Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 475 (2003) 19–27
www.elsevier.com/locate/ejphar

The anti-inflammatory effect of honokiol on neutrophils: mechanisms in the inhibition of reactive oxygen species production

Kuo-Tong Liou[a], Yuh-Chiang Shen[b], Chieh-Fu Chen[b], Cheng-Ming Tsao[c], Shen-Kou Tsai[d,e,*]

[a] *Institute of Clinical Medicine, National Yang-Ming University School of Medicine, Taipei, Taiwan*
[b] *National Research Institute of Chinese Medicine, Taipei, Taiwan*
[c] *Department of Anesthesiology, Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan*
[d] *Department of Anesthesiology, Taipei-Veterans General Hospital, Taipei, Taiwan*
[e] *Department of Anesthesiology, College of Medicine, National Taiwan University and National Yang-Ming University, Taipei, Taiwan*

Received 24 April 2003; received in revised form 7 July 2003; accepted 11 July 2003

Abstract

Reactive oxygen species produced by neutrophils contribute to the pathogenesis of focal cerebral ischemia/reperfusion injury and signal the inflammatory response. We have previously shown that honokiol, an active principle extracted from *Magnolia officinalis*, has a protective effect against focal cerebral ischemia/reperfusion injury in rats that paralleled a reduction in reactive oxygen species production by neutrophils. To elucidate the underlying mechanism(s) of the antioxidative effect of honokiol, peripheral neutrophils isolated from rats were activated with phorbol-12-myristate-13-acetate (PMA) or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) in the presence or absence of honokiol. In this study, we found that honokiol inhibited PMA- or fMLP-induced reactive oxygen species production by neutrophils by three distinct mechanisms: (1) honokiol diminished the activity of assembled-NADPH oxidase, a major reactive oxygen species producing enzyme in neutrophils by 40% without interfering with its protein kinase C (PKC)-dependent assembly; (2) two other important enzymes for reactive oxygen species generation in neutrophils, i.e., myeloperoxidase and cyclooxygenase, were also inhibited by honokiol by 20% and 70%, respectively; and (3) honokiol enhanced glutathione (GSH) peroxidase activity by 30%, an enzyme that triggers the metabolism of hydrogen peroxide (H_2O_2). These data suggested that honokiol, acting as a potent reactive oxygen species inhibitor/scavenger, could achieve its focal cerebral ischemia/reperfusion injury protective effect by modulating enzyme systems related to reactive oxygen species production or metabolism, including NADPH oxidase, myeloperoxidase, cyclooxygenase, and GSH peroxidase in neutrophils.

Keywords: Honokiol; Reactive oxygen species; NADPH oxidase; Myeloperoxidase; Cyclooxygenase; Glutathione peroxidase; Protein kinase C

1. Introduction

Honokiol ($C_{18}H_{18}O_2$, MW = 266.33, Fig. 1), an active component isolated from the herb 'Houpo' (*Magnolia officinalis* Rehd. et Wils.), is an effective antioxidant (Taira et al., 1993), protects animal tissues against lipid peroxidation (Lo et al., 1994; Chiu et al., 1997), serves as an anti-platelet drug (Teng et al., 1988), and displays an anti-inflammatory effect in activated macrophages (Matsuda et al., 2001; Son et al., 2000). We have previously reported that honokiol can reduce myocardial infarct size and exhibit anti-arrhythmic effect in rats subjected to coronary artery occlusion (Tsai et al., 1996, 1999). Recently, we found that honokiol can ameliorate focal cerebral ischemia/reperfusion-induced brain infarction (Liou et al., 2003).

Cerebral infarction is closely associated with inflammatory responses in the progression of stroke or post-ischemic reperfusion injury, in which reactive oxygen species generation by primed/activated neutrophils plays an important role in brain damage (Dirnagl et al., 1999; Williams, 1994). Reactive oxygen species produced by neutrophils not only contribute to the pathogenesis of ischemia/reperfusion injury but also exaggerate the ischemia/reperfusion injury by further activating these inflammatory target cells (Finkel, 1998). The release of reactive oxygen species and reactive oxygen species-related free radicals by neutrophils depends

* Corresponding author. Department of Anesthesiology, College of Medicine, National Taiwan and Yang-Ming Universities, 201 Shih-Pai Road, Sec. 2, Pei-Tou, Taipei 112, Taiwan. Tel.: +886-2-28757001; fax: +886-2-28751597.
E-mail address: sktsai@vghtpe.gov.tw (S.-K. Tsai).

0014-2999/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0014-2999(03)02121-6

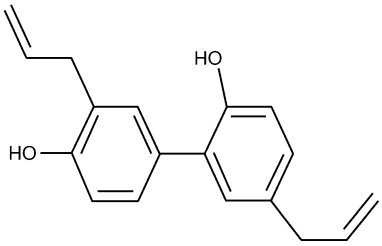

Fig. 1. Chemical structure of honokiol ($C_{18}H_{18}O_2$, MW = 266.33).

upon the activation of reactive oxygen species-producing enzyme systems, including NADPH oxidase, myeloperoxidase, and cyclooxygenase, etc. Reactive oxygen species produced by the abovementioned enzyme systems are detoxified mainly via peroxisomal catalase or glutathione (GSH) peroxidase, by catalyzing the conversion of hydrogen peroxide (H_2O_2) to oxygen and water (Chan, 2001).

NADPH oxidase, a powerful oxidant-producing enzyme complex consisting of membrane-bound components (e.g., gp91-phox, p22-phox) and cytosolic components (e.g., p47-phox, p67-phox), is assembled on the surface membrane of neutrophils and generates enormous amounts of superoxide anion ($O_2^{\cdot -}$) when activated by inflammatory stimulants (Casimir and Teahan, 1994). Superoxide dismutates $O_2^{\cdot -}$ to form H_2O_2, which can be further metabolized to more cytotoxic radical 'hypochlorous acid (HOCl)' by myeloperoxidase, an abundant chloride peroxidase in azurophil granules in neutrophils (Weiss, 1989). NADPH oxidase can be activated by at least two distinct mechanisms: (1) through direct protein kinase C (PKC) activation by phorbol esters, e.g., phorbol-12-myristate-13-acetate (PMA), which may trigger the phosphorylation of cytosolic components (e.g., p47-phox) that are essential for the assembly of other NADPH oxidase subunits (e.g., p67-phox) to activate this oxidase (Casimir and Teahan, 1994); and (2) through receptor/G protein coupling activation by receptor-coupled inflammatory stimuli, e.g., *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), which may activate NADPH oxidase through a calcium-dependent pathway (Suzuki et al., 1985; Lew et al., 1984). Recent studies showed that cyclooxygenase (also prostaglandin H synthase) is another crucial enzyme in reactive oxygen species generation during ischemia/reperfusion injury (Rieger et al., 2002), and inhibition of cyclooxygenase activity is beneficial for cerebral infarction (Sugimoto and Iadecola, 2003). With anti-ischemia/reperfusion injury potential, honokiol could exert its protective effect by modulating the reactive oxygen species producing-enzyme systems in neutrophils, as mentioned above.

