inhibition of any specific AA metabolites production. Magnolol decreased the PGs formation at low concentrations, whereas it reduced both COX and 5-LO metabolic capacities at high concentrations, suggesting that magnolol inhibited both COX and 5-LO activities in rat neutrophils. Cytotoxic effects did not likely cause the inhibition of eicosanoid production by magnolol, since cell viability was almost not changed during the incubation of cells with 30 μM magnolol. The possibility of magnolol acts as a dual COX/LO inhibitor was corroborated by the results of inhibition of ram seminal vesicles COX and human recombinant 5-LO activity in a cell-free system, although higher concentrations of magnolol were required to inhibit enzyme activity than to inhibit their metabolic capacities in neutrophils. This variation could be explained by differences in the experimental conditions and the enzyme source. Magnolol was more active against COX than against 5-LO, since a significantly lower concentration of magnolol was required to inhibit COX than 5-LO, consistent with the inhibitory activity of magnolol on PGs and LTs production (Figs. 1 and 2). This could also explain the low concentration of magnolol stimulated A23187-induced LTB_4 formation, shown in Fig. 1B, because of the elevation of AA in the 5-LO pathway after COX inhibition. It has been demonstrated that COX exists as two genetically distinct isoforms. COX-1 is constitutively expressed and mediates physiological responses. COX-2 is encoded by an immediate-early gene and involved in pathological processes. COX-2 is not detectable in freshly isolated neutrophils [33]. A similar IC_{50} value was observed in the inhibition of COX-1 and COX-2 by magnolol indicates that magnolol acts as a non-selective COX inhibitor. Magnolol was also reported to inhibit A23187-induced LTs formation in human neutrophils [34], but at a concentration lower than used in the present study, and did not

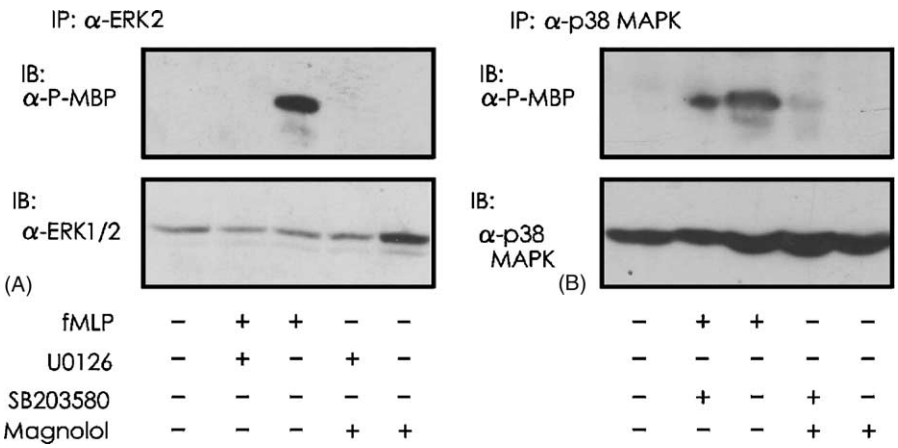

Fig. 8. Effect of magnolol on MAPK activation. Cells were preincubated with DMSO, 3 μM U0126 or 30 μM SB203580 for 10 min before the addition of 1 μM fMLP with 5 μg/mL of cytochalasin B for 1 min, or 30 μM magnolol for 20 min. Cell lysates were immunoprecipitated with (A) anti-ERK2 or (B) anti-p38 MAPK antibody, and assayed for MAPK activity using MBP as substrate. To confirm each loading, the blots were probed by anti-pan ERK or anti-p38 MAPK antibody. Similar results were obtained from 3 independent experiments.

directly inhibit 5-LO activity in rat basophilic leukemia-2H3 cells [35]. The discrepancy may also relate to differences between species or cell types.

5-LO is the principal lipoxygenase in neutrophils. A23187 treatment results in a Ca^{2+}-dependent translocation of 5-LO from the cytosol to a membrane-bound site where it interacts with 5-LO-activating protein and associates with $cPLA_2$ for LTs biosynthesis [11]. Thus, membrane translocation of 5-LO may be an important initial step in the chain of events leading to full activation of this enzyme in the intact leukocyte. However, it is unlikely that magnolol blocks this step because this compound did not affect A23187-induced 5-LO translocation, and magnolol alone stimulated the membrane association of 5-LO. Unlike 5-LO, COX is a membrane-associated glycoprotein in resting cells [12]. Phenolic compounds inhibit COX activity and have been proposed to compete for the AA binding site and competitively reduce the enzyme [36]. The decrease in hydroperoxide tone by phenolic compounds is probably the key element in the inhibition of COX activity. The inhibition of 5-LO activity by phenolic compounds is probably due to the reduction of catalytically active Fe^{3+} enzyme to the inactive Fe^{2+} form [37] or modulation of the hydroperoxide tone [38]. Magnolol, a hydroxylated biphenyl compound, is an effective antioxidant and suppresses lipid peroxidation [15,39]. It is plausible that magnolol acts as a redox type inhibitor, however, the precise mechanisms underlying the effect of magnolol on COX and 5-LO remain to be determined.

