Effect of Hwao-tang on Superoxide Generation and Neutrophil Functions

Soo Young Park, Han Geu Kim, Soo Kyung Lee, Jong Chan Ahan, Tae Wook Chung, Jin-Young Moon[1], Sun Dong Park[2], June Ki Kim[3], Dall Yeong Choi[3], Cherl Ho Kim[4], Won Hwan Park*

Department of Diagnostics, 1:Department of AM-Pointology, 2:Department of Prescription
3:Department of Pathology, 4:Department of Biochemistry, College of Oriental Medicne, Dongguk University

We investigated that Hwao-tang had various effects on stimulus-induced superoxide generation in human neutrophils. Hwao-tang significantly inhibited N-formyl-methionyl-leucyl-phenylalanine-induced superoxide generation in a concentration-dependent manner, but not that induced by arachidonic acid. Also, Hwao-tang significantly reduced mouse paw oedema induced by carrageenan. The results suggest that protein tyrosine kinase participates in fMLP-mediated superoxide generation by Hwao-tang-treated human neutrophils. Also, the results indicate that Hwao-tang exerts anti-inflammatory effects related to the inhibition of neutrophil functions and of NO and prostaglandin E2 production, which could be due to a decreased expression of iNOS and COX-2.

Key words : Hwao-tang(huàyūtāng, 化瘀湯), neutrophils, superoxide generation, iNOS, COX-2

Introduction

The traditional korean therapeutic system has been used for the treatment of various disease for hundreds of years, including the clinical treatment of hypercholesterolimia, diabetes and obesity[1]. Hwao-tang has been reported to have a hypolipidemic effect in patients with hypercholesterolemia[2], and in cholesterol-induced experimental models. Hwao-tang is consisted of *Angelica gigantis Radix, Rehmanniae Radix, Paeoniae Radix, Ciniamomi Cortex, Cnidii Rhizoma, Persicae Semen* and *Carthami Flos* (Scheme 1)[3]. According to the ancient chinese medicinal literature 《NaShiYakEuiKyng(羅氏會約醫鏡)》, Hwao-tang has been known to exhibit activating blood circulation, removing blood stasis and abdominal pain, and has been widely used in Chinese and Korean traditional medicine[1)3)]. Hwao-tang is also activate vital energy and regulate menstruation, and sudden loss of vision caused by retinal hemorrhage[2]. In the experimental study, effects of Hwao-tang in experimentally induced blood stasis and hyperlipidemia in rats have been reported(Park et al., not shown)[4]. And inhibitory effects of Hwao-tang on the atherosclerosis and the venous thrombosis have also been reported(Hong et al., not shown)[5].

However, the pharmaceutical effect of these Hwao-tang on platelet aggregation was not investigated. Superoxide generation in human neutrophils is stimulated during phagocytosis and by treatment of cells with various stimuli, such as certain chemoattractants and activators of protein kinase[6)7)8)]. However, resting neutrophils in the blood circulation are poorly responsive to agonists. Neutrophil function can be primed by a variety of pro-inflammatory stimuli[9-14)]. When the cells are exposed to priming agents, such functions as respiratory burst, phagocytosis and degranulation are greatly enhanced. However, the mechanism of such priming has not been clearly defined.

Prostaglandins and nitric oxide (NO) exert numerous vascular and inflammatory effects. Production of prostaglandins or NO by the constitutive isoenzymes, cyclooxygenase-1 (COX-1), or endothelial NO synthase (eNOS), is implicated in regulation of vascular tone and homeostatic functions. In contrast, COX-2 and inducible NO synthase (iNOS) are not generally expressed in resting cells, but are induced following appropriate stimulation with pro-inflammatory agents such as cytokines and lipopolysaccharide[15]. The activity of these inducible enzymes results in overproduction of prostaglandins and NO, which play key roles in the pathophysiology of arthritis and other inflammatory conditions[16]. NO is also able to enhance the production of tumour necrosis factor-α and interleukin-1β, which participate in the macrophage-dependent inflammation[17].

Neutrophils are essential for host defense and their contribution to the propagation and maintenance of acute and chronic inflammation includes several mechanisms. Activated neutrophils release granule constituents[18] and produce

* To whom correspondence should be addressed at : Won Hwan Park, Department of Diagnostics, College of Oriental Medicne, Dongguk University, Sukjang-Dong 707, Kyung-Ju 780-714, Korea.
E-mail: diapwh@mail.dongguk.ac.kr, Tel: 054-770-2373
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leukotrienes, which participate in the inflammation through stimulation of leukocyte functions and vascular permeability[19]. Thus, the suppression of neutrophil functions could control the inflammation. Little is known of the biological activity of Hwao-tang and previous studies have focused mainly on their anti-thrombosis[5]. There is a growing interest in the pharmacological potential of the Hwao-tang due to the recent finding by our group that a series of Hwao-tang were able to inhibit NO and prostaglandin E2 synthesis in murine peritoneal macrophages stimulated with bacterial endotoxin.

In the present study, we investigated the effects of two novel Hwao-tang, and found that they had an inhibitory effect on platelet aggregation, as well as various effects on stimulus-induced superoxide generation in human neutrophils. The present study was also undertaken to examine the effects of a new Hwao-tang on murine macrophage and human neutrophil functions as well as on several enzymes relevant to the inflammatory process. The results demonstrated the *in vitro* inhibitory effects on cell functions exerted by this Hwao-tang, which also exhibited anti-inflammatory activity *in vivo*.

Materials and Methods

1. Hwao-tang Extracts

Hwao-tang is a dried decoctum of a mixture of 7 herbal drugs. Hwao-tang prescription was consisted of *Angelica gigantis Radix* 16.0 g, *Rehmanniae Radix* 10.0 g, *Paeoniae Radix* 8.0 g, *Ciniamomi Cortex* 8.0 g, *Cnidii Rhizoma* 4.0 g, *Persicae Semen* 4.0 g and *Carthami Flos* 3.2 g.

A total of 53.2 g of Hwao-tang was added to 500 ml of water and boiled for 2 hrs, filtered and then concentrated to 200 ml. This decoction was spray-dried to give a powdered extract. The yield was 5.2 g, which represents one human dose/day.

