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Inhibition by Magnolol of Formylmethionyl-leucyl-phenyl alanine-induced Respiratory Burst in Rat Neutrophils

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Abstract

The influence of the plant product magnolol on neutrophil superoxide anion ($O_2^{-\cdot}$) generation has been investigated in the rat.

Intraperitoneal injection of magnolol ($30\,mg\,kg^{-1}$) significantly inhibited the formyl-methionyl-leucyl-phenylalanine (fMLP)-induced respiratory burst in rat whole blood ex-vivo. Magnolol also inhibited the $O_2^{-\cdot}$ generation with an IC50 (concentration resulting in 50% inhibition) of $15{\cdot}4 \pm 1{\cdot}6\,\mu M$ and O_2 consumption in rat neutrophils in-vitro. Magnolol weakly inhibited the $O_2^{-\cdot}$ generation in the xanthine-xanthine oxidase system, decreased cellular cyclic AMP level and had no effect on cyclic GMP levels. It weakly inhibited neutrophil cytosolic protein kinase C activity but did not alter porcine heart protein kinase A activity. Magnolol attenuated fMLP-induced protein tyrosine phosphorylation with an IC50 of $24{\cdot}0 \pm 1{\cdot}9\,\mu M$ and the phosphorylation of mitogen-activated protein kinase p42/44 with an IC50 of $28{\cdot}5 \pm 4{\cdot}5\,\mu M$. However, magnolol alone activated neutrophil phospholipase D activity as determined by the formation of phosphatidic acid and phosphatidylethanol in the presence of ethanol. In the presence of NADPH, the arachidonate-activated NADPH oxidase activity in a cell-free system was weakly suppressed by magnolol.

These results suggest that the inhibition of respiratory burst in fMLP-activated neutrophils by magnolol is probably attributable mainly to the attenuation of protein tyrosine phosphorylation and p42/44 mitogen-activated protein kinase activation, and partly to the suppression of protein kinase C and NADPH oxidase activities.

Neutrophils play an important role in host defence mechanisms and inflammatory responses. Neutrophils are activated in response to various soluble and particulate stimuli. One of the events in neutrophil activation is respiratory burst in which the O_2 uptake from the surrounding medium is increased and large amounts of superoxide anion ($O_2^{-\cdot}$), which subsequently leads to the formation of other toxic O_2 metabolites, are generated (Badwey & Karnovsky 1980). Reactive O_2 species are believed to serve as bactericidal agents, as evinced by the susceptibility of patients with chronic granulomatous disease to severe recurrent infections (Smith & Curnutte 1991). Under certain circumstances, the excessive or inappropriate release of these highly reactive O_2 species can result in undesirable tissue damage. This is probably involved in the pathogenesis of many diseases (Halliwell & Gutteridge 1990).

The activation mechanisms of neutrophils are not fully elucidated. It is known that stimulation of neutrophils by receptor-binding ligands results in the production of several intracellular signals. Phospholipase C is rapidly activated by a G-protein-mediated process, leading to the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate the second messenger inositol trisphosphate and diacylglycerol that result in increase of intracellular Ca^{2+} concentration and activation of protein kinase C, respectively (Berridge 1987). These two second messengers act synergistically for $O_2^{-\cdot}$ generation (Robinson et al 1984). Phospholipase D is also activated by certain agonist in neutrophils and appears to be functionally linked to $O_2^{-\cdot}$ generation

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(Bonser et al 1989). Moreover, the chemotactic factor stimulates respiratory burst in neutrophils, which is accompanied by increased protein tyrosine phosphorylation and activation of mitogen-activated protein kinase (Torres et al 1993; Downey et al 1998; Zu et al 1998). Upon neutrophil activation, the cytosolic components of NADPH oxidase (mainly $p47^{phox}$ and $p67^{phox}$) translocated from the cytosol to the cell membranes where they associate with a flavocytochrome b_{558}, forming a functional active complex responsible for the production of $O_2^{-\cdot}$ in neutrophils (Segal & Abo 1993).

Magnolol, a hydroxylated biphenyl compound isolated from the Chinese herb Hou p'u, cortex of *Magnolia officinalis* (Fujita et al 1973), has been found to relax rat vascular smooth muscle (Teng et al 1990) and scavenge hydroxyl radicals (Fujita & Taira 1994) in-vitro, and suppress plasma extravasation caused by inflammatory mediators (Wang et al 1993) and protect rat heart against injury during ischaemia-reperfusion (Hong et al 1996) in-vivo. A preliminary study indicated that magnolol inhibited the formylmethionyl-leucyl-phenylalanine (fMLP)-induced $O_2^{-\cdot}$ generation in neutrophils. In the current study we have found that attenuation of protein tyrosine phosphorylation and p42/44 mitogen-activated protein kinase activation by magnolol probably plays a major role, whereas suppression of protein kinase C and NADPH oxidase activities by magnolol play only a minor role in the inhibition of the respiratory burst in rat neutrophils.