To elucidate the possible mechanism(s) for the protective effect of honokiol, we undertook the current study to explore whether honokiol could prevent PMA- or fMLP-induced reactive oxygen species production by peripheral rat neutrophils in an intact cell system or a cell-free system, paying special attention to the activity of NADPH oxidase, myeloperoxidase, cyclooxygenase, and GSH peroxidase.

2. Materials and methods

2.1. Animal preparation

The animals used in the present investigation were handled according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication no. 85-23, revised 1996). All experimental procedures and protocols used in this investigation were reviewed and approved by the Hospital Animal Research Committee. Male Long-Evans rats (National Lab. Animal Breeding and Research Center, Taipei, Taiwan) weighing 250–350 g were kept under a 12:12-h light–dark cycle in temperature- (24 ± 1 °C) and humidity- ($55 \pm 5\%$) controlled animal breeding rooms. All animals were allowed free access to food and water.

2.2. Neutrophil preparation and drug treatments

Peripheral whole blood was collected from anesthetized Long-Evans rats (250–350 g) into syringes containing heparin (20 U/ml blood). Blood samples were mixed with an equal volume of 6% dextran solution in a 50-ml centrifuge tube and incubated in an upright position for 30 min at room temperature to allow sedimentation of erythrocytes. The upper layer, rich in neutrophils, was collected and subjected to centrifugation at $250 \times g$ for 15 min at 4 °C. Peripheral blood neutrophils were then separated by the Ficoll gradient centrifugation method, followed by lysis of contaminating erythrocytes, and washed three times with cold phosphate-buffered saline (PBS) (Shen et al., 2001). The neutrophils were mixed with 0.1, 1, or 10 μM of honokiol or vehicle control [0.05% dimethylsulfoxide (DMSO)] in Hanks' balanced saline solution (HBSS) for 10 min at 37 °C before the addition of PMA or fMLP. Cells were incubated with test drugs in 96-well cell culture plates (FALCON®, Becton Dickinson, USA) or 5-ml polystyrene round-bottomed tubes (FALCON®, Becton Dickinson) depending on the experiments.

2.3. Measurement of extracellular $O_2^{\cdot -}$ generation by neutrophils

Extracellular $O_2^{\cdot -}$ generation was evaluated according to Korchaks (1984) method. The production of $O_2^{\cdot -}$ induced by PMA (2.5 μg/ml) or fMLP (1 μM) by neutrophils pretreated with honokiol (0.1–10 μM) was determined as superoxide dismutase-inhibitable reduction of cytochrome *c* (80 μM), by measuring the changes in absorbance at 550 nm (ΔOD_{550}) in the presence or absence of superoxide dismutase (133 U/ml) at 37 °C for 1 h. Staurosporine (Stau), a PKC inhibitor, was included as a positive control for the inhibition of extracellular $O_2^{\cdot -}$ production. Data are expressed as $O_2^{\cdot -}$ nmol/2×10^5 cells/h, using the molar extinction coefficient of 2.1×10^4 l/mol/cm for cytochrome *c*, with a path length of 8 mm.

2.4. Flow cytometric analysis of intracellular reactive oxygen species production in neutrophils

Intracellular accumulation of reactive oxygen species by neutrophils was measured by a flow cytometer (FACSCalibur™; Becton Dickinson) according to our previous report (Shen et al., 1998). Briefly, neutrophils (2×10^6/ml) were incubated at 37 °C for 20 min with 20 μM of 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes, USA). The acetate moieties of DCFH-DA were cleaved off intracellularly by esterase, liberating the membrane-impermeable 2′,7′-dichlorofluorescin (DCFH), which fluoresces when oxidized to 2′,7′-dichlorofluorescein (DCF) by reactive oxygen species. After labeling, cells were treated with 0.1, 1, or 10 μM of honokiol or 0.2 μM of staurosporine for 20 min and then stimulated with fMLP (1 μM) or PMA (100 ng/ml). The production of intracellular reactive oxygen species was then determined 30 min later by flow cytometry (FACSCalibur™; Becton Dickinson) with an emission wavelength of 525 nm (FL1) for DCF. Data are expressed as mean channel fluorescence for each sample (1×10^4 cells auto-selected from 2×10^6 cells by flow cytometer) as calculated by CellQuest® software (Becton Dickinson) on a Power Macintosh 7300/200 computer.

2.5. Measurement of NADPH oxidase activity in subcellular fractions

Neutrophil–particulate fractions were prepared according to the method of Clark et al. (1990) with some modifications. Neutrophils (2×10^7/ml) were incubated for 10 min at 37 °C in the presence of PMA (0.1 μg/ml) or an equivalent concentration of DMSO. After incubation, the cell suspension was centrifuged at 4 °C at $300 \times g$ for 6 min, and the pellet was resuspended in sample buffer containing 100 mM KCl, 3 mM NaC1, 3.5 mM $MgC1_2$, 1.25 mM EGTA, 10 mM PIPES, 2 mM phenylmethyl sulfonyl fluoride, 33 μM leupeptin, 35 μM antipain, 24 μg/ml chymostatin, 0.035 μM pepstatin, and 0.08 μM aprotinin at pH 7.3. The cells were disrupted using a microprobe sonicator at low power (10%) three times for 10 s at 4 °C. Intact cells were removed by centrifugation at $500 \times g$ for 5 min. The resulting supernatant was centrifuged at 4 °C for 20 min at $115{,}000 \times g$, after which the supernatant was discarded and the pellet (particulate fractions) was washed in sample buffer and recentrifuged for 20 min at $115{,}000 \times g$. Following the removal of the supernatant, the pellet was gently resuspended in assay buffer without protease inhibitors. NADPH oxidase activity was determined in the particulate fractions in the presence of 400 μM NADPH, and $O_2^{\cdot -}$ generation was monitored for 20 min at 25 °C as superoxide dismutase-inhibitable cytochrome *c* reduction (Korchak et al., 1984). Honokiol was added to the PMA pre-assembled particulate fractions 20 min before the addition of NADPH (for subcellular NADPH oxidase activity study). Alternatively, neutrophils were preincubated with honokiol or staurosporine for 20 min at 37 °C before PMA stimulation (for NADPH oxidase assembly study). Data are expressed as nmol $O_2^{\cdot -}$/2×10^6 cell equivalents/20 min.