2. Chemicals

NADPH, ferricytochrome c (cyt. c), superoxide dismutase, N-formyl-methionyl-leucyl-phenylalanine (fMLP), arachidonic acid (AA) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO, USA). Genistein was from Wako Pure Chemicals (Osaka, Japan). The APTT assay kit was purchased from Dade International (Aguade, PR, USA). All other reagents used were of analytical grade and were purchased from Nacalai Tesque (Osaka, Japan) unless otherwise stated. Hwao-tang were extracted according to the methods of W.H., Park et al., as reported[1)4)].

3. Isolation of neutrophils

Human peripheral blood polymorphonuclear leukocytes (HPPMNs) were isolated from the peripheral blood of healthy humans by Ficoll-Hypaque (Flow Laboratories) density gradient centrifugation[21] and were washed twice with Krebs-Ringer-phosphate solution, pH 7.4 (KRP)[19]. The cells were counted and resuspended in KRP at a concentration of 1 $\times 10^8$ cells/ml.

4. Assay of superoxide generation

Superoxide generation was assayed by measuring the reduction of cyt. c at 37℃ using a dual-beam spectrophotometer (Shimadzu UV-3000; Shimadzu, Kyoto, Japan) under constant stirring conditions[22]. The standard assay mixture consisted of 1×10^6 cells/ml, 1 mmol/l $CaCl_2$, 20 mmol/l cyt. c, 10 mmol/l glucose, 0-50 mmol/l steroidal saponin and a stimulus (12.5 nmol/l fMLP, 1 nmol/l PMA or 10 mmol/l AA) in a final volume of 2 ml of KRP. After preincubation for 3 min with a steroidal saponin, the reaction was started by adding a stimulus and the absorbance change at 550-540 nm (A550-540) was monitored for 5 min.

5. Detection of tyrosyl phosphorylation of neutrophil proteins

Neutrophils (1×10^6 cells/ml) were incubated in 1 ml of KRP containing 1 mmol/l $CaCl_2$, 10 mmol/l glucose and 0-100 mmol/l of timosaponins E1 and E2 for 3 min at 37℃, and then 0.5 ml of ice-cold 45% trichloroacetic acid (final concentration, 15%) containing 1 mmol/l sodium vanadate and 2 mmol/l phenylmethylsulfonylfluoride was added to stop the reaction. After incubation for 30 min at 4℃, the mixture was centrifuged at 10,000 g for 15 min at 4℃. The precipitate was washed twice with ice-cold diethyl ether:ethanol (1:1, v/v), dissolved in 50 ml of 62.5 mmol/l Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 0.7 mol/l -mercaptoethanol and 10% glycerol and was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 7.5% gel. The electrophoresed proteins were transferred onto Immobilon-P membranes (Nippon Millipore) using a semidry blotting apparatus (Sartorius) for 60 min at 2 mA/cm2, and the tyrosyl phosphorylated proteins were detected using phosphotyrosine-specific monoclonal antibody (PY-20; ICN Biochemicals), peroxidase-conjugated rabbit anti-mouse immunoglobulin G antibody (E.Y. Laboratories) and the ECL Western Blotting Detection System (Amersham, Japan)[21]. The apparent molecular masses of the proteins were determined using prestained molecular weight standards (14,300-200,000 molecular weight range; Gibco-BRL). To estimate the phosphorylation level, the lanes were scanned using an Epson GT 8000 (Seiko Epson Co., Japan) and the intensity of the 58

kDa band was analysed using NIH Image software (Nayne Rasband, National Institute of Health, USA).

6. Preparation of human neutrophils

Venous blood was obtained, with informed consent, from healthy volunteers. Leukocytes were obtained and purified as previously described[23]. Viability was more than 95% according to the trypan blue exclusion test. The mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan24) was used to assess the possible cytotoxic effect of Hwao-tang on human neutrophils.

7. Isolation and culture of mouse peritoneal macrophages

Female Swiss mice weighing 25-30 g were used to obtain highly purified peritoneal macrophages. Cells were harvested by peritoneal lavage 4 days after i.p. injection of 1 ml of 10% thioglycolate broth. Cells were resuspended in culture medium (120 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2 \times 7H2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, 1 mM arginine, and 10 mM glucose) supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 g/ml streptomycin, and incubated at 37℃ for 2 hrs. The adherent cells were used to perform the experiments described below. Cytotoxicity was assessed by the reduction of MTT [24].

8. Elastase release by human neutrophils

Neutrophils (2.5×10^6 cells/ml) were preincubated with test Hwao-tang or vehicle for 5 min and then stimulated with cytochalasin B (10 μM) and N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 10 nM) or platelet-activating factor (PAF) (0.5 μM) for 10 min. Elastase activity was estimated in supernatants, using N-tert-butoxy-carbonyl-alanine p-nitrophenyl ester (200 μM) as substrate and p-nitrophenol release was measured. Possible direct inhibitory effects on elastase activity were also assessed[25].

9. Synthesis and release of leukotriene B4 by human neutrophils

A suspension of human neutrophils (5×10^6 cells/ml) was preincubated with test compound or vehicle for 5 min and then stimulated with calcium ionophore A23187 (1 μM) for 10 min at 37℃. Leukotriene B4 levels in supernatants were measured by radioimmunoassay[26]. High-speed (100,000×g) supernatants from sonicated human neutrophils were obtained and incubated under appropriate conditions with 10 μM arachidonic acid to assess 5-lipoxygenase activity[27].

10. Nitrite and prostaglandin E2 production in mouse peritoneal macrophages

Peritoneal macrophages(4×10^5/well) were incubated with Escherichia coli [serotype 0111:B4] lipopolysaccharide (10 μg/ml) at 37℃ for 24 hrs in the presence of test compounds or vehicle. Nitrite and prostaglandin E2 levels were determined in culture supernatants by a fluorimetric method[28] and by radioimmunoassay[27], respectively. In another set of experiments, lipopolysaccharide-stimulated cells were collected to determine iNOS and COX-2 expression by Western blot analysis as described below.