Materials and Methods

Materials

Magnolol was isolated and purified from the cortex of *Magnolia officinalis* (Magnoliaceae) as described elsewhere (Fujita et al 1973). The purity of magnolol (>99%) was determined by high-performance liquid chromatography. Other materials were purchased from Sigma (St Louis, MO) except for dextran T-500 (Pharmacia, Uppsala, Sweden), Hanks' balanced salt solution (HBSS) and protein kinase A assay kit (Gibco, Gaithersburg, MD), M&B 22948 (Rhône-Poulenc Rorer, Essex, UK), DE-52 cellulose (Whatman, Singapore), [γ-^{32}P]ATP, 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine, cyclic AMP and cyclic GMP enzyme immunoassay kits, protein kinase C assay kit and enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK), KT 5720 (Biomol Res., Plymouth Meeting, PA), Immobilon-P membrane (Millipore, Bedford, MA), mouse monoclonal antibodies to phosphotyrosine (PY-20), p38 mitogen-activated protein kinase, extracellular signal-regulated kinases 1 and 2 (Transduction, Lexington, KY), rabbit polyclonal antibodies to phospho-p38 mitogen-activated protein kinase and phospho-p42/44 mitogen-activated protein kinase (New England Biolabs, Beverly, MA). Magnolol was dissolved in a solvent mixture (40% polyethylene glycol 40, 10% ethanol, 20% normal saline, 20% distilled water and 10% dimethylsulphoxide (DMSO)) for intraperitoneal administration. DMSO was the solvent used for the inhibitors in the in-vitro test and the final volume of DMSO in the reaction mixture was 0·5%.

Measurement of luminol-dependent chemiluminescence of whole blood

A sample containing 10 μL of heparinized whole blood (withdrawn from the jugular vein of anaesthetized rats), 0·1 mM sodium azide, 33 μg mL^{-1} of horseradish peroxidase and 166 μM luminol, in a final volume of 0·6 mL, was preincubated at 37°C for 2 min. Then 1 μM fMLP plus 5 μg mL^{-1} of dihydrocytochalasin B were added to start the reaction. Sodium azide was included to inhibit myeloperoxidase, which was replaced by a controlled amount of azide-insensitive horseradish peroxidase (Wymann et al 1987). Chemiluminescence was monitored in a luminometer (LKB, Model 1251) and the peak height was recorded in mV. Chemiluminescence assays were initiated within a few minutes of collecting each blood sample.

Preparation of neutrophils and their subcellular fractions

Blood was collected from the abdominal aorta of pentobarbital-anaesthetized rats, and neutrophils were purified by dextran sedimentation, hypotonic lysis of erythrocytes and centrifugation through Ficoll-Hypaque (Wang et al 1997). Purified neutrophils with >95% viability were resuspended in Hanks' balanced salt solution (HBSS) containing 10 mM HEPES (pH 7·4) and 4 mM $NaHCO_3$, and kept in an ice-bath before use. Subcellular fractions were prepared as described elsewhere (Wang et al 1997). Briefly, neutrophils (1×10^8 cells mL^{-1}) were treated with 2·5 mM diisopropyl fluorophosphate for 30 min at 4°C, disrupted in Tris buffer (0·34 mM sucrose, 10 mM Tris–HCl (pH 7·0), 10 mM benzamidine and 2 mM phenylmethylsulphonyl fluoride) by sonication, and then centrifuged. Supernatants were pooled as the cytosol fraction, and pellets were resuspended in Tris

buffer as the membrane fraction for assay the $O_2^{-\cdot}$ generation in a cell-free system.

Measurement of $O_2^{-\cdot}$ generation and O_2 consumption

The generation of $O_2^{-\cdot}$ in neutrophil suspensions, xanthine-xanthine oxidase system and cell-free system was determined by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (Wang et al 1997). Absorbance changes of the reduction of ferricytochrome *c* at 550 nm were monitored continuously in a double-beam spectrophotometer. In the experiments to assess the xanthine oxidase activity, ferricytochrome *c* was omitted from the reaction mixture. The amount of uric acid formation was monitored continuously at 292 nm and calculated by using an absorbance-uric acid standard curve. The O_2 consumption of neutrophil suspensions was measured continuously with a Clark-type oxygen electrode using a YSI biological oxygen monitor (Model 5300) (Ingraham et al 1982).

Determination of cyclic AMP and cyclic GMP levels

Neutrophils (2×10^6 cells mL^{-1} for cyclic AMP, or 2×10^7 cells mL^{-1} for cyclic GMP) in HBSS were preincubated with test drugs for 9·5 min at 37°C. Thirty seconds after addition of fMLP, the reaction mixture was immediately added to 1·0 mL of 0·05 M acetate buffer (pH 6·2) containing 0·05 mM 3-isobutyl-1-methylxanthine for the cyclic AMP assay or 0·05 mM M&B 22948 for the cyclic GMP assay. After boiling for 5 min, the suspension was kept on ice, sonicated and followed by sedimentation (Simchowitz et al 1980). Supernatants were acetylated by addition of 0·025 volumes of triethylamine : acetic anhydride (2 : 1, v/v). The cyclic AMP and cyclic GMP content in aliquots of acetylated samples were assayed by using enzyme immunoassay kits.

Protein kinase C and protein kinase A assays

For the preparation of cytosolic protein kinase C, neutrophils were disrupted by sonication. After centrifugation, the supernatant was subjected to a DE-52 cellulose column to obtain partially purified protein kinase C (Wang et al 1997). Protein kinase C was preincubated with test drugs at 25°C for 3 min before addition of 50 μM ATP (0·2 μCi [γ-^{32}P]ATP) to start the reaction. Reactions were terminated after incubation for 15 min. Protein kinase C activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into the peptide substrate by use of a protein kinase C assay kit, based on the mixed-micelle method as described elsewhere (Hannun et al 1986). Porcine heart protein kinase A was preincubated with test drugs at 30°C for 3 min before addition of 100 μM ATP (0·3 μCi [γ-^{32}P]ATP) and 10 μM cyclic AMP to start the reaction. Reactions were terminated after incubation for 5 min. Protein kinase A activity was assayed by measuring the incorporation of ^{32}P into kemptide in the presence of cyclic AMP by use of a protein kinase A assay kit (Roskoski 1983).