2.6. Protein kinase(s) activity assay

Neutrophils were resuspended to a concentration of 2×10^7/ml in ice-cold extraction buffer. The extraction buffer consisted of 50 mM Tris–HC1 (pH 7.5), 50 mM EGTA, 1 mM phenylmethyl sulfonyl fluoride, protease inhibitor cocktail (Calbiochem®, USA), and 50 mM 2-mercaptoethanol. Protease inhibitor cocktail consisted of 0.1 mM leupeptin, 1 μM pepstatin A, 5 μM bestatin, 0.08 μM aprotinin, and 1.5 μM cysteine protease inhibitor. The cell suspension was sonicated for 10 s at 4 °C five times and the cell lysate was separated into the cytosol (as cytosolic fraction) and pellet fractions by centrifugation at $100{,}000 \times g$ for 60 min at 4 °C. The pellet was solubilized by resuspension in extraction buffer with 0.1% Triton X-100 (Calbiochem®, Germany) vortexed and incubated at 4 °C for 60 min. The solubilized extract as particulate fraction was separated by centrifugation at $10{,}000 \times g$ for 5 min. Both cytosolic and particulate fractions were stored at 4 °C and assayed for kinase activity on the same day. Protein concentration was determined with a protein assay reagent (Bio-Rad, USA). Protein kinase C (PKC) or adenosine-3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase (PKA) activity was measured with a nonradioactive protein kinase assay kit (Calbiochem®, Germany). This assay kit was based on an enzyme-linked immunosorbent assay that used a synthetic PKC/PKA pseudosubtrate and a monoclonal antibody that recognized the phosphorylated peptide. PKC or PKA phosphorylates pseudosubstrate (peptide) on serine through a Ca^{2+}/phosphatidylserine- or a cAMP-dependent mechanism, respectively. Ca^{2+}/phospholipid-dependent PKC was assayed in the presence of Ca^{2+} and phosphatidylserine, and the negative control was measured in the presence of 20 mM EGTA. In some experiments, PMA (100 ng/ml) was added to live neutrophils for stimulation and translocation of PKC prior to sonication, and cytosolic PKC activity from this preparation was used as another negative control as most of the cytosolic PKC had translocated to the particulate fraction. In the PKA activity assay, the negative control was measured in the absence of cAMP. Data are expressed as $100 \times OD_{492}/12$ μl cell lysate from 2×10^7 cells.

2.7. Estimation of myeloperoxidase activity

Myeloperoxidase activity was evaluated according to the method of Bani et al. (1998) with some modifications. Briefly, myeloperoxidase activity was determined in the presence or absence of honokiol by mixing 0.1 ml of cytosolic fraction (from 2×10^6 cells in HBSS) with 2.9 ml of potassium phosphate buffer (PPB, 50 mM, pH 6) containing 0.19 mg/ml of *o*-dianisidine chloride and

0.0005% H_2O_2 as a substrate for myeloperoxidase. Oxidized *o*-dianisidine formed a soluble chromophore and absorbance (OD_{460}) was determined by spectrophotometry (Hitachi, Japan) over 2 min. Myeloperoxidase activity (ΔOD_{460}) was calculated by subtracting the value of OD_{460} at time 0 min from that at 2 min for each sample. Data are expressed as $100 \times \Delta OD_{460}/2 \times 10^6$ cell equivalents.

2.8. Glutathione (GSH) peroxidase activity assay

Neutrophils were resuspended at a concentration of 2×10^7/ml in ice-cold extraction buffer as stated above. Cells were sonicated for 10 s at 4 °C three times and the cytosolic fraction was separated by centrifugation at 10,000 × *g* for 20 min at 4 °C. Protein concentration was determined with a protein assay reagent (Bio-Rad). GSH peroxidase activity (Arun et al., 1999) was determined in the supernatants by mixing 0.1 ml of cytosolic fraction with 0.9 ml of reaction mixture containing 100 mM phosphate buffer ($NaHPO_4$/Na_2HPO_4, pH 7.5), 2 mM glutathione (GSH), 1 mM sodium azide, 1 U glutathione reductase, 0.12 mM NADPH, and 2 mM H_2O_2. The GSH peroxidase activity was assayed using the conversion of NADPH to NAD by recording the changes in absorbance at 340 nm over 3 min (ΔOD_{340}) using H_2O_2 (Se-dependent activity) as substrate against a reference that did not contain substrates. An additional blank containing all components except glutathione reductase was determined to correct for non-enzymatic oxidation of GSH and NADPH by substrates (H_2O_2). Data are expressed as the rate of change in absorbance ($100 \times \Delta OD_{340}$).

2.9. Cyclooxygenase activity assay

Cytosolic fraction of rat neutrophils was prepared from cells (2×10^7/ml) activated by PMA (0.1 μg/ml) for 20 min. Cyclooxygenase activity was measured with a chemiluminescent-based cyclooxygenase assay kit (Calbiochem®, Germany). This assay kit used a specific chemiluminescent substrate to detect the peroxidative activity of cyclooxygenase enzymes. Briefly, after pretreatment with honokiol (0.1, 1.0, or 10 μM), 50 μM of NS-398 (a cyclooxygenase-2 inhibitor), 100 μM of resveratrol (a cyclooxygenase-1/cyclooxygenase-2 inhibitor), or 1 mM of ibuprofen (a cyclooxygenase-1 inhibitor), the direct residual activity of cyclooxygenase was measured by addition of a proprietary luminescent substrate and arachidonic acid. Light emission began immediately and was proportional to the cyclooxygenase activity in the sample. The chemiluminescent signal was measured over 5 s by a microplate luminometer reader (Orion®, Germany). Data are expressed as relative light units (RLU).

2.10. Estimation of cell viability

Cell viability was determined according to Ormerod's (2000) method after incubation of cells (2×10^6/ml) with test drugs for 1 h in 5-ml polystyrene round-bottomed tube (FALCON, Becton Dickinson). This method can be adapted for a flow cytometer by adding propidium iodide (10 μg/ml), which is excluded by viable cells but which, when taken up by dead or dying cells, binds to nucleic acids and fluoresces red. The viable cells can be further identified by the addition of fluorescein diacetate (100 ng/ml), which is not fluorescent and which is taken up by cells and is converted to fluorescein by an intracellular esterase. Fluorescein is retained by the cell if the plasma membrane is intact. After incubation with test drugs, cells suspension were further incubated with propidium iodide and fluorescein diacetate at room temperature for 10 min and analyzed immediately on a flow cytometer (FACSCalibur™; Becton Dickinson) by recording forward and light scatter, red (>630 nm) and green (520 nm) fluorescence. After gating for light scatter to include single cells and to exclude clumps and debris, cell populations were displayed by green (viable) versus red (dead) fluorescence. Cell viability (%) was calculated by CellQuest® software (Becton Dickinson) on a Power Macintosh 7300/200 computer. Alternatively, cell viability was further compared by using a cytotoxicity detection kit (Roche®, Germany). This kit measures cytotoxicity and cell lysis by detecting lactate dehydrogenase (LDH) activity released from damaged cells.