11. iNOS and COX-2 activity in intact cell

Twenty-four-hours lipopolysaccharide-stimulated macrophages (4×10^5/well) were washed and fresh medium supplemented with L-arginine (0.5 mM) and arachidonic acid (10 μM) was added for a further 2 hrs incubation with test compounds to assess the effects of compounds on induced enzyme activity. Supernatants were collected for the measurement of nitrite and prostaglandin E2 accumulation for the last 2 hrs. Nitrite concentration, as reflection of NO released, was assayed fluorometrically and prostaglandin E2 levels were assayed by radioimmunoassay.

12. COX-2 activity in broken cell preparations

Murine peritoneal macrophages stimulated with E. coli lipopolysaccharide (10 μg/ml) at 37℃ for 24 h were collected and sonicated at 4℃ in an ultrasonicator at maximum potency, microsomes were prepared by centrifugation at 2000×g for 5 min at 4℃ followed by centrifugation of the supernatant at 100,000×g for 100 min at 4℃. Microsomes (40 μg protein/tube) were incubated for 30 min at 37℃ in 50 mM Tris HCl, pH 7.4, with arachidonic acid (5 M) and test compound or vehicle in the presence of 2 μM hematin and 1 mM L-tryptophan[29]. The reaction was stopped by boiling the samples for 5 min and prostaglandin E2 synthesis was determined by radioimmunoassay[26].

13. iNOS activity in broken cell preparations

High-speed (100,000×g) supernatants from peritoneal macrophages stimulated with E. coli lipopolysaccharide were obtained as described above. Aliquots of supernatants were used to determine NO synthase activity by monitoring the conversion of L-[3H]arginine to L-[3H]citrulline[30]. Briefly, supernatants (100 g protein/tube) were incubated at room temperature for 60 min with NADPH (1 mM) and a mixture of unlabeled and L-[3H]arginine (10 M, 1 μCi/ml). Incubations were terminated by adding of 20 mM HEPES (1 ml, pH 5.5) containing 1 mM EGTA and 1 mM EDTA. L-[3H]citrulline was separated from arginine by adding 1.5 ml of a 1:1 suspension

of DOWEX (50 W) in water. Radioactivity was measured in supernatants by liquid scintillation counting.

14. COX-1 activity in human platelets microsomes

Human platelets were sonicated at 4℃ in an ultrasonicator at maximum potency. Microsomes were prepared by centrifugation at 2000 ×g for 5 min at 4℃ follwoed by centrifugation of the supernatant at 100,000×g for 100 min at 4℃. Microsomes (20 μg protein/tube) were incubated for 30 min at 37℃ in 50 mM Tris-HCl (pH7.4) with arachidonic acid and 1 mM L-tryptopha[18]. The reaction was stopped by boiling the samples for 5 min, and thromboxane B2 levels were determined by radioimmunoassay.

15. Wetern blot analysis

iNOS or COX-2 protein expression was studied in the cytosolic or microsomal fractions, respectively, from lipopolysaccharide-stimulated peritoneal macrophages and cell pellets obtained by centrifugation of air pouch exudates. Equal amounts of protein were loaded on 12.5% polyacrylamide gel electrophoresis-sodium dodecyl sulphate (PAGE-SDS) and transferred onto polyvinylidene difluoride membranes for 90 min at 125 mA. Membranes were blocked in phosphate buffer saline (0.02 M, pH7.0)-Tween-20 (0.1%) containing 3% w/v unfatted milk. For iNOS, membranes were incubated with specific anti-iNOS polyclonal antiserum (1/1000); for COX-2, membranes were incubated with specific anti-COX-2 polyclonal antiserum (1/1000). Both membranes were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (1/20,000) and peroxidase-conjugated rabbit anti-goat /sheep IgG (1/20,000), respectively. The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL, Amersham Korea, Korea).

16. Carrageenan paw oedema

The anti-inflammatory activity of Hwao-tang was assessed by the carrageenan paw oedema test in mice according to the method of Sugishita et al. (1981)[31]. Hwao-tang (10, 50, 100, 300 mg/100g by oral administration), indomethacin (5mg/kg), or vehicle (tween 80/saline 1:99, v/v) was administered intraperitoneally 1 hr before injection of carrageenan (0.05 ml; 3% w/v in saline) into the subplantar area of the right hind paw. The volumes of injected and contralateral paws were measured 1, 3 and 5 hrs after induction of oedema by using a plethysmometer. The volume of oedema was expressed in each animal as the difference between the carrageenan-injected and contralateral paws. After the last determination of paw oedema (5 hrs), the animals were killed by cervical dislocation and the right hind paws were homogenized in 2 ml of saline. Aliquots of supernatants were used to determine prostaglandin E2 levels and elastase activity as above. Stomachs were homogenized in 2 ml of methanol and the content of prostaglandin E2 was measured in supernatants after centrifugation.

17. Materials

L[9,10-3H]oleic acid and L-3-phosphatidylcholine 1-palmitoyl-2-arachidonyl [arachidonyl-1-14C] were purchased from Du Pont (Itisa, Madrid, Spain). iNOS and COX-2 specific polyclonal antisera, N-(2-cyclohexyloxy-4-nitrophenyl) methane sulfonamide (NS398) and N-(3-(aminomethyl)benzyl) acetamidine dihydrochloride (1400W) were purchased from Cayman Chem. (MI, USA). The rest of reagents were from Sigma Chem. (MO, USA).

18. Statistical analysis

The results are presented as means±standard eroor; Inhibitory concentration 50% (IC_{50}) or inhibitory dose 50% (ID_{50}) values were calculated from at least four concentrations (n=6, n represents the number of experiments). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's t-test for multiple comparisons.

Results

1. Effect of Hwao-tang on fMLP- (A) and PMA- (B) induced superoxide generation in human neutrophils

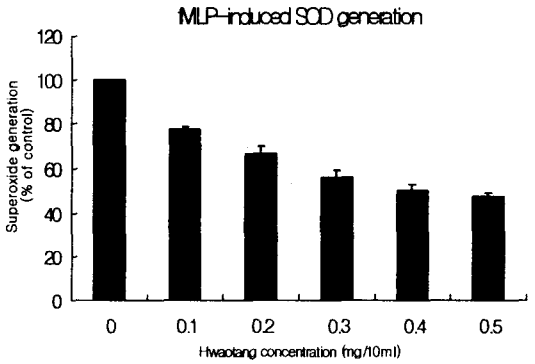

Fig. 1. Effect of Hwao-tang on fMLP-induced superoxide generation in human neutrophils. The cells were preincubated with Hwao-tang (0-0.5 mg/10 ml) for 3 min prior to the addition of 12.5 nmol/l fMLP. The experimental conditions are as described in Material and Methods. Results are expressed as the mean±S.E. from three independent experiments.