Western blot analysis of proteins tyrosine phosphorylation and cellular mitogen-activated protein kinases

Neutrophils (1×10^7 cells mL^{-1}) in HBSS were preincubated with DMSO or test drugs at 37°C for 5 min before initiating the reaction by adding fMLP/dihydrocytochalasin B. One minute later, the reaction was quenched by adding stop solution (20% trichloroacetic acid, 1 mM phenylmethylsulphonyl fluoride, 7 μg mL^{-1} of aprotinin and pepstatin, 2 mM *N*-ethylmaleimide, 100 mM NaF, 5 mM diisopropyl fluorophosphate, 2 mM Na_3VO_4, 10 mM *p*-nitrophenyl phosphate). Proteins were separated through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to an Immobilon-P membrane, and probed with mouse monoclonal anti-phosphotyrosine antibody (1 : 1000 dilution) or rabbit polyclonal anti-phospho-p42/44 mitogen-activated protein kinase antibody (1 : 2000 dilution) in TST buffer (10 mM Tris-HCl (pH 8·0), 150 mM NaCl and 0·05% Tween 20) with 0·5% non-fat milk, or with rabbit polyclonal anti-phospho-p38 mitogen-activated protein kinase antibody (1 : 2000 dilution) in TST buffer with 1% bovine serum albumin. Detection was made using the enhanced chemiluminescence kit. The approximate molecular mass of the phosphotyrosine-containing proteins was interpolated from a standard curve constructed using standard proteins of known molecular weight. To confirm that the same amount of cellular proteins was loaded on each lane, the membranes were stripped, after detection of phospho-p38 mitogen-activated protein kinase and phospho-p42/44 mitogen-activated protein kinase, with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62·5 mM Tris–HCl, pH 6·8) for 30 min at 50°C, and then reprobed with mouse monoclonal anti-p38 mitogen-activated protein kinase (1 : 250 dilution) and anti-extracellular signal-regulated kinase 1 (1 : 1000 dilution) or anti-extracellular signal-regulated kinase 2 (1 : 2500

dilution) antibody in TST buffer with 0·5% non-fat milk, respectively.

Measurement of phospholipase D activity

Neutrophils (5×10^7 cells mL^{-1}) were suspended in HEPES buffer and loaded with 10 μCi 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine (150 Ci $mmol^{-1}$) at 37°C for 75 min, then washed and resuspended in HEPES buffer with 0·05% bovine serum albumin. The assay mixture containing test drugs and 1 mM $CaCl_2$, with or without 0·5% ethanol, was incubated for 3 min at 37°C before initiating the reaction by adding fMLP/dihydrocytochalasin B or magnolol. Lipids in the reaction mixture were then extracted and subsequently resolved by thin-layer chromatography on a silica gel 60 plate (Wang et al 1997). The lipids were located by staining with iodine vapour. The radioactivity of [^{3}H]products was directly quantified with a PhosphorImager (Molecular Dynamics, 445 SI) using ImageQuaNT software.

Statistical analysis

Statistical analyses were performed using the Bonferroni *t*-test method after analysis of variance. A P value less than 0·05 was considered significant for all tests. Analysis of the regression line test was used to calculate IC50 values.

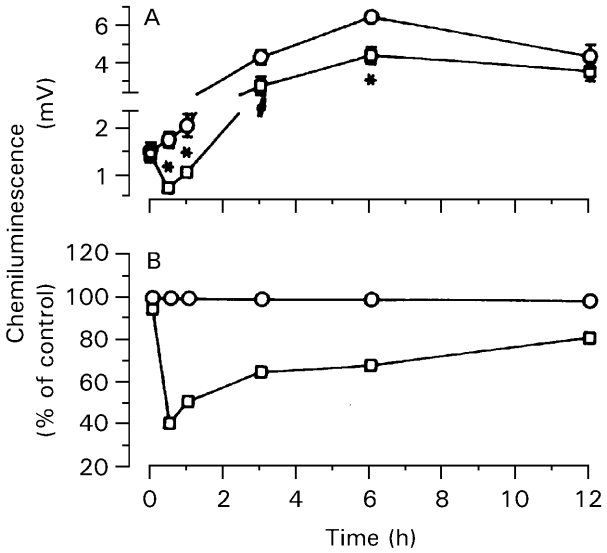

Figure 1. Time course of ex-vivo inhibition of formylmethionyl-leucyl-phenylalanine (fMLP) plus dihydrocytochalasin B-induced respiratory burst in rat whole blood by magnolol. At various times after intraperitoneal injection of vehicle (○) or 30 mg kg^{-1} of magnolol (□), blood was withdrawn and the luminol-dependent chemiluminescence was induced by the addition of 1 μM fMLP plus 5 μg mL^{-1} of dihydrocytochalasin B. (A) The peak chemiluminescence in mV was used to measure the respiratory burst. (B) The levels of chemiluminescence were presented as percentages of the corresponding vehicle control values. Values are expressed as means ± s.e.m. of nine to ten separate experiments. $P < 0.05$, $^*P < 0.01$ compared with the corresponding vehicle control values.