2.11. Honokiol and other chemicals

Honokiol was purchased from Nacalai (Japan). It was first dissolved in DMSO as a stock solution (20 mM) and then serially diluted in PBS immediately prior to experiments. Stock solution was used within 1 week after preparation. For examination of the effect of these drugs, 10 μl of drug solution was added to 1.0 ml of neutrophil suspension and incubated at 37 °C for 10 min prior to the addition of 100 ng/ml PMA (Sigma, USA) or 1 μM fMLP (Sigma). Other chemicals, except where indicated, were purchased from Sigma.

2.12. Statistical analysis

All values in the text and figures are given as means ± S.E.M. Parametric data were analyzed by analysis of variance (ANOVA) followed by post hoc Dunnett's *t*-test for multiple comparisons. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Effect of honokiol on fMLP- or PMA-induced extracellular $O_2^{\cdot -}$ production by rat neutrophils

To examine whether extracellular $O_2^{\cdot -}$ production could be inhibited by honokiol, neutrophils were stimulated with

fMLP (a receptor-mediated activator) or PMA (a non-receptor-mediated but direct PKC activator) in the presence or absence of honokiol. Both fMLP- and PMA-induced extracellular $O_2^{\cdot -}$ production was concentration dependently inhibited by honokiol (Table 1). Staurosporine, a PKC inhibitor, used in this test as a positive control for the inhibition of extracellular $O_2^{\cdot -}$ production, significantly inhibited fMLP- or PMA-induced extracellular $O_2^{\cdot -}$ production (Table 1).

3.2. Effect of honokiol on fMLP- or PMA-induced intracellular reactive oxygen species accumulation by rat neutrophils

To examine whether intracellular reactive oxygen species accumulation could be diminished by honokiol, neutrophils were stimulated with fMLP or PMA in the presence or absence of honokiol. FMLP or PMA triggered the prompt accumulation of intracellular reactive oxygen species (as reflected by increased DCF fluorescence) to 4.8- or 4.3- fold the basal level, respectively (Table 2). Honokiol concentration dependently decreased the generation of intracellular reactive oxygen species induced by fMLP or PMA. Staurosporine (0.2 μM) completely abolished the intracellular reactive oxygen species production induced by fMLP or PMA.

3.3. Effect of honokiol on the assembly or the activity of assembled NADPH oxidase

To examine whether the machinery for the assembly of NADPH oxidase or the activity of assembled NADPH oxidase could be modulated by honokiol, oxidase activity was determined in particulate fractions. PMA enhanced NADPH oxidase activity to 204% or 157% in 'drug treatment before PMA' or 'drug treatment after PMA' groups, respectively. Pretreatment with honokiol did not interfere with the assembly of NADPH oxidase (Table 3). Staurosporine (0.1 μM), a PKC inhibitor, used to contrast the PKC-dependent assembly of NADPH oxidase, caused a

Table 1
Effect of honokiol on fMLP- or PMA-induced extracellular $O_2^{\cdot -}$ production by neutrophils

Agonists (inducers)	Extracellular $O_2^{\cdot -}$ production (nmol/2 × 10^5 cells/h)[a]	
	FMLP	PMA
Agonist only	0.96 ± 0.31	1.20 ± 0.26
+ HK (0.1 μM)	0.76 ± 0.16	1.15 ± 0.08
+ HK (1.0 μM)	0.64 ± 0.08[b]	0.92 ± 0.06[c]
+ HK (10 μM)	0.52 ± 0.07[b]	0.81 ± 0.06[c]
+ RVT (10 μM)	0.76 ± 0.13	1.02 ± 0.10
+ Stau (0.5 μM)	0.19 ± 0.18[b]	0.20 ± 0.12[c]

[a] Extracellular $O_2^{\cdot -}$ production was determined as superoxide dismutase-inhibitable reduction of cytochrome *c*, as described in Materials and methods. FMLP (1 μM)- or PMA (2.5 μg/ml)-induced production of $O_2^{\cdot -}$ by peripheral rat neutrophils was examined in the presence or absence of honokiol (HK). Values represent the means ± S.E.M. of six experiments performed on different days using cells from different rats. Resveratrol (RVT), a plant-derived antioxidant, was included as reference compound. Staurosporine (Stau), a PKC inhibitor, was included as a positive control for the inhibition of extracellular reactive oxygen species production.

[b] $P < 0.05$ as compared to samples treated with fMLP alone, respectively.

[c] $P < 0.05$ as compared to samples treated with PMA alone, respectively.

Table 2
Effect of honokiol on intracellular reactive oxygen species accumulation

Agonists (inducers)	Intracellular reactive oxygen species accumulation[a] (DCF mean channel fluorescence/1 × 10^4 cells)	
	fMLP-activated	PMA-activated
Control (vehicle)	10.0 ± 0.6[b]	9.9 ± 0.2[c]
Agonist alone	48.3 ± 4.0	43.5 ± 3.4
+ HK (0.1 μM)	45.5 ± 6.0	43.0 ± 3.0
+ HK (1.0 μM)	38.3 ± 3.7[b]	42.2 ± 4.1
+ HK (10 μM)	30.6 ± 4.0[b]	31.5 ± 2.6[c]
+ RVT (10 μM)	46.3 ± 6.0	42.0 ± 5.4
+ Stau (0.2 μM)	13.0 ± 1.0[b]	12.9 ± 0.5[c]

[a] Neutrophils (2 × 10^6/ml) were incubated with 20 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA). After labeling, cells were treated with honokiol (HK), resveratrol (RVT) or staurosporine (Stau) followed by fMLP (1 μM) or PMA (100 ng/ml) stimulation. Accumulation of intracellular reactive oxygen species was then determined after 30 min on a flow cytometer with measurement of emission at 525 nm (FL1) for 2′,7′-dichlorofluoroscein (DCF). Data are expressed as mean channel fluorescence of DCF for each sample (1 × 10^4 cells auto-selected from 2 × 10^6 cells by cytometer). Values represent the means ± S.E.M. of 6–10 experiments performed on different days using cells from different rats.

[b] $P < 0.05$ as compared to samples treated with fMLP alone, respectively.

[c] $P < 0.05$ as compared to samples treated with PMA alone, respectively.

Table 3
Effect of honokiol on subcellular NADPH oxidase activity

	NADPH oxidase activity [$O_2^{\cdot -}$ production (nmol)/2 × 10^6cell equivalents/20 min][a]	
	Drug treatment before PMA-activated[b]	Drug treatment after PMA-activated[c]
Control (vehicle)	0.52 ± 0.06	0.45 ± 0.08
PMA alone	1.06 ± 0.09	0.71 ± 0.12
+ HK (0.1 μM)	1.05 ± 0.11	0.62 ± 0.15
+ HK (1.0 μM)	1.03 ± 0.14	0.46 ± 0.09[e]
+ HK (10 μM)	1.01 ± 0.16	0.40 ± 0.11[e]
+ Stau (0.1 μM)	0.59 ± 0.07[d]	ND

ND, sample not detected.