The effect of Hwao-tang on fMLP- and PMA- induced superoxide generation in human neutrophils was investigated by measuring superoxide generation by HPPMNs in order to

examine the pharmacological action of Hwao-tang. fMLP-induced superoxide generation was inhibited by Hwao-tang in a concentration-dependent manner (Fig. 1). On the other hand, PMA-induced superoxide generation was enhanced by Hwao-tang in a concentration-dependent manner (Fig. 2). The effect of Hwao-tang on AA-induced superoxide generation could not be observed (data not shown).

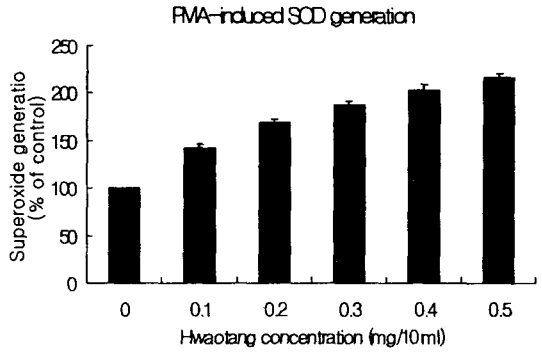

Fig. 2. Effect of Hwao-tang on PMA-induced superoxide generation in human neutrophils. The cells were preincubated with Hwao-tang (0-0.5 mg/10 ml) for 3 min prior to the addition of 1 nmol/l PMA. The experimental conditions are as described in Material and Methods. Results are expressed as the mean±S.E. from three independent experiments.

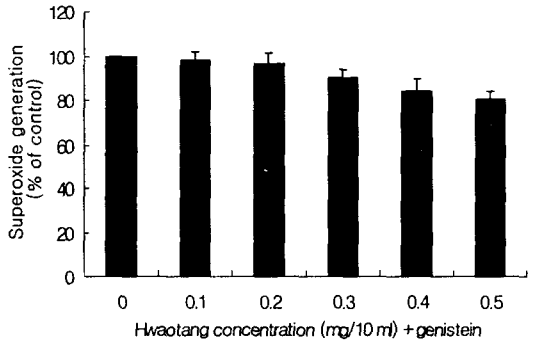

Fig. 3. Effect of protein kinase inhibitor, genistein, on PMA-induced superoxide generation by Hwao-tang-treated HPPMNs. The cells were preincubated with 0.4 mg/10 ml Hwao-tang in the presence or absence of a protein kinase inhibitor for 3 min at 37°C prior to the addition of PMA. The data were obtained at 4 min after the addition of PMA. Other conditions were the same as in Fig. 1. Results are expressed as the mean±S.E. from three independent experiments. Effect of genistein on Hwao-tang-induced superoxide generation

2. Effects of protein kinase inhibitors on PMA-induced superoxide generation by Hwao-tang-treated HPPMNs

To determine if protein kinase C and protein tyrosine kinase participate in the mechanism for priming of HPPMNs by Hwao-tang, the effects of protein kinase inhibitors on PMA-induced superoxide generation were examined. PMA-induced superoxide generation by Hwao-tang was inhibited by staurosporine, an inhibitor of protein kinase C, in a concentration-dependent manner. On the other hand, the effect of genistein, an inhibitor of protein tyrosine kinase, on superoxide generation was negligible (Fig. 3, Fig. 4).

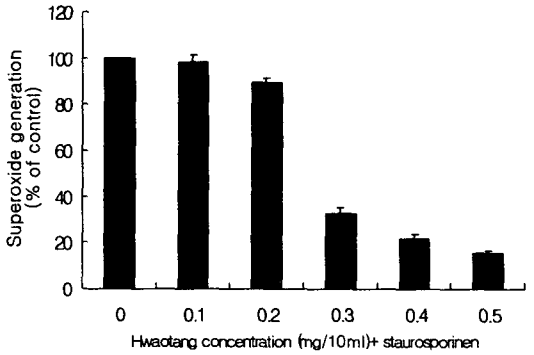

Fig. 4. Effect of protein kinase inhibitor, staurosporine on PMA-induced superoxide generation by Hwao-tang-treated HPPMNs. The cells were preincubated with 0.4 mg/10 ml Hwao-tang in the presence or absence of a protein kinase inhibitor for 3 min at 37°C prior to the addition of PMA. The data were obtained at 4 min after the addition of PMA. Other conditions were the same as in Fig. 1. Results are expressed as the mean±S.E. from three independent experiments. Effect of staurosporinen on Hwao-tang -induced superoxide generation.

3. Effect of Hwao-tang on fMLP-induced tyrosyl phosphorylation of HPPMN proteins

The effect of Hwao-tang on the phosphorylation of tyrosine residues of HPPMN protein in fMLP-induced neutrophils was examined. When the fMLP-induced neutrophils were incubated with Hwao-tang, Hwao-tang inhibited the phosphorylation of tyrosine residues of the 58 kDa protein in fMLP-induced neutrophils, in parallel with a decrease in superoxide generation (Fig. 5, Table 1).

Table 1. Effect of Hwao-tang on fMLP-induced tyrosyl phosphorylation of HPPMN proteins

Treatment	Intensity	% of control
Control (without Hwao-tang)	104.0	100
25 nmol/l fMLP	232.0	224.6
25 nmol/l fMLP + 0.2 mg Hwao-tang/10 ml	213.0	212.6
25 nmol/l fMLP + 0.5 mg Hwao-tang/10 ml	186.0	174.7
25 nmol/l fMLP + 1.0 mg Hwao-tang/10 ml	173.0	167.5

The extent of tyrosyl phosphorylation was estimated by densitometry. The results are expressed as the mean from three independent experiments.