Results

Ex-vivo effect of magnolol on respiratory burst in whole blood

Reactive oxygen metabolites produced during the respiratory burst can be detected by their ability to oxidize luminol to a chemiluminescent product. Purified cell preparations are normally used for chemiluminescence studies, but a chemiluminescence emission, which is dependent primarily on neutrophil activity, can be detected in whole blood (Faden & Maciejewski 1981). This technique provides a convenient and more physiological approach for monitoring neutrophil function (Eckardt et al 1986). In rat whole blood, fMLP/dihydrocytochalasin B-induced chemiluminescence occurred rapidly and peaked within 1 min (data not shown). In a time course study of the effect of magnolol on neutrophil respiratory burst, 30 mg kg^{-1} (a dosage which significantly inhibits inflammation (Wang et al 1996)) was intraperitoneally injected and then blood was withdrawn at various time intervals. Significant reduction of fMLP/dihydrocytochalasin B-induced chemiluminescence in the ex-vivo test was observed at 30 min and persisted for at least 6 h after injection of magnolol (Figure 1).

In-vitro effect of magnolol on respiratory burst in neutrophil suspension

In order to evaluate whether magnolol directly inhibited neutrophil function, the effect of magnolol on respiratory burst in neutrophil suspension in-vitro was also determined. Addition of 0·3 μM fMLP plus 5 μg mL^{-1} of dihydrocytochalasin B into neutrophil suspension evoked a rapid and transient $O_2^{-\cdot}$ generation (5·9 ± 0·8 nmol $(10\,min)^{-1}$ per 10^6 cells). Magnolol concentration-dependently inhibited fMLP/dihydrocytochalasin B-induced $O_2^{-\cdot}$ generation (Figure 2) with an IC50 value of 15·4 ± 1·6 μM. Addition of 0·1 μM fMLP plus 5 μg mL^{-1} of dihydrocytochalasin B into neutrophil suspension in the presence of 1 mM NaN_3 evoked non-mitochondrial O_2 consumption (14·5 ± 1·9 nmol $(5\,min)^{-1}$ per 6×10^6 cells), and this response was attenuated by magnolol in a concentration-dependent manner (Figure 2). More than 95% viability was observed with trypan blue exclusion in cells treated with 100 μM magnolol for

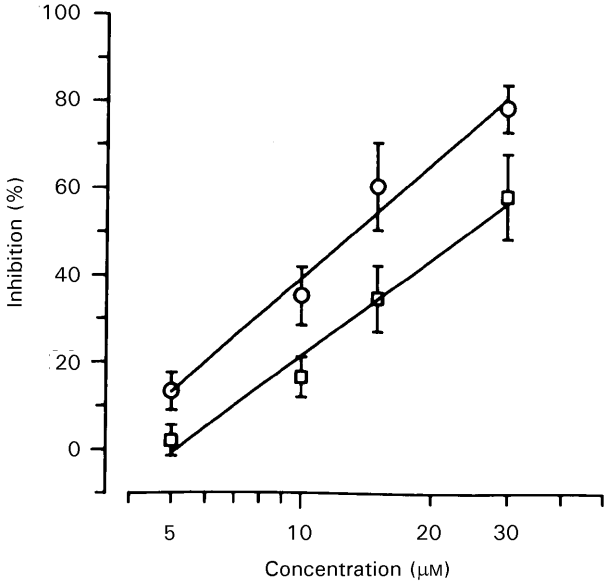

Figure 2. Effect of magnolol on formylmethionyl-leucyl-phenylalanine (fMLP)- plus dihydrocytochalasin B-induced $O_2^{-\cdot}$ generation and O_2 consumption in rat neutrophils. Neutrophils were preincubated with 5–30 μM magnolol at 37°C for 3 min before addition of 0·3 μM fMLP plus 5 μg mL^{-1} of dihydrocytochalasin B for $O_2^{-\cdot}$ generation (○), or 0·1 μM fMLP plus 5 μg mL^{-1} of dihydrocytochalasin B for O_2 consumption (□). Values are expressed as means ± s.e.m. of four to five separate experiments.

5 min. Magnolol (30 μM) also inhibited the $O_2^{-\cdot}$ generation induced by 300 μg mL^{-1} of concanavalin A and 1 mg mL^{-1} of opsonized zymosan (7·7 ± 0·5 as control vs 5·8 ± 0·7 nmol (10 min)$^{-1}$ per 10^6 cells and 21·2 ± 2·6 as control vs 11·8 ± 1·3 nmol (10 min)$^{-1}$ per 10^6 cells, respectively, both $P < 0·01$).

Effect of magnolol on $O_2^{-\cdot}$ generation in xanthine-xanthine oxidase system

The scavenging ability of magnolol was assessed using a xanthine-xanthine oxidase $O_2^{-\cdot}$ generating system. When xanthine is converted by xanthine oxidase to uric acid, $O_2^{-\cdot}$ is released. Unlike superoxide dismutase, which markedly attenuated the $O_2^{-\cdot}$ generation at 1 μg mL^{-1} (Wang et al 1994), a significant inhibition of $O_2^{-\cdot}$ generation by magnolol was observed only at high concentration (3·51 ± 0·15 as control vs 2·93 ± 0·17 nmol (10 min)$^{-1}$ at 100 μM magnolol, 15·1 ± 6·6% inhibition, $P < 0·05$). At this concentration, magnolol also inhibited uric acid formation (20·1 ± 7·7% inhibition, $P < 0·05$).