Neutrophils may contain several types of PLA_2 enzymes. Different isoforms of PLA_2 in neutrophils might mobilize AA for different functions [40]. $sPLA_2$ plays important microbicidal roles in neutrophils. $iPLA_2$ releases AA for the generation of superoxide anion. However, $cPLA_2$ plays a central role in the release of AA in cells in response to a wide variety of stimuli [4]. Magnolol had no inhibitory effect on A23187-induced AA release in rat neutrophils. Moreover, magnolol alone stimulates AA release. EGTA and $AACOCF_3$, a $cPLA_2$ inhibitor [27], attenuated both magnolol- and A23187-induced responses, suggesting that the same PLA_2 was activated by magnolol and A23187. $cPLA_2$ is required for [^{3}H]AA release and eicosanoid production induced by A23187 [41]. The observation that BEL reduced the magnolol-induced AA release suggests the involvement of $iPLA_2$, but this remains to be investigated.

Activation of $cPLA_2$ involves its translocation from cytosol to membrane to provide access to phospholipid [4]. The observation that magnolol alone, like A23187, stimulated the membrane recruitment of $cPLA_2$ supports the idea that magnolol activates PLA_2. Both A23187- and magnolol-induced response were blockade by EGTA, suggesting the involvement of $[Ca^{2+}]_i$ changes. Ca^{2+} is required for promoting the binding of $cPLA_2$ to membrane phosphocholine headgroups through a C_2 domain of $cPLA_2$ [4]. Our previous report demonstrated that magnolol induces $[Ca^{2+}]_i$ elevation in rat neutrophils [42]. This could explain the magnolol stimulation of the membrane association of 5-LO and $cPLA_2$, and why both effects were reduced by EGTA.

Full activation of $cPLA_2$ requires both increased $[Ca^{2+}]_i$ and $cPLA_2$ phosphorylation [43]. $cPLA_2$ is phosphorylated on multiple sites, however, only Ser^{505} phosphorylation partially contributes to $cPLA_2$ activation and results in a characteristic retardation of its electrophoretic mobility [5]. A23187 induced a partial $cPLA_2$ gel shift in rat neutrophils. A similar result was also demonstrated in rat macrophages [43]. Like A23187, magnolol induced a partial retardation in the electrophoretic mobility of $cPLA_2$, suggesting a similar mechanism of activation underlying the effect of A23187 and magnolol. It has been reported that the phosphorylation of $cPLA_2$ by p42 MAPK (ERK2), p38 MAPK and PKC results in an increase in catalytic activity [5,6]. However, the kinase involved in phosphorylation of $cPLA_2$ and AA release depend on the stimuli [44]. Our previous reports demonstrated that magnolol alone at high concentration (100 μM) stimulates the phosphorylation of p38 MAPK, while inhibiting PKC activity and having no effect on the phosphorylation of p42/44 MAPK [16,17]. Results of the immunoprecipitation MAPK activity experiments, revealed that magnolol at 30 μM did not increase in ERK2 and p38 MAPK enzyme activity. Thus, further study will be required to clarify the kinase participated in magnolol-stimulated $cPLA_2$ phosphorylation.

$cPLA_2$ phosphorylation in itself is not sufficient for AA release [41]. Ser^{505} phosphorylation is not essential for AA release in response to agonists that induce a sustained increase in $[Ca^{2+}]_i$, but does appear to be required when there is only a transient increase in $[Ca^{2+}]_i$ [43]. A sustained increase in Ca^{2+} is sufficient for inducing AA release. These reports are in line with our observation that the treatment of cells with U0126, a MEK inhibitor [29], SB203580, a p38 MAPK inhibitor [30], and GF109203X, a protein kinase C inhibitor [31] did not affect either magnolol- or A23187-induced AA release. A23187-induced AA release was also not suppressed by U0126 in mouse macrophages [41]. It was clear that fMLP induces transient $[Ca^{2+}]_i$ elevation. Neither a transient increase in $[Ca^{2+}]_i$ nor phosphorylation of $cPLA_2$ alone are sufficient for inducing AA release [43]. PD98059, a MEK inhibitor, and SB203580 significantly decrease the AA release from fMLP-stimulated human neutrophils [44]. However, U0126 but not SB203580 attenuated the fMLP-induced AA release in the present study. The discrepancy may relate to the differences in species and the experimental conditions, since we used 1 μM instead of 15 μM SB203580 to pretreat cells. Effects of SB203580 at concentrations above 1–2 μM were unrelated to p38 MAPK activity [45].

In conclusion, magnolol suppression of TXB_2 and LTB_4 formation in A23187-stimulated rat neutrophils at low concentrations is attributed to the direct inhibition of COX and 5-LO activities, respectively. Magnolol alone at high concentration stimulates the membrane translocation

of 5-LO and $cPLA_2$ via the sustained elevation of cellular free Ca^{2+}, and evokes AA release probably through the activation of $cPLA_2$ and $iPLA_2$.

Acknowledgments

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