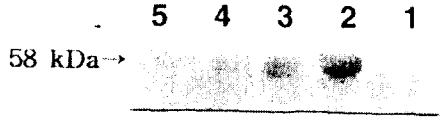

Fig. 5. Western analysis of Hwao-tang on fMLP-induced tyrosyl phosphorylation of HPPMN proteins. Proteins with phosphorylated tyrosine residues were detected by immunoblotting with anti-phosphotyrosine antibody. The results are expressed as the mean from three independent experiments.

4. Effect of Hwao-tang on fMLP-induced p47 phox synthesis in human neutrophils

The effect of Hwao-tang on 47 kDa protein in neutrophils by using human monoclonal antibody to human p47 phox was examined. When the fMLP-induced neutrophils were incubated with Hwao-tang, the amount of p47 phox protein decreased in a concentration-dependent manner (Fig. 6, Table 2). Hwao-tang inhibited the synthesis of p47 phox protein in fMLP-induced neutrophils.

Table 2. Effect of Hwao-tang on fMLP-induced p47 phox synthesis in human neutrophils

Treatment	Intensity	% of control
Control (without Hwao-tang)	385.0	100
25 nmol/l fMLP	396.0	105.6
25 nmol/l fMLP + 0.2 mg Hwao-tang/10 ml	387.0	211.5
25 nmol/l fMLP + 0.5 mg Hwao-tang/10 ml	246.0	68.4
25 nmol/l fMLP + 1.0 mg Hwao-tang/10 ml	156.0	44.2

The extent of p47 phox proteins was estimated by densitometry. The results are expressed as the mean from three independent experiments.

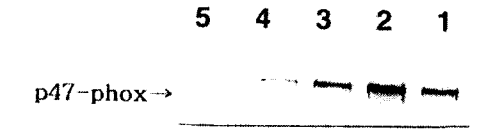

Fig. 6. Western blot of Hwao-tang on fMLP-induced p47 phox synthesis in human neutrophils. p47 phox proteins were detected by immunoblotting with human monoclonal antibody. The results are expressed as the mean from three independent experiments.

5. Elastase release by human neutrophils

We assayed Hwao-tang in the degranulation process of human neutrophils activated by two different stimuli. Preincubation of isolated human neutrophils with the test compound elicited a concentration-dependent inhibition of cytochalasin B+fMLP and cytochalasin B+PAF-induced degranulation measured as elastase release. The IC_{50} was 4.6 ug/ml (Fig. 7). Direct inhibitory effects on elastase activity were not observed (data not shown).

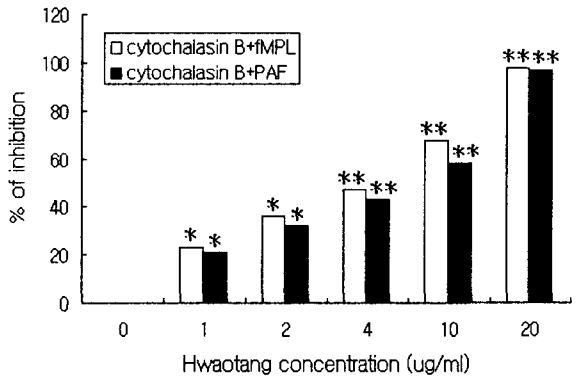

Fig. 7. Inhibition by Hwao-tang of neutrophil activation. Elastase release induced by cytochalasin B+fMPL or cytochalasin B+PAF. Data represent means ±S.E., n=4-5. * : P<0.05, ** : P<0.01.

6. Synthesis and release of leukotriene B4 by human neutrophils

Hwao-tang at 10 ug/ml completely abolished leukotriene B4 release by human neutrophils stimulated with ionophore A23187. The concentration-dependent study showed an IC50 value of 5.6 ug/ml. Nevertheless Hwao-tang failed to modify leukotriene B4 synthesis by high-speed supernatants from human neutrophils at concentrations up to 1 ug/ml (Fig. 8). Thus, it appears that the reduction of leukotriene B4 release by Hwao-tang in intact neutrophils is not due to direct inhibition of 5-lipoxygenase activity.

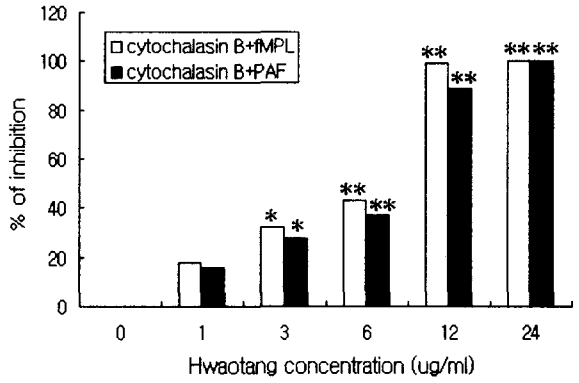

Fig. 8. Inhibition by Hwao-tang of release of leukotriene B4 by human neutrophils. Leukotriene B4 release induced by cytochalasin B+fMPL or cytochalasin B+PAF. Data represent means±S.E., n=3-5. * : P<0.05, ** : P<0.01.

7. Production of nitrite and prostaglandin E2 in stimulated mouse peritoneal macrophages

Incubation of 24 hrs lipopolysaccharide-stimulated mouse peritoneal macrophages with DTD caused a concentration -dependent inhibition of nitrite (as index of NO generation) and prostaglandin E2 production. Table 3 shows the IC_{50} values of test Hwao-tang for nitrite and prostaglandin E2, respectively. As expected, 1400W (selective inhibitor of iNOS activity) reduced nitrite levels and NS398 (COX-2 inhibitor) showed a high inhibitory potency on prostaglandin E2 production, whereas dexamethasone inhibited both metabolites at nM concentrations. None of these compounds affected cellular viability, as assessed by mitochondrial reduction of MTT after 24 h (data not shown) indicating that they were not cytotoxic.