Effect of magnolol on the activity of protein kinase C and protein kinase A and the cellular level of cyclic nucleotide

In the presence of Ca^{2+}, phosphatidylserine and phorbol 12-myristate 13 acetate, the incorporation of ^{32}P from [γ-^{32}P]ATP into peptide substrate was demonstrated in neutrophil cytosolic protein kinase C preparations. Protein kinase C activity was greatly inhibited by 3 nM staurosporine (0·55 ± 0·01 as control vs 0·16 ± 0·01 nmol ^{32}P min^{-1} (mg protein)$^{-1}$, $P < 0·01$), whereas it was weakly inhibited by magnolol (15·0 ± 2·1 and 31·2 ± 2·3% inhibition at 30 and 100 μM, respectively, both $P < 0·01$). Porcine heart protein kinase A induced kemptide phosphorylation in the presence of ATP and 10 μM cyclic AMP as activator. This response was significantly reduced by 30 μM KT 5720, a protein kinase A inhibitor (Kase et al 1987) (45·4 ± 3·2 as control vs 24·0 ± 1·5 pmol ^{32}P min^{-1} (μg protein)$^{-1}$, $P < 0·01$), whereas magnolol had negligible effect whether the cyclic AMP was present or absent (7·0 ± 0·6 as control vs 6·1 ± 0·8 pmol ^{32}P min^{-1} (μg protein)$^{-1}$ at 100 μM magnolol in the absence of cyclic AMP, $P > 0·05$). Magnolol reduced the cellular cyclic AMP level (0·55 ± 0·06 as control vs 0·33 ± 0·03 pmol per 2×10^6 cells at 30 μM; about 40·1 ± 4·5% inhibition, $P < 0·05$), whereas it had no appreciable effect on cyclic GMP level even up to 100 μM magnolol.

Effect of magnolol on protein tyrosine phosphorylation

Protein tyrosine phosphorylation was assessed by immunoblotting with a specific monoclonal anti-phosphotyrosine antibody. Figure 3 depicts the pattern of tyrosine phosphorylation in whole-cell lysates prepared from neutrophils. Several proteins were labelled to variable extent in unstimulated cells. fMLP (0·1 μM) plus dihydrocytochalasin B (5 μg mL^{-1}) enhanced the tyrosine phosphorylation state of a number of proteins and prominent bands migrating at about 118 and 62 kDa (arrows). Genistein (10 μg mL^{-1}), a tyrosine kinase inhibitor (Akiyama et al 1987), concomitantly attenuated the fMLP/dihydrocytochalasin B-induced tyrosine phosphorylation of both 118 and 62 kDa proteins (about 48·4 ± 4·0 and 56·6 ± 5·9% inhibition, respectively). Magnolol inhibited the tyrosine phosphorylation of these two proteins in a concentration-dependent manner (IC50 about 23·1 ± 2·8 and 24·0 ± 1·9 μM for 118 and 62 kDa proteins, respectively).

Effect of magnolol on mitogen-activated protein kinase activation

Immunoblot analysis with specific anti-phospho-p42/44 or anti-phospho-p38 mitogen-activated protein kinase antibody was performed to determine the effect of magnolol on mitogen-activated

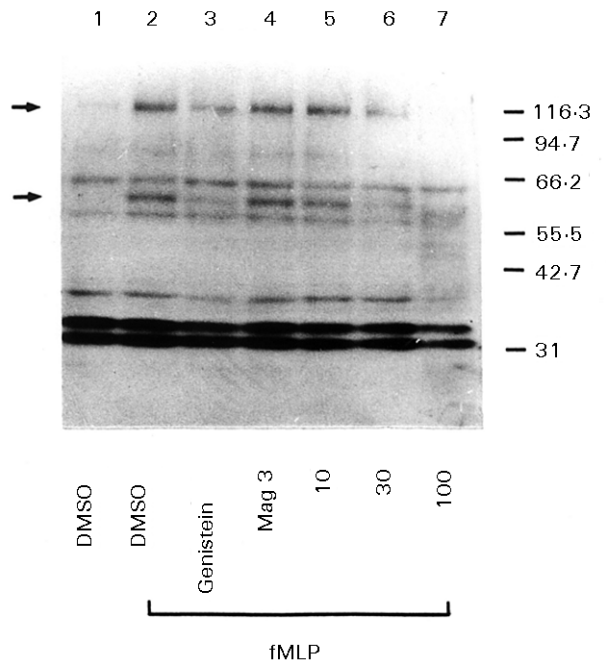

Figure 3. Effect of magnolol on protein tyrosine phosphorylation. Neutrophils were preincubated with dimethylsulphoxide (DMSO, lanes 1–2), 10 μg mL^{-1} of genistein (lane 3) or 3–100 μM magnolol (Mag, lanes 4–7) for 5 min at 37°C before addition of DMSO (lane 1) or 0·1 μM formylmethionyl-leucyl-phenylalanine plus 5 μg mL^{-1} of dihydrocytochalasin B (fMLP, lanes 2–7). One minute later, cells were rapidly sedimented, boiled in Laemmli sample buffer, and subjected to SDS-PAGE. Each lane contains sample protein (50 μg). Analysis was performed by immunoblotting with a monoclonal antibody to phosphotyrosine. The arrows point to the proteins of about 118 and 62 kDa. The results shown are from an experiment representative of three separate experiments.