[a] NADPH oxidase activity was measured as $O_2^{\cdot -}$ production in particulate fractions isolated from PMA-activated neutrophils (2 × 10^6 cells), expressed as superoxide dismutase-inhibitable cytochrome *c* reduction in 20 min at 37 °C. Values represent the means ± S.E.M. of five experiments performed on different days using cells from different rats.

[b] Neutrophils were preincubated with honokiol (HK) or staurosporine (Stau) for 10 min at 37 °C before PMA stimulation followed by isolation of the particulate fraction (drug treatment before PMA-activated).

[c] HK was added 10 min to the isolated particulate fractions before addition of the NADPH (drug treatment after PMA-activated).

[e] $P < 0.05$ as compared with PMA alone, respectively.

[d] $P < 0.05$ as compared with PMA alone, respectively.

significant inhibition of this assembly process. In contrast, addition of honokiol to PMA-preactivated particulate fraction concentration dependently inhibited the activity of assembled NADPH oxidase (Table 3).

3.4. Effect of honokiol on protein kinase(s) activity

To examine whether modulation of protein kinase(s) activity mediates the inhibitory effect of honokiol on reactive oxygen species production, we measured the activity of PKC and PKA with an enzyme-linked immunosorbent assay (ELISA). Cytosolic PKC activity was not inhibited by honokiol (Table 4). In the positive control groups, PKC activity was significantly reduced to 64% in the presence of 20 mM EGTA (for negation of calcium effects) or reduced to 54% in PMA-pretreated samples (in which most of the cytosolic PKC was translocated to the particulate fraction). PKA activity was enhanced to 138% in the presence of cyclic AMP (20 μM). Honokiol also did not modulate PKA activity (Table 4).

3.5. Effect of honokiol on myeloperoxidase or GSH peroxidase activity

To study whether modulation of myeloperoxidase or GSH peroxidase activity contributed to the antioxidant effect of honokiol, we examined the effect of honokiol on cytosolic myeloperoxidase or GSH peroxidase activity. The myeloperoxidase activity in the control (vehicle only) sample was 7.12 ± 0.05 ($100 \times OD_{460}/2 \times 10^6$ cells). Honokiol significantly inhibited myeloperoxidase activity of neutrophils at concentrations of 1 and 10 μM (Fig. 2); however, at 1 and 10 μM, honokiol enhanced GSH peroxidase activity by 20–30% (Fig. 3). These data illustrate that inhibition of reactive oxygen species production or activation of reactive oxygen species metabolism are possible mechanisms responsible for the reactive oxygen species scavenging effect of honokiol.

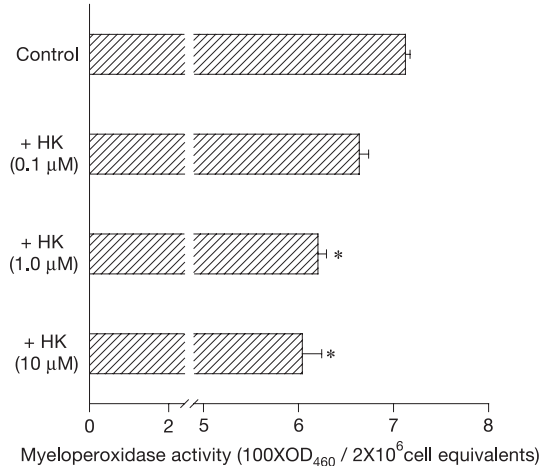

Fig. 2. Effect of honokiol on myeloperoxidase activity of peripheral neutrophils. Myeloperoxidase activity was determined in the presence or absence of honokiol (HK) by mixing 0.1 ml of cytosolic fractions (from 2×10^6 cells) with 2.9 ml of potassium phosphate buffer containing 0.19 mg/ml of *o*-dianisidine chloride and 0.0005% H_2O_2 as a substrate for myeloperoxidase. Oxidized *o*-dianisidine formed a soluble chromophore and the absorbance (OD_{460}) was determined by spectrophotometry over 2 min. Myeloperoxidase activity (ΔOD_{460}) was calculated by subtracting the value of OD_{460} at time 0 min from that at 2 min for each sample. Data are expressed as $100 \times \Delta OD_{460}/2 \times 10^6$ cell equivalents. Values represent the means ± S.E.M. of three experiments performed on different days using cells from different rats. *$P < 0.05$ as compared to control (vehicle).

Table 4
Effect of honokiol on the protein kinase(s) activity

	Protein kinase(s) activity ($100 \times OD_{492}/12$ μl cell lysate from 2×10^7 cells)[a]	
	PKC	PKA
Control (vehicle only)	291.6 ± 17.7	224.1 ± 20.2
+HK (0.1 μM)	287.6 ± 21.4	223.2 ± 18.5
+HK (1.0 μM)	284.6 ± 18.6	222.5 ± 16.5
+HK (10 μM)	279.4 ± 16.2	229.7 ± 24.6
+EGTA (20 mM)	186.5 ± 20.2[b]	ND
PMA-treated[c]	157.1 ± 14.7[b]	ND
cAMP (20 μM)[d]	ND	309.2 ± 21.4[e]

ND, sample not detected.

[a] Cytosolic proteins were extracted from neutrophils (2×10^7) for the determination of the protein kinase C (PKC) or adenosine-3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase (PKA) activity in the presence of honokiol (HK), using a nonradioactive protein kinase assay kit (Calbiochem®, Germany) based on an enzyme-linked immunosorbent assay that uses a synthetic PKC or PKA pseudosubtrate and a monoclonal antibody that recognizes the phosphorylated peptide. Negative control for PKC was determined in the presence of 20 mM EGTA. Data are expressed as $100 \times OD_{492}/12$ μl cell lysate from 2×10^7 cells. Values represent the means ± S.E.M. of three experiments performed on different days using cells from different rats.

[b] $P < 0.05$ as compared with control, respectively.

[c] In some experiments, PMA (100 ng/ml) was added to live neutrophils for stimulation/translocation of PKC prior to sonication, and cytosolic PKC activity from this preparation was used as another negative control as most of the cytosolic PKC had been translocated to the particulate fraction.

[d] In PKA activity assay, PKA activity was measured in the presence of cAMP (20 μM).

[e] $P < 0.05$ as compared with control, respectively.