Table 3. IC_{50} values for inhibition of nitrite and prostaglandin E2 accumulation in stimulated macrophages

Treatment	IC_{50}[a] nitrite	IC_{50}[a] prostaglandin E2
Hwao-tang	6.4 ug/ml	1.2ug/ml
1400W	2.1 uM	N.D.
NS398	N.D.	2.6 nM
Dexamethasone	32.5 nM	0.9 nM

Peritoneal macrophages stimulated with lipopolysaccharide for 24 hrs produced 978.3 ng nitrite/ml and 2.7 ng PGE2/ml, compared with 83.6 ng nitrite/ml and 0.4 ng PGE2/ml in untreated cells. a : Fifty % Inhibitory concentration of each drug in producing nitrite and PGE2.

8. Effect of Hwao-tang on iNOS and COX-2 activity in mouse peritoneal macrophages

The following experiments were designed to determine if the inhibition of nitrite and prostaglandin E2 production in macrophages was due either to interference with enzyme induction or to direct inhibition of enzyme activities. Twenty-four-hours lipopolysaccharide-treated cells were washed and Hwao-tang was added at 10 ug/ml and all other test products were added at 10 uM, followed by 2 hrs incubation in fresh culture medium supplemented with L-arginine and arachidonic acid. No significant reduction of either nitrite or prostaglandin E2 levels was observed for Hwao-tang after this 2 hrs period (Table 4). Nevertheless, 1400W and NS398 caused a very significant reduction of nitrite (66%) and prostaglandin E2 (68%) production respectively.

Table 4. Effect of Hwao-tang and enzyme inhibitors on iNOS and COX-2 activities in intact peritoneal macrophages after 24 hrs of lipopolysaccharide stimulation

Treatment	iNOS (ng/ nitrite/ml)	COX-2 (ng PGE2/ml)
Basal	1.6±0.2**	2.3±0.2**
Control	42.5±1.7	8.3±0.4
Hwao-tang	40.3±3.5	6.7±0.5
1400W	15.3±1.3**	N.D.
NS398	N.D.	4.5±0.3**

All compounds except for Hwao-tang (10 ug/ml) were incubated at 10 uM for 2 hrs after the stimulation period. Data shown, means±S.E. (n=4-6). N.D. : not determined. Basal: cells not stimulated with lipopolysaccharide. ** ; P<0.01.

9. iNOS and COX-2 activity in broken cells preparations

To confirm the results obtained with intact cells, we examined the effects of this Hwao-tang on iNOS and COX-2 activity in broken cell preparations (Table 5). Hwao-tang at 20 ug/ml was inactive in all the enzymatic activities assayed. In contrast, 1400W and NS398 reduced significantly the production of citrulline (86% inhibition), and prostaglandin E2 (53% inhibition) respectively, in these subcellular preparations.

Table 5. Effects of Hwao-tang and enzyme inhibitors on iNOS and COX-2 activities in high speed supernatants or microsomes of 24 hrs lipopolysaccharide-stimulated macrophages, respectively, and on COX-1 activity in human platelet microsomes

Treatment	iNOS (pmol citrulline /mg protein, min)	COX-2 (ng PGE2/ml)	COX-1 (ng TBX B2/mg protein)
Control	15.2±1.2	17.6±0.6	118.5±6.4
Hwao-tang	13.7±0.5	16.2±1.1	113.6±5.3
1400W	1.9±0.3**	N.D.	N.D.
NS398	N.D.	7.4±0.6**	N.D.
Indomethacin	N.D.	N.D.	35.6±0.8**

Data shown, means±S.E. (n=4-5). N.D. : not determined. Hwao-tang was assayed at 20 ug/ml and at 10 uM. TBX : thromboxane. ** : P<0.01.

10. Synthesis of thromboxane B2 by human platelet microsomes

Synthesis of thromboxane B2 by COX-1 present in microsomes from human platelets was significantly inhibited by the reference compound, indomethacin (86%), whereas Hwao-tang was inactive (data not shown), suggesting that Hwao-tang does not reduce prostaglandin E2 generation by inhibition of COX-1 activity

11. Carrageenan paw oedema

After i.p. administration, Hwao-tang caused a dose-dependent reduction in carrageenan-induced oedema at 3 and 5 hrs after induction of inflammation (Table 6). The greatest effect was observed 3 hrs later, the inhibition percent of 23.4%, 34.6% and 41.3% at the doses of 50, 100 and 300 mg/100g respectively. Indomethacin (5 mg/kg i.p.) was assayed as reference compound, showing a significant reduction in swelling 3 (56.4%) and 5 hrs (44.6%) after the administration of carrageenan.

Table 6. Effects of Hwao-tang and indomethacin on carrageenan mouse paw oedema, 1, 3 and 5 hrs after the induction of inflammation

	Time	Control	Indomethacin (5mg/kg)	Hwao-tang(mg/100g) 50	100	300
Paw oedema (μl)	1 hr	9.6±0.9	8.3±0.7	7.4±0.4	6.4±0.4	4.7±0.6*
	3 hrs	20.9±0.6	8.9±0.5**	15.7±0.6*	11.1±0.8*	7.1±0.8**
	5 hrs	16.0±0.3	8.9±0.4**	9.5±0.8**	7.4±0.4**	5.2±0.4**

Control : vehicle (tween 80/saline 1:99, v/v) was administered intraperitoneally 1 h before injection of carrageenan (0.05 ml; 3% w/v in saline) into the subplantar area of the right hind paw. * : P<0.05, ** : P<0.01 (n=6-7 animals). Hwao-tang at the doses of 50, 100 and 300 mg/100g was administrated orally. Others were i.p. administrated.

12. Inhibition by Hwao-tang and indomethacin of elastase activity and PGE2 levels inflamed paws

Table 7. Inhibition by Hwao-tang and indomethacin of elastase activity (control value 154.7±3.6 nmol p-nitrophenol released/ml) and prostaglandin E2 levels in homogenates of inflamed paws or stomachs (control values 92.2±5.4 and 16.4±1.4 ng/ml, respectively)

Dose	% Inhibition		
	Inflamed paws		Stomachs
	Elastase	PGE2	PGE2
Hwao-tang (50 mg/100g) oral	32.3±2.5**	12.5±3.5	0
Hwao-tang (100 mg/100g) oral	49.3±3.5**	28.2±4.1*	9.3±1.2
Hwao-tang (300 mg/100g) oral	57.4±2.1**	37.6±3.5**	18.3±1.6
Indomethacin (5.0mg/kg) i.p	40.3±2.4**	87.3±2.5**	72.2±0.8

Data represent means±S.E. (n=5-6 animals). * ; P-value<0.05, ** ; P-value<0.01.