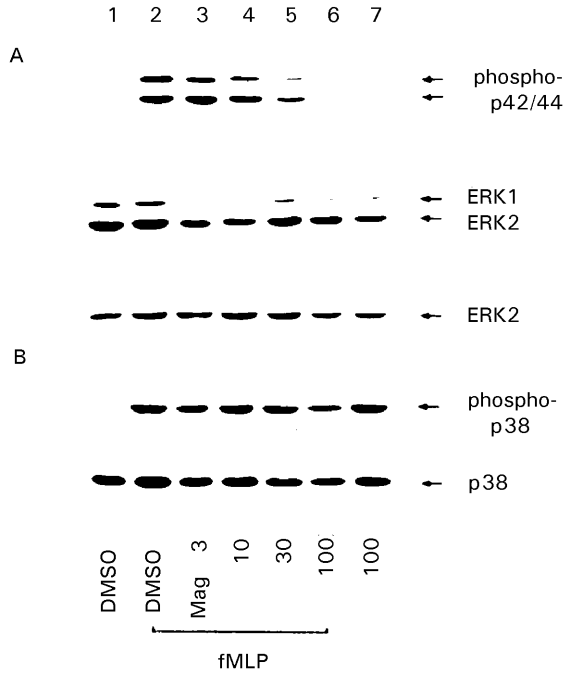

Figure 4. Effect of magnolol on mitogen-activated protein kinase activation. Neutrophils were preincubated with dimethylsulphoxide (DMSO, lane 2) or 3–100 μM magnolol (Mag, lanes 3–6) for 5 min at 37°C and then stimulated with 0·1 μM formylmethionyl-leucyl-phenylalanine plus 5 μg mL^{-1} of dihydrocytochalasin B (fMLP, lanes 2–6) for 1 min. Effect of DMSO (lane 1) or 100 μM magnolol (lane 7) alone on the cells was also studied for total 6 min reaction time. After termination of the reaction, cells were rapidly sedimented, boiled in Laemmli sample buffer, and subjected to SDS-PAGE. Each lane contains sample protein (60 μg). Analysis was performed by immunoblotting with (A) anti-phospho-p42/44 antibody, antibodies to extracellular signal-regulated kinases 1 or 2 (ERK1, ERK2), and (B) anti-phospho-p38 or anti-p38 mitogen-activated protein kinase antibody. The results shown are representative of four separate experiments.

protein kinase pathway in neutrophils. Following stimulation with fMLP/dihydrocytochalasin B, as shown in Figure 4, cellular p42/44 and p38 mitogen-activated protein kinases become phosphorylated and hence activated. Magnolol specifically inhibited p42/44 but not p38 mitogen-activated protein kinase in a concentration-dependent manner (IC50 about 28·5 $\pm$ 4·5 μM). However, magnolol alone at high concentration (100 μM) significantly increased the phosphorylation of p38 mitogen-activated protein kinase. The differences observed for the induced protein phosphorylation of cellular mitogen-activated protein kinases did not result from differences in loading or from cellular protein digestion. The anti-extracellular signal-regulated kinase 1 that we used also recognizes extracellular signal-regulated kinase 2 (p42).

Effect of magnolol on phospholipase D activity

Phospholipase D catalyses the hydrolysis primarily of phosphatidylcholine to produce phosphatidic acid (Billah et al 1989). In the presence of ethanol, phosphatidic acid yields phosphatidylethanol via a transphosphatidylation reaction. Addition of 1 μM fMLP plus 5 μg mL^{-1} of dihydrocytochalasin B to the [^{3}H]alkyl-lysophosphatidylcholine-loaded neutrophils significantly increased the phosphatidic acid and phosphatidylethanol mass in the presence of 0·5% (v/v) ethanol (Figure 5A, B). Magnolol alone, at 100 but not 30 μM, stimulated phosphatidic acid and phosphatidylethanol formation. This activation response was in a time-dependent manner (Figure 5C, D, E) with a significant increase of phosphatidic acid ($P < 0·05$) and phosphatidylethanol ($P < 0·01$) mass at 3 min reaction time. Pretreatment of cells with 30 μM magnolol for 3 min did not affect the fMLP-induced response (data not shown).

Effect of magnolol on arachidonate-activated NADPH oxidase activity

In a cell-free system, addition of arachidonate to the mixture of neutrophil cytosol and membrane frac-

