行政院國家科學委員會專題研究計畫　成果報告

Honokiol 在大花鼠腦梗塞/ 再灌注性傷害動物模式中之保護作用及其作用機轉之探討(3/3)

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中文摘要及關鍵詞：

在本三年計劃的前兩年計劃中，實驗發現中藥厚朴所含的一個有效成分 honokiol，對於大花鼠受到大腦局部缺血及再灌注傷害具有神經保護作用，在於 honokiol 改善大花鼠受到大腦局部缺血及再灌注傷害的作用機轉，我們認為是藉由抑制 leukocyte 的黏著以及減少 leukocyte 細胞內以及細胞外的活性氧屬的產生進而抑制 leukocyte 的浸潤而來的，在缺血以及再灌注傷害時，leukocyte 產生的活性氧屬是缺血以及再灌注傷害時發炎反應的一個訊息，其會先活化 NADPH 氧化酵素（一個位在 leukocyte 細胞模的過氧化物生成酵素），研究發現 honokiol 會抑制 NADPH 氧化酵素的活性，然而對於 protein kinase C (PKC)依賴性的氧化酵素沒有作用，honokiol 也會抑制 myeloperoxidase (MPO) 和 cyclooxygenase (COX)產生活性氧屬，相反的，honokiol 會增加 glutathione peroxidase (GPX)的活性。此外，honokiol 會抑制 *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), fluoroaluminate ion (AlF_4^-)，和 thapsigargin 所引起鈣離子灌流進細胞內。

由研究結果我們認為，honokiol 會藉由抑制 NADPH 氧化酵素的活性，抑制 myeloperoxidase (MPO) 和 cyclooxygenase (COX)的活性，以及抑制 G protein 調節鈣離子灌流而抑制活性氧屬的產生，此外，honokiol 也會增加 glutathione peroxidase (GPX)的活性，進而抑制 leukocyte 的功能，而能夠抑制 leukocyte 浸潤到大腦局部缺血及再灌注傷害的區域，保護大花鼠大腦對抗大腦局部缺血及再灌注傷害。

關鍵字： honokiol ；活性氧屬；NADPH oxidase; myeloperoxidase; cyclooxygenase; glutathione peroxidase; protein kinase C; G protein

英文摘要及關鍵詞：

We have previously showed that honokiol (HK) can inhibit leukocyte infiltration via impairment of their adhesion and reduce subsequent production of intracellular and extracellular reactive oxygen species (ROS) that accounts for the amelioration of focal cerebral ischaemia-reperfusion (FCI)-induced injury. ROS produced by leukocytes contributes to the pathogenesis of FCI injury as well as signals the inflammatory responses. This begins with the activation and assembly of NADPH oxidase, a superoxide-producing enzyme complex located on the cell membrane of leukocytes. At maximal effects in all studies, HK inhibited the activity of assembled oxidase by 40% but not the protein kinase C (PKC)-dependent assembling of the oxidase. HK also inhibited other enzymatic sources of ROS including myeloperoxidase (MPO) and cyclooxygenase (COX) by 20% and 70%, respectively. In contrast, glutathione peroxidase (GPX) activity, an enzyme catabolizing the metabolism of hydrogen peroxide (H_2O_2), was 20 % enhanced by HK. Mobilization of intracellular calcium mediates the ligand-initiated ROS production. HK impeded about 40% of the calcium influx induced by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), fluoroaluminate ion (AlF_4^-), and thapsigargin. We conclude that (1) the ROS production is diminished through interfering NADPH oxidase, and/or inhibition of MPO and COX activities, as well as antagonizing G protein-mediated calcium influx, and (2) the promotion of GPX activity by HK may account for the inhibition of leukocyte functions that confers on HK the ability to abolish leukocyte infiltration in FCI injury.