The last evaluation of oedema (5 hrs) was followed by killing of the animals and the paws injected with carrageenan were homogenized to determine the levels of elastase and prostaglandin E2 (Table 7). The results indicate that elastase activity was significantly and dose dependently decreased by the three doses of Hwao-tang assayed. Indomethacin also significantly reduced elastase activity in homogenates of inflamed paws. This reference compound strongly reduced the levels of prostaglandin E2 at the dose assayed, whereas

Hwao-tang reduced the levels of this prostanoid at the doses of 100 mg/100g and 300 mg/100g. On the other hand, the content of prostaglandin E2 in stomach homogenates (Table 7) was not significantly affected by the administration of Hwao-tang, in contrast to indomethacin, which clearly reduced the levels of this metabolite.

Discussion

Nowadays, the occurrence rate of the blood circulation system disease has been increased because that the increase of under exercise, fatness, adding the stress, advanced age etc. And the thrombosis importantly came to the front as the risk factor of these circulation system's disease[32)33)]. Thrombosis is the formation of a blood clot in the heart or a blood vessel. Contributing factors include injury to a blood vessel's lining from inflammation(thrombophlebitis) or atherosclerosis, blood flow that is turbulent (e.g., from an aneurysm) or sluggish (e.g., from prolonged bed rest), or coagulation abnormalities (e.g., from high numbers of platelets or excessive fats in the blood). Thrombosis, especially in deep veins of the leg, is a particular danger after major surgery. A thrombus can block blood flow at the point of clot formation or break free to block it elsewhere[34)]. In oriental medical, the thrombosis belong to the category of blood stasis. Blood stasis is a term in oriental medicine. It refers to blood stasis in the body resulting from unsmooth circulation or stagnation of blood and retention of blood in channels or zang-fu organs[1)2)]. This blood stasis present the generalize or local blood circulation disturbance that generated by all kinds of pathological fact or blood stream retention accompanying with a series of syndrome. As the syndrome, stabbing pain fixed at certain region and aggravated by pressing on at night, squamous and dry skin, fullness and pain of the chest and hypochondrium, firmness and fullness of the lower abdomen and so forth have been created. And it becomes the pathopoiesis cause that the symtom complex with a mass or swelling in the abdomen, apoplexy etc[4)]. Moreover, the drugs for invigorating blood circulation and eliminating blood stasis or drugs for removing blood stasis are used for all kinds of syndrome through the blood stasis[35)36)]. Hwao-tang has been used for the treatment of various blood circulation disease, including the clinical treatment of thrombosis[1)]. HOT is additional prescripton of 'Decoction Containing Four Drugs with Persicae and Carthami(桃紅四物湯)' from 《Golden Mirror of Medicine(醫宗金鑑)》, and 'Decoction Containing Four Drugs with Persicae and Carthami(桃紅四物湯)' is an alias of Decoction Containing Four Drugs with Addition(加味四物湯)' from 《OkGiMiEui(玉機微義)》[3)]. HOT is consisted of Angelicae gigantis Radix, *Rehmanniae Radix*, *Paeoniae Radix*, *Ciniamomi Cortex*, *Cnidii Rhizoma*, *Persicae Semen* and *Carthami Flos*[3)]. Hwao-tang has been reported to have a hypolipidemic effect in patients with hypercholesterolemia, and in cholesterol-induced experimental models[4)]. Hwao-tang has also been reported to have an inhibitory effets of Hwao-tang on the atherosclerosis and the venous thrombosis. On this study, Hwao-tang was used for investigating reaction of enzymes acted on mechanism of thrombosis formation.

When the cells were preincubated with Hwao-tang for 3 min, inhibition of fMLP-induced superoxide generation was observed. According to the time course experiment, 3 min was sufficient for the preincubation time (data not shown). fMLP-induced superoxide generation was inhibited by Hwao-tang in a concentration-dependent manner. On the other hand, PMA-induced superoxide generation was enhanced by Hwao-tang in a concentration-dependent manner (Fig. 2). The results indicate that Hwao-tang are typical priming factors for the agonist-mediated respiration burst of neutrophils. The rates of the priming effects of Hwao-tang on fMLP- and PMA-induced superoxide generation were different from each other. The reverse was true of the rates of enhancement of PMA-induced superoxide generation. PMA-induced superoxide generation by Hwao-tang was inhibited by staurosporine, an inhibitor of protein kinase C. However, genistein, an inhibitor of protein tyrosine kinase did not inhibited the superoxide generation (Fig. 3 and Table 1), suggesting that Hwao-tang enhance superoxide generation in neutrophils via activation of protein kinase C. It was previously reported that the phosphorylation process of tyrosine residues of HPPMN proteins was inhibited by genistein and herbimycine A, inhibitors of tyrosine protein kinase[14)37)]. Therefore, the effect of Hwao-tang on the phosphorylation of tyrosine residues of HPPMN protein in fMLP-induced neutrophils was examined. Hwao-tang inhibited the phosphorylation of tyrosine residues of the 58 kDa protein in fMLP-induced neutrophils (Fig. 3). These results indicate that Hwao-tang decrease superoxide generation in neutrophils via inhibition of tyrosine protein kinase. It was reported detecting several priming factors for the phosphorylation of tyrosine residues in a 45 kDa protein of human neutrophils, and that the phosphorylation was inhibited by the tyrosine kinase inhibitors, genistein and herbimycin A, but not by the protein kinase C inhibitors, staurosporine and H-7. The priming factors enhanced superoxide generation induced by PMA and AA. The function of Hwao-tang in agonist-mediated superoxide generation in human neutrophils was different from that of other priming factors investigated in previous papers. At present, the mechanism for inhibition of