3.6. Effect of honokiol on cyclooxygenase activity

Cyclooxygenase is an important enzyme in the generation of reactive oxygen species (Pepicelli et al., 2002; Rieger et al., 2002). Our results demonstrated that honokiol concentration (0.1–10 μM) dependently inhibited cyclooxygenase activity (Fig. 4). Ibuprofen (1 mM), a relative cyclooxygenase-1 inhibitor, did not cause a significant inhibition of cyclooxygenase activity. Resveratrol (100 μM), a cyclooxygenase-1 (Johnson and Maddipati, 1998) and cyclooxygenase-2 inhibitor (Subbaramaiah et al., 1998), and NS-398 (50 μM), a selective cyclooxygenase-2 inhibitor, both significantly inhibited cyclooxygenase activity in this study (Fig. 4).

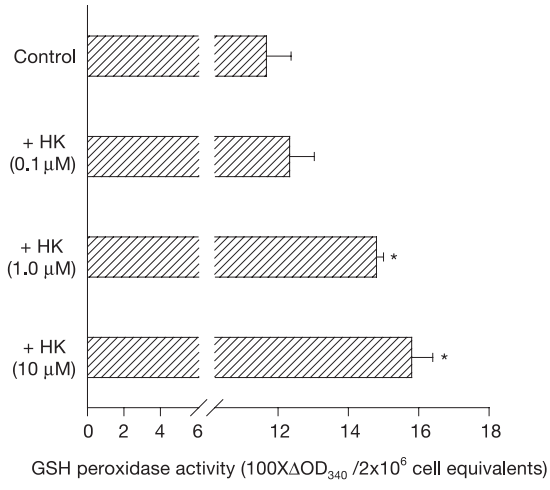

Fig. 3. Effect of honokiol on GSH peroxidase activity of peripheral neutrophils. Glutathione (GSH) peroxidase activity was determined in the supernatants by mixing 0.1 ml of cytosolic fraction (from 2×10^6 cells) with 0.9 ml of reaction mixture containing 0.1–10 μM honokiol (HK). The GSH peroxidase activity was assayed using the conversion of NADPH to NAD by recording the changes in absorbance at 340 nm over 3 min (ΔOD_{340}) using H_2O_2 (Se-dependent activity) as substrate against a reference that did not contain substrate. An additional blank containing all components except glutathione reductase was used to correct for non-enzymatic oxidation of GSH and NADPH by substrates (H_2O_2). Data are expressed as the rate of change in absorbance ($100 \times \Delta OD_{340}/2 \times 10^6$ cell equivalents). Values represent the means ± S.E.M. of three experiments performed on different days using cells from different rats. *$P < 0.05$ as compared to control (vehicle).

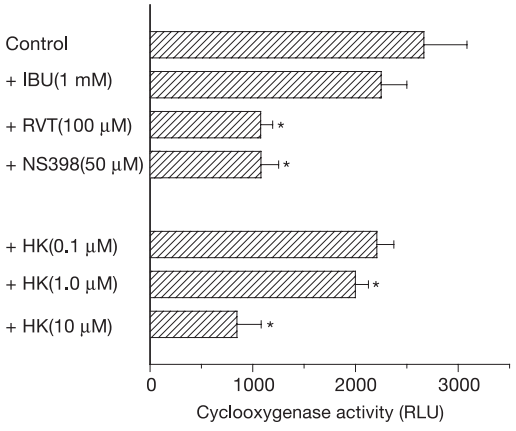

Fig. 4. Effect of honokiol on cyclooxygenase activity of peripheral neutrophils. Cyclooxygenase activity was determined with a chemiluminescent-based cyclooxygenase assay kit (Calbiochem®, Germany). Cytosolic fractions (from 2×10^7 cells/ml) were treated with vehicle (control), 0.1–10 μM honokiol (HK), 50 μM NS-398 (a cyclooxygenase-2 inhibitor), 100 μM resveratrol (RVT, a cyclooxygenase-1/cyclooxygenase-2 inhibitor) or 1 mM ibuprofen (IBU, a cyclooxygenase-1 inhibitor). The cyclooxygenase activity was then measured via addition of a proprietary luminescent substrate and arachidonic acid. The chemiluminescent signal was measured over 5 s in a microplate luminometer (Orion®) and expressed in relative light units (RLU). Values represent the means ± S.E.M. of three experiments performed on different days using cells from different rats. *$P < 0.05$ as compared to control.

Table 5
Cell viability

	Cell viability (%)[a]
Control (drug free)	98.2 ± 0.3
Vehicle control (0.05% DMSO)	97.6 ± 0.3
HK (0.1 μM)	97.8 ± 0.4
HK (1.0 μM)	97.7 ± 0.6
HK (10 μM)	96.8 ± 0.2
FMLP (1 μM)	92.1 ± 3.5
PMA (100 ng/ml)	89.6 ± 2.8

[a] Cell viability was measured by a propidium iodide exclusion assay as described in Materials and methods. After incubation of cells (2×10^6/ml) with honokiol (HK) or test drugs for 1 h, cell suspension was further incubated with propidium iodide (10 μg/ml) and fluorescein diacetate (100 ng/ml) at room temperature for 10 min. Cell suspension was analyzed immediately on a flow cytometer (FACSCalibur™; Becton Dickinson) by recording forward and light scatter, red (>630 nm) and green (520 nm) fluorescence. After gating for light scatter to include single cells and to exclude clumps and debris, cell populations (1×10^4 cells) were displayed as green (viable) versus red (dead) fluorescence. Cell viability (%) was calculated by the CellQuest® software (Becton Dickinson) on a Power Macintosh 7300/200 computer. Values represent the means ± S.E.M. of five experiments performed on different days using cells from different rats.

3.7. Effect of honokiol on the cell viability

Our results demonstrated that honokiol in concentrations ranging from 0.1 to 10 μM did not show significant cytotoxicity as compared to control (drug-free or 0.05% DMSO) after incubation with cell suspensions for 1 h (Table 5). Combination of honokiol with fMLP or PMA had a similar effect to that of fMLP or PMA alone. Detection of lactate dehydrogenase (LDH) activity, to quantify cytotoxicity, yielded comparable results (data not shown).

4. Discussion

When tissue suffers from ischemia and reperfusion injury, adhesion of circulating neutrophils to endothelial cells and the generation of reactive oxygen species by these inflammatory target cells could enhance neutrophil infiltration into the site of injury, mediating the pathogenesis of ischemia/reperfusion injury (Fraticelli et al., 1996; Williams, 1994). Evidence shows that inhibition of reactive oxygen species production could reduce the infiltration and recruitment of neutrophils in cerebral ischemia/reperfusion injury (Fabian and Kent, 1999). This can be further illustrated by the following reports: probucol, an antioxidant, improved post-myocardial infarction by reducing oxidative stress and expression of pro-inflammatory cytokines (Bonnefont-Rousselot et al., 1999; Sia et al., 2002), and attenuated reperfusion-induced cardiac injury (Rabkin et al., 1999). Similar results have been observed in our recent studies showing that honokiol, an effective antioxidant, protected animal against myocardial and cerebral ischemia/reperfusion injury (Tsai et al., 1996, 1999; Liou et al., 2003) although the mechanism(s) of action remained unclear. In

this study, honokiol significantly prevented extracellular $O_2^{\cdot -}$ generation and intracellular reactive oxygen species accumulation in activated rat neutrophils without having a significant cytotoxic effect (Table 5), suggesting that the anti-inflammatory potential of honokiol may underlie its ischemia/reperfusion injury protective effect.