Key words: honokiol; reactive oxygen species; NADPH oxidase; myeloperoxidase; cyclooxygenase; glutathione peroxidase; protein kinase C; G protein

目　　錄

報告內容：

前言：

Honokiol (HK), an active component isolated from the herb 'Houpo' (*Magnolia officinalis*), had been demonstrated to be an effective anti-oxidant [1]. We have previously reported that HK can reduce myocardial infarct size and display anti-arrhythmic effect in rats subjected to coronary artery occlusion [2,3]. Recently, we found that HK ameliorated focal cerebral ischaemic reperfusion (I/R)-induced brain infarction [4] possibly through prevention of leukocytes activation/adhesion and ROS production (unpublished results). However, reactive oxygen species (ROS) produced by leukocytes contributes to the pathogenesis of I/R injury and signals the inflammatory responses [5].

Release of ROS and ROS-related free radicals by leukocytes mainly depends upon the activation of NADPH oxidase and myeloperoxidase (MPO). NADPH oxidase is a powerful oxidant-producing enzyme complex located on the surface membrane of leukocytes which generates superoxide anion ($O_2^{\cdot -}$), hydroxy radical (OH^-) and hydrogen peroxide (H_2O_2) [6]. Furthermore, H_2O_2 can be metabolized to more cytotoxic radical 'hypochlorous acid (HOCl)' by MPO, an abundant chloride peroxidase in leukocyte granules [7]. There are at least two types of activator that can activate NADPH oxidase: (1) a direct protein kinase C (PKC) activator, e.g., phorbol-12-myristate-13-acetate (PMA), which may trigger the phosphorylation of some cytosolic components that is essential for the assembling of active NADPH oxidase and (2) a receptor/G-protein coupling activator, e.g., fMLP, which may activate oxidase through calcium-dependent pathway [8,9].

研究目的：

In this study, PMA- or fMLP-induced ROS production in the intact cell and cell free system was established as *in vitro* models to examine the anti-inflammatory effect of HK. With antioxidative potential, whether HK could effectively inhibit the activity of NADPH oxidase, MPO activity, or modulate other cellular targets, e.g., glutathion peroxidase (GPX) and cyclooxygenase (COX), in regulation of ROS production were investigated in our laboratory.

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研究方法：

Animal preparation

The animals used in the present investigation follow 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 to 350 grams were fed in 12:12 hour light-dark cycle, temperature (24±1℃) and humidity (55±5%) controlled animal breeding rooms. All animals were allowed free access to food and water.

Leukocyte preparation and drug treatments

Peripheral whole blood was collected from anesthetized Long-Evans rats (250 to 350 grams) and collected into syringes containing heparin (20 U/ml blood). Blood samples were mixed with equal volumes of 3% 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 leukocytes, was collected and subjected to centrifugation at 250 × *g* for 15 min at 4°C. Peripheral blood leukocytes were then separated by lysis of contaminating erythrocytes and washed tree times with cold phosphate-buffered saline (PBS) [10]. The leukocyte were mixed with the various concentrations of HK or solvent control (vehicle, 0.05% DMSO) in Hank´s balanced saline solution (HBSS) for 10 min at 37 °C before the addition of PMA or fMLP.

Measurement of NADPH oxidase activity in subcellular fractions

Leukocyte-particulate fractions were prepared according to a modified method of Clark et al. [11]. Leukocytes (2×10^7/ml) were incubated for 10 min at 37°C in the presence of either PMA (0.1 μg/ml) or an equivalent concentration of DMSO. After incubation, the cell suspension was centrifuged at 4°C at 300 ×*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 PMSF, 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. Unbroken cells were removed by centrifugatiion at 500 ×*g* for 5 min. The resulting supernatant was centrifuged at 4°C for 20 min at 115,000 ×*g* after which the supernatant was discarded and the pellet (particulate fractions) washed in sample buffer and recentrifuged for 20 min at 115,000 ×*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 SOD-inhibitable cytochrome *c* reduction [12]. HK was added 20 min in the PMA

pre-assembled particulate fractions before the addition of the NADPH (for subcellular oxidase activity study). Alternatively, leukocytes were preincubated with HK or staurosporine (STAU, a PKC inhibitor) for 20 min at 37°C before PMA stimulation (for oxidase assembling study). Results were expressed as % of control by calculation the $O_2^{\cdot -}$ production ($O_2^{\cdot -}$ nmol/2×10^5 cell equivalents/20