fMLP-induced superoxide generation, for inhibition of protein tyrosine kinase and for activation of PMA-induced superoxide generation by Hwao-tang is unknown. Whether Hwao-tang alter the properties of fMLP- and PMA-receptors by direct binding or indirectly by changing the membrane environment is also unknown. It has been reported that a 47 kDa protein, one of the cytosolic proteins for activation of NADPH oxidase in human neutrophils, was phosphorylated in human neutrophils after stimulation with fMLP or PMA, based on an analysis using SDS-PAGE38). The mechanism for inhibition of protein synthesis p47 phox by Hwao-tang is not clear. In the present work, the respiratory burst elicited in human neutrophils by TPA was potently inhibited by Hwao-tang, showing a minor scavenging action in the cell-free system. Hwao-tang reduced the degranulation induced by cytochalasinB+fMLP or cytochalasinB+PAF, as well as the leukotriene B4 synthesis induced by ionophore A23187, thus exerting inhibitory effects on neutrophil functions triggered by structurally divergent agonists. Hwao-tang may either prevent or slow the progression of neutrophil-mediated tissue injury. Hwao-tang seems to affect cell activation at a site common to different signaling pathways as it inhibited responses induced by fMLP, PAF, TPA or ionophore A23187. The induction of NO synthase and COX-2 greatly increases the synthesis of NO and prostaglandins. iNOS inhibition results in modulation of the inflammatory response and delayed paw swelling induced by carrageenan in mice[39]. Furthermore, NO has been shown, in *in vitro* and *in vivo* studies, to increase the production of pro-inflammatory prostaglandins[40]. On the other hand, overproduction of prostaglandins by COX-2 expression *in vivo* has been reported for chronic inflammatory conditions such as rheumatoid arthritis[16] and experimental models of inflammation[41]. Hwao-tang inhibited the production of NO and prostaglandin E2 in murine peritoneal macrophages stimulated by lipopolysaccharide. The inhibition was dose-dependent without any evidence of a cytotoxic effect. Western blot analysis of mouse peritoneal macrophages lysates showed that iNOS and COX-2 protein expression was reduced by the presence of Hwao-tang during lipopolysaccharide treatment, indicating that Hwao-tang inhibits the induction rather than the activity of both enzymes. This hypothesis was confirmed by the fact that Hwao-tang was inactive on iNOS and COX-2 activity in a cell-free system (broken cell preparations). In addition, Hwao-tang did not modify the arachidonic acid pathway by a direct action on the activity of enzymes such as phospholipase A2, 5-lipoxygenase, or COX-1.

In a model of inflammation, the mouse paw oedema induced by carrageenan, Hwao-tang exerted potent inhibitory effects. Interestingly, Hwao-tang reduced the elastase content in the inflamed paw, an index of migration. In addition, the inhibition of COX-2 expression by Hwao-tang may account for the anti-inflammatory effects of this on mouse paw oedema, as evidenced by the observed reduction of prostaglandin E2 levels in the inflamed paw. NO or prostaglandin E2 overproduction can be controlled by NO synthase or COX-2 inhibitors, respectively. Nevertheless, at the doses normally used they can also inhibit constitutive isoforms, which leads to detrimental effects. Drugs such as glucocorticoids, able to inhibit iNOS and COX-2 expression are potent anti-inflammatory agents[42]. Our results indicate that Hwao-tang can control NO and prostaglandin E2 overproduction by selective inhibition of the enhanced expression of both enzymes, thus providing a possible strategy in the treatment of inflammatory diseases.

In sum, the present study demonstrated that Hwao-tang can control NO and prostaglandin E2 overproduction by selective inhibition of the enhanced expression of both enzymes. Hwao-tang inhibited the oxidative burst in human neutrophils and murine peritoneal macrophages. Hwao-tang exerts acute anti-inflammatory effects by reduction of leukocyte activation and inhibition of iNOS and COX-2 expression. Reactive oxygen species and reactive nitrogen intermediates have been implicated in the synthesis of different pro-inflammatory mediators and thus it is known that these species do not operate solely as end-stage effector molecules, but also as mediators regulating cytokine gene expression[43]. Besides, reactive oxygen species can participate in the activation of nuclear factors such as nuclear factor-kappaB (NF-κB)[44]. This transcription factor is essential in the enhanced expression of iNOS[45] and COX-2[46] genes in lipopolysaccharide-treated macrophages. Further studies are required to find if the inhibition of enzyme expression by Hwao-tang is related to an effect on the generation of reactive oxygen species and/or on the regulation of transcription factors such as NF-κB.

Conclusion

Hwao-tang is a dried decoctum of a mixture of 7 herbal, consisting of *Angelica gigantis Radix, Rehmanniae Radix, Paeoniae Radix, Ciniamomi Cortex, Cnidii Rhizoma, Persicae Semen* and *Carthami Flos*. We have reported that Hwao-tang had various effects on stimulus-induced superoxide generation in human neutrophils. The effects of these Hwao-tang on superoxide generation in human neutrophils were investigated. Hwao-tang significantly inhibited N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced superoxide generation in a concentration-

dependent manner, but not that induced by arachidonic acid (AA). On the other hand, both Hwao-tang enhanced superoxide generation induced by phorbol 12-myristate 13-acetate (PMA) in a concentration-dependent manner. The superoxide generation induced by PMA with Hwao-tang was suppressed by staurosporine, an inhibitor of protein kinase C, but was not suppressed by genistein, an inhibitor of protein tyrosine kinase. Tyrosyl phosphorylation of a 58 kDa protein, which was increased by fMLP, was inhibited by Hwao-tang. Hwao-tang also inhibited the generation of a 47 kDa protein and platelet aggregation in human blood. The results suggest that protein tyrosine kinase participates in fMLP-mediated superoxide generation by Hwao-tang-treated human neutrophils. A Hwao-tang inhibited neutrophil functions, including degranulation, superoxide generation, and leukotriene B4 production, without any effect on 5-lipoxygenase activity. This Hwao-tang reduced nitric oxide (NO) and prostaglandin E2 production in mouse peritoneal macrophages stimulated with lipopolysaccharide, whereas no influence on the activity of iNOS, COX-2 or COX-1 was observed. Hwao-tang significantly reduced mouse paw oedema induced by carrageenan. Western blot analysis showed that Hwao-tang reduced the expression of iNOS and COX-2. The results indicate that Hwao-tang exerts anti-inflammatory effects related to the inhibition of neutrophil functions and of NO and prostaglandin E2 production, which could be due to a decreased expression of iNOS and COX-2.

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