The production of reactive oxygen species by neutrophils predominantly comes from the activation of NADPH oxidase to generate $O_2^{\cdot -}$. This process involves the assembly of PKC-mediated phosphorylation and translocation of cytosolic components (e.g., p47-phox, p67-phox) to membrane-associated components (gp9l-phox and p22-phox) to form an active NADPH oxidase enzyme complex (Clark et al., 1990; Kadri-Hassani et al., 1995; Segal, 1989; Heyworth et al., 1989). In this study, a particulate fraction isolated from cells pretreated with PMA completed NADPH oxidase assembly and generated $O_2^{\cdot -}$ in the presence of NADPH (400 μM) to 1.5- to 2.0-fold that of control cells (vehicle only). Honokiol treatment before PMA activation did not inhibit the NADPH oxidase activity in the particulate fraction, indicating that the NADPH oxidase assembly machinery was not interfered with by honokiol. In samples pretreated with staurosporine (a broad spectrum PKC inhibitor) or cromolyn, an inhibitor of the assembly of NADPH oxidase (Kilpatrick et al., 1995), NADPH oxidase activity was significantly suppressed (data not shown). Since honokiol also did not inhibit PKC activity, these results suggest that honokiol did not prevent the PKC-dependent phosphorylation and translocation of cytosolic component(s) that is essential for NADPH oxidase activation. In addition to PKC, activation of PKA could negatively regulate reactive oxygen species production, possible by increasing cytosolic cAMP (Orlic et al., 2002; Derian et al., 1995). Here, we found that honokiol did not directly modulate PKA activity. Whether cytosolic cAMP could be enhanced by honokiol, to down-regulate reactive oxygen species production, awaits further study.

In addition to NADPH oxidase and protein kinase(s), there are several other possible targets for the regulation of reactive oxygen species production in neutrophils that could be modulated by honokiol. These possible targets include: (1) myeloperoxidase, which generates the more potent oxidant HOCl from H_2O_2, (2) cyclooxygenase, another important enzyme in the generation of oxygen radicals (Rieger et al., 2002), and (3) catalase or GSH peroxidase, which convert $O_2^{\cdot -}$ to water and oxygen. Honokiol significantly inhibited myeloperoxidase and cyclooxygenase but enhanced GSH peroxidase activity without affecting catalase activity (data not shown). Based on these findings, we propose that multiple mechanisms for inhibiting reactive oxygen species production and enhancing reactive oxygen species metabolism might underlie the antioxidative effect of honokiol, leading to the amelioration of focal cerebral ischemia/reperfusion injury through the inhibition of neutrophil activation. Besides, based on the potency of honokiol on the different cellular enzyme systems examined in this study, we speculate that cyclooxygenase could be the preferred site targeted by honokiol. Whether the effect of honokiol on cyclooxygenase activity is due to the inhibition of cyclooxygenase-1 or cyclooxygenase-2 activity awaits further study. However, in view of the finding that honokiol had a potency similar to that of resveratrol (100 μM) and NS-398 (50 μM) at low concentrations (1–10 μM) (Fig. 4), we propose that the inhibition of cyclooxygenase activity by honokiol might involve cyclooxygenase-2 rather than cyclooxygenase-1 in our assay system.

In conclusion, our data suggest that honokiol could impair reactive oxygen species production through, at least in part, three distinct mechanisms: (1) inhibition of NADPH oxidase activity without interfering with the PKC- or PKA-dependent signaling pathway, (2) inhibition of myeloperoxidase and cyclooxygenase activities, and (3) enhancement of GSH peroxidase activity. As a powerful antioxidant acting by multiple mechanisms, honokiol could protect animals against focal cerebral ischemia/reperfusion injury by inhibiting reactive oxygen species-related inflammatory responses that predominantly feature neutrophil activation in injured tissue.

Acknowledgements

The authors thank Mr. C.H. Hsu for his help in the preparation of the animal study and the assay of neutrophil functions. This study was supported by grant NSC 90-2314-B-002-102 from the National Science Council, and Academic Sinica, Taipei, Taiwan, to S.K. Tsai.

References

Arun, S., Krishnamoorthy, P., Subramanian, P., 1999. Properties of glutathione peroxidase from the hepatopancreas of freshwater prawn *Macrobrachium malcolmsonii*. Int. J. Biochem. Cell Biol. 3, 1725–1732.

Bani, D., Masini, E., Bello, M.G., Bigazzi, M., Sacchi, T.B., 1998. Relaxin protects against myocardial injury caused by ischaemia and reperfusion in rat heart. Am. J. Pathol. 152, 1367–1376.

Bonnefont-Rousselot, D., Segaud, C., Jore, D., Delattre, J., Gardes-Albert, M., 1999. Antioxidant effect of probucol on $RO_2^{\cdot}/O_2^{(\cdot -)}$-induced peroxidation of human low-density lipoproteins. Radiat. Res. 151, 343–353.

Casimir, C.M., Teahan, C.G., 1994. The respiratory burst of neutrophils and its deficiency. In: Hellewell, P.G., Williams, T.J. (Eds.), Immunopharmacology of Neutrophils. Academic Press, London, pp. 27–54.

Chan, P.H., 2001. Reactive oxygen radicals in signaling and damage in the ischemic brain. J. Cereb. Blood Flow Metab. 21, 2–14.

Chiu, J.H., Ho, C.T., Wei, Y.H., Liu, W.Y., Hong, C.Y., 1997. In vitro and in vivo protective effect of honokiol or rat liver from lipid peroxidative injury. Life Sci. 61, 1967–1971.

Clark, R.A., Volpp, B.D., Leidal, K.G., Nauseef, W.M., 1990. Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. J. Clin. Invest. 85, 714–721.

Derian, C.K., Santulli, R.J., Rao, P.E., Solomon, H.F., Barrett, J.A., 1995. Inhibition of chemotactic peptide-induced neutrophil adhesion to vascular endothelium by cAMP modulators. J. Immunol. 154, 308–317.

Dirnagl, U., Iadecola, C., Moskowitz, M.A., 1999. Pathology of ischemic stroke: an integrated view. Trends Neurosci. 22, 391–397.

Fabian, R.H., Kent, T.A., 1999. Superoxide anion production during reperfusion is reduced by an anti-neutrophil antibody after prolonged cerebral ischaemia. Free Radic. Biol. Med. 26, 355–361.

Finkel, T., 1998. Oxygen radicals and signaling. Curr. Opin. Cell Biol. 10, 248–253.

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.