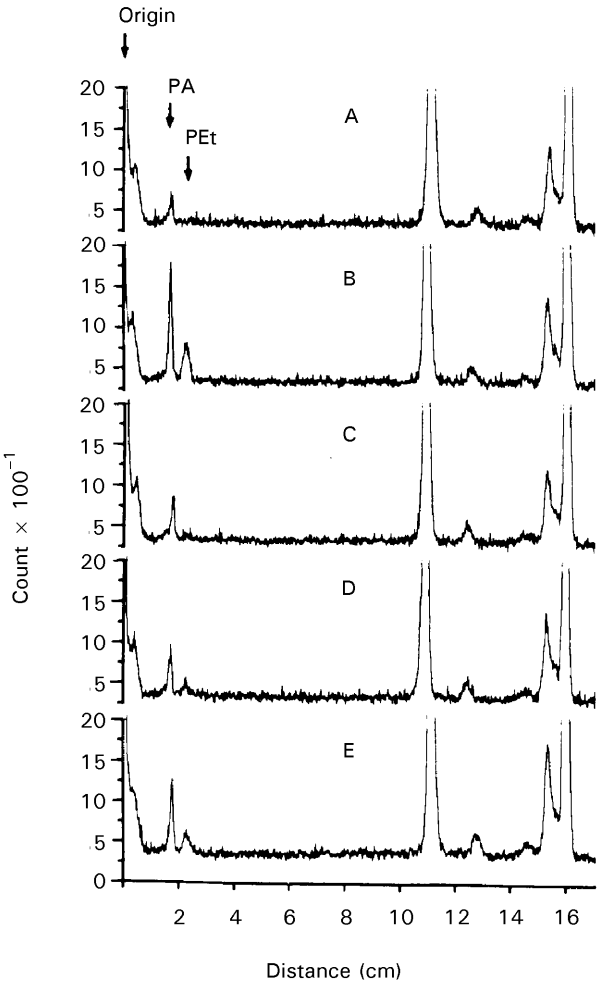

Figure 5. Effect of magnolol on phospholipase D activity of rat neutrophils. [^{3}H]Alkyl-lysophosphatidylcholine-loaded cells were activated with (A) dimethylsulphoxide for 3 min, (B) 1 μM formylmethionyl-leucyl-phenylalanine plus 5 μg mL^{-1} of dihydrocytochalasin B for 30 s, or 100 μM magnolol for (C) 30 s, (D) 1 min or (E) 3 min at 37°C in the presence of 0·5% (v/v) ethanol. Lipids were extracted from the reaction mixture, separated on silica gel 60 plates, and the [^{3}H]products were visualized and quantified by phosphor screen autoradiography. The location of phosphatidic acid (PA) and phosphatidylethanol (PEt) are indicated. The results shown are from an experiment representative of three separate experiments.

tions led to assembly and activation of NADPH oxidase. Trifluoperazine (60 μM), an NADPH oxidase inhibitor (Bellavite et al 1983), greatly reduced the $O_2^{-\cdot}$ generation, whereas magnolol was only a weak inhibitor (26·7 ± 5·8% inhibition at 100 μM) (Table 1).

Table 1. Effect of magnolol on $O_2^{-\cdot}$ generation in arachidonate-activated cell-free system.

	NADPH oxidase activity (nmol $O_2^{-\cdot}$ (10 min)$^{-1}$ (10^7 cells)$^{-1}$)
Control	2·57 ± 0·11
Magnolol 10 μM	2·51 ± 0·27
Magnolol 30 μM	2·10 ± 0·16
Magnolol 100 μM	1·90 ± 0·10*
Trifluoperazine 60 μM	0·87 ± 0·20**

Neutrophil cytosol and membrane fractions, 10 μM FAD, 3 μM GTPγS and 0·25 mg mL^{-1} of ferricytochrome *c* were preincubated with dimethylsulphoxide (vehicle control), magnolol (10–100 μM) or trifluoperazine (60 μM) in the presence of 50 μM NADPH at 28°C for 3 min before addition of 100 μM arachidonate to start the reaction. $O_2^{-\cdot}$ generation was measured by continuously detecting the absorbance changes of ferricytochrome *c*. Values are expressed as means ± s.e.m. of six to seven separate experiments. *$P < 0·05$, **$P < 0·01$ compared with the control value.

Discussion

We have demonstrated that magnolol inhibited luminol-dependent chemiluminescence in rat whole blood in response to fMLP ex-vivo. The pharmacokinetics analyses of plasma concentration-time profiles of magnolol after intravenous injection is fitted by a two-compartment open model with constant elimination half-life ($t_{1/2}$ = 2·5 and 50 min) (Tsai et al 1996). Thus, the plasma concentration of magnolol at 30 min after intraperitoneal injection of 30 mg kg^{-1} was estimated to be 35 μM. Over the same concentration range, magnolol also inhibited the respiratory burst in fMLP-, concanavalin A- and opsonized zymosan-activated rat neutrophils in-vitro. This suggested that magnolol directly inhibited the neutrophil respiratory burst through interaction with certain signal transduction steps that follows cell activation. Although magnolol weakly reduced the amount of $O_2^{-\cdot}$ in the xanthine–xanthine oxidase system, the inhibition of $O_2^{-\cdot}$ generation by magnolol in neutrophils could not be due to the scavenging of generated $O_2^{-\cdot}$, based on the consideration that much higher concentrations of magnolol had to be used in the inhibition of the former than the latter system. Furthermore, magnolol at high concentration also inhibited xanthine oxidase activity as evidenced by the reduction of uric acid formation, a finding also made in other reports (Chang et al 1994).

It is well recognized that agents which increase cellular cyclic AMP inhibit $O_2^{-\cdot}$ generation (Nielson 1987). However, the role of cyclic GMP in respiratory burst is uncertain. The inhibitory action of cyclic AMP on neutrophils appears to depend on the activation of protein kinase A. Magnolol had no effect on porcine heart protein kinase A activity whether or not the cyclic AMP was present, indicating that magnolol might not activate protein kinase A.

However, the cellular protein kinase A activity is assumed to be decreased because of the reduction of cellular cyclic AMP levels by magnolol. Thus, there is no indication that inhibition of the respiratory burst by magnolol is acting via the increase in cellular cyclic AMP level or protein kinase A activity. It is generally accepted that fMLP-induced activation of oxidase takes place via protein kinase C-dependent and -independent signal transduction pathways. Protein kinase C plays a role in phosphorylation of membrane-associated $p47^{phox}$ and the assembly of an active NADPH oxidase complex (Majumdar et al 1993). The observations that magnolol only weakly inhibited the neutrophil cytosolic protein kinase C activity suggest that this effect did not play a major role in the inhibition of respiratory burst.

Tyrosine phosphorylation plays a regulatory role in the signal transduction pathway leading to the respiratory burst induced by fMLP (Torres et al 1993). Several proteins were labelled to variable extent as assessed by immunoblotting with monoclonal anti-phosphotyrosine antibody, the most prominent phosphotyrosine-containing proteins were about 118 and 62 kDa after stimulation with fMLP. Like the tyrosine kinase inhibitor genistein (Akiyama et al 1987), magnolol also attenuated fMLP-induced tyrosine phosphorylation of both 118 and 62 kDa protein bands. Mitogen-activated protein kinases have been demonstrated to play an important role in mediating intracellular signal transduction and regulating cellular functions. Activation of cellular p42/44 and p38 mitogen-activated protein kinases are probably indispensable for fMLP-induced $O_2^{-\cdot}$ generation (Downey et al 1998; Zu et al 1998). Because both p38 and p42/44 mitogen-activated protein kinases phosphorylated $p47^{phox}$ at the same site and at similar rates (El Benna et al 1996), mitogen-activated protein kinases might have a role in the translocation of this oxidase component to the plasma membrane and activation of the respiratory burst. Magnolol attenuated fMLP-induced phosphorylation of p42/44 mitogen-activated protein kinase but did not reduce the immunoreactivity of phospho-p38 mitogen-activated protein kinase. The inhibition of protein tyrosine phosphorylation and p42/44 mitogen-activated protein kinase phosphorylation occurred over the same range of concentrations at which the neutrophil respiratory burst was inhibited, suggesting that the blockade of these pathways might play an important role.

There is evidence that fMLP-induced $O_2^{-\cdot}$ generation is a Ca^{2+}-dependent process (Lehmeyer et al 1979). In the presence of extracellular Ca^{2+}, elevation of intracellular Ca^{2+} concentration in response to fMLP results in the release of Ca^{2+} from inositol trisphosphate-sensitive intracellular stores and from the influx of extracellular Ca^{2+}. It is plausible that magnolol might exert its effect through the inhibition of fMLP-induced intracellular Ca^{2+} changes. Unexpectedly, magnolol alone increases the intracellular Ca^{2+} concentration through an inositol trisphosphate-dependent (but pertussis toxin-insensitive) pathway (Wang & Chen 1998). However we could not exclude the possibility that magnolol might desensitize the fMLP-induced response. To clarify this point we examined the effect of the Ca^{2+} ionophore ionomycin on fMLP-induced $O_2^{-\cdot}$ generation. Pretreatment of cells with 0·1 μM ionomycin, which greatly elevates intracellular Ca^{2+} levels (Wang 1996), for 3 min did not affect the fMLP-induced $O_2^{-\cdot}$ generation. A longer preincubation with ionomycin is necessary for the inhibition of fMLP-induced response (data not shown). Thus the desensitization of fMLP-induced changes in intracellular Ca^{2+} levels might not play a major role in the inhibition of fMLP-induced $O_2^{-\cdot}$ generation after a brief pretreatment with magnolol. Although magnolol increased intracellular Ca^{2+} and enhanced the phosphorylation of p38 mitogen-activated protein kinase, these two actions alone are probably not sufficient to evoke $O_2^{-\cdot}$ generation (Korchak et al 1988; Nick et al 1997). In this study, magnolol by itself did not activate neutrophil $O_2^{-\cdot}$ generation.

Since phosphatidic acid, the product of phospholipase D activation, could act on the respiratory burst through the activation of protein kinase C or NADPH oxidase (Bellavite et al 1988), it is plausible to assume that magnolol might exert its effect through inhibition of fMLP-activated phospholipase D activity. However, magnolol alone at high concentration activated phospholipase D activity as evidenced by the detection of phosphatidic acid and phosphatidylethanol formation. Although the mechanism by which phospholipase D is activated in neutrophils is not fully defined, the fMLP-activation of phospholipase D has been reported to be Ca^{2+} dependent (Planat et al 1996). Whether the magnolol-induced elevation of intracellular Ca^{2+} contributed to phospholipase D activation needs further investigation. Because magnolol at a concentration which significantly inhibited $O_2^{-\cdot}$ generation did not affect the fMLP-induced phospholipase D activation, the possibility of the involvement of this signalling pathway in the inhibition of respiratory burst by magnolol was excluded.

The $O_2^{-\cdot}$-generating NADPH oxidase complex in neutrophils constitutes a heterodimeric flavocytochrome b_{558} and cytosolic factors, mainly

$p47^{phox}$ and $p67^{phox}$ (Segal & Abo 1993). Upon activation, $p47^{phox}$ is phosphorylated and its polyproline motif is rendered accessible to the C-terminal SH3 domain of $p67^{phox}$ (Finan et al 1994). This interaction changes the overall structure of the complex and makes it able to recognize the membrane flavocytochrome b_{558} through the polyproline motif of $p22^{phox}$ and one of the SH3 domains of $p47^{phox}$ (Sumimoto et al 1994), favourable to electron transport, and therefore the univalent reduction of O_2 (Segal & Abo 1993). Because arachidonate could mimic the effect of phosphorylation that occurs in neutrophil activation, addition of arachidonate to the cytosol and membrane fractions produced assembly and activation of NADPH oxidase (Fuchs et al 1995). The finding that magnolol weakly inhibited the oxidase activity in the presence of NADPH suggested that inhibition of oxidase might not play a crucial role in the inhibition of respiratory burst by magnolol.

To summarize, we found that magnolol inhibited the fMLP-activated respiratory burst in whole blood ex-vivo and in neutrophil suspension in-vitro. Magnolol did not function via scavenging of generated $O_2^{-\cdot}$, decreasing the concentration of intracellular Ca^{2+} phospholipase D activity, or increasing cellular cyclic AMP levels. Inhibition of respiratory burst by magnolol is probably attributable mainly to the blockade of protein tyrosine phosphorylation and mitogen-activated protein kinase activation, and also partly to the suppression of protein kinase C and NADPH oxidase activities.

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