Heyworth, P.G., Shrimpton, C.F., Segal, A.W., 1989. Localization of the 47 kDa phosphoprotein involved in the respiratory-burst NADPH oxidase of phagocytic cells. Biochem. J. 260, 243–248.

Johnson, J.L., Maddipati, K.R., 1998. Paradoxical effects of resveratrol on the two prostaglandin H synthases. Prostaglandins Other Lipid Mediat. 56, 131–143.

Kadri-Hassani, N., Leger, C.L., Descomps, B., 1995. The fatty acid bimodal action on superoxide anion production by human adherent monocytes under phorbol 12-myristate 13-acetate or diacylglycerol activation can be explained by the modulation of protein kinase C and p47-phox translocation. J. Biol. Chem. 270, 5111–5118.

Kilpatrick, L.E., Jakabovics, E., McCawley, L.J., Kane, L.H., Korchak, M., 1995. Cromolyn inhibits assembly of the NADPH oxidase and superoxide anion generation by human neutrophils. J. Immunol. 154, 3429–3436.

Korchak, H., Vienne, M.K., Rutherford, L.E., Wilkenfeld, C., Finkelstein, M.C., Weissmann, G., 1984. Stimulus–response coupling in the human neutrophil: II. Differential requirements for receptor occupancy in neutrophil responses to a chemoattractant. J. Biol. Chem. 259, 4076–4082.

Lew, P.D., Wolheim, C.B., Waldvogel, F.A., Pozzan, T., 1984. Modulation of cytosolic free calcium transients by changes in intra-cellular calcium buffering capacity; correlation with exocytosis and $O_2^{\cdot -}$ production in human neutrophils. J. Cell Biol. 99, 1212–1220.

Liou, K.T., Lin, S.M., Huang, S.S., Chih, C.L., Tsai, S.K., 2003. Honokiol ameliorates cerebral infarction on rats subjected to ischaemia–reperfusion injury. Planta Med. 69, 130–134.

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.

Matsuda, H., Kageura, T., Oda, M., Morikawa, T., Sakamoto, Y., Yoshikawa, M., 2001. Effects of constituents from the bark of *Magnolia obovata* on nitric oxide production in lipopolysaccharide-activated macrophages. Chem. Pharm. Bull. 49, 716–720.

Orlic, T., Loomis, W.H., Shreve, A., Namiki, S., Junger, W.G., 2002. Hypertonicity increases cAMP in PMN and blocks oxidative burst by PKA-dependent and -independent mechanisms. Am. J. Physiol., Cell Physiol. 282, C1261–C1269.

Ormerod, M.G., 2000. Further applications to cell biology. In: Ormerod, M.G. (Ed.), Flow Cytometry, 3rd ed. Oxford Univ. Press, UK, pp. 249–250.

Pepicelli, O., Fedele, E., Bonanno, G., Raiteri, M., Ajmone-Cat, M.A., Greco, A., Levi, G., Minghetti, L., 2002. In vivo activation of *N*-methyl-D-aspartate receptors in the rat hippocampus increases prostaglandin E(2) extracellular levels and triggers lipid peroxidation through cyclooxygenase-mediated mechanisms. J. Neurochem. 81, 1029–1034.

Rabkin, D.G., Jia, C.X., Spotnitz, H.M., 1999. Attenuation of reperfusion injury with probucol in the heterotopic rat cardiac isograft. J. Heart Lung Transplant. 18, 775–780.

Rieger, J.M., Shah, A.R., Gidday, J.M., 2002. Ischaemia–reperfussion injury of retinal endothelium by cyclooxygenase- and xanthine oxidase-derived superoxide. Exp. Eye Res. 74, 493–501.

Segal, A.W., 1989. The electron transport chain of the microcidal oxidase of phagocytic cells and its involvement in the molecular pathology of chronic granulomatous disease. J. Clin. Invest. 83, 1785–1793.

Shen, Y.C., Sung, Y.J., Chen, C.F., 1998. Magnolol inhibits Mac-1 (CD11b/CD18)-dependent neutrophil adhesion: relationship with its antioxidant effect. Eur. J. Pharmacol. 343, 79–86.

Shen, Y.C., Chou, C.J., Chiou, W.F., Chen, C.F., 2001. Anti-inflammatory effects of the partially purified extract of *Radix stephaniae tetrandrae*: comparative studies with its active principles tetrandrine and fangchinoline on human polymorphonuclear leukocyte functions. Mol. Pharmacol. 60, 1083–1090.

Sia, Y.T., Parker, T.G., Liu, P., Tsoporis, J.N., Adam, A., Rouleau, J.L., 2002. Improved post-myocardial infarction survival with probucol in rats: effects on left ventricular function, morphology, cardiac oxidative stress and cytokine expression. J. Am. Coll. Cardiol. 39, 148–156.

Son, H.J., Lee, H.J., Yun-Choi, H.S., Ryu, J.H., 2000. Inhibitors of nitric oxide synthesis and TNF-alpha expression from *Magnolia obovata* in activated macrophages. Planta Med. 66, 469–471.

Subbaramaiah, K., Chung, W.J., Michaluart, P., Telang, N., Tanabe, T., Inoue, H., Jang, M., Pezzuto, J.M., Dannenberg, A.J., 1998. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J. Biol. Chem. 273, 21875–21882.

Sugimoto, K., Iadecola, C., 2003. Delayed effect of administration of COX-2 inhibitor in mice with acute cerebral ischemia. Brain Res. 960, 273–276.

Suzuki, H., Pabst, M.J., Johnston, R.B.JR., 1985. Enhancement by Ca^{2+} or Mg^{2+} of catalytic activity of the superoxide-producing NADPH oxidase in membrane fractions of human neutrophils and monocytes. J. Biol. Chem. 260, 3635–3639.

Taira, J., Ikemoto, T., Miruma, K., Hagi, A., Murakami, A., Makino, K., 1993. Effective inhibition of hydroxyl radicals by hydroxylated biphenyl compounds. Free Radic. Res. 19, S71–S77.

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

Tsai, S.K., Huang, S.S., Hong, C.Y., 1996. Myocardial protective effect of honokiol: an active component in *Magnolia officinalis*. Planta Med. 62, 503–506.

Tsai, S.K., Hong, C.Y., Huang, S.S., Hung, L.M., Hong, C.Y., 1999. Anti-arrhythmic effect of magnolol and honokiol during acute phase of coronary occlusion in anesthetized rats: influence of L-NAME and aspirin. Pharmacology 59, 227–233.

Weiss, S.J., 1989. Tissue destruction by neutrophils. N. Engl. J. Med. 320, 365–376.

Williams, F.M., 1994. Role of neutrophils in reperfusion injury. In: Hellewell, P.G., Williams, T.J. (Eds.), Immunopharmacology of Neutrophils. Academic Press, London, pp. 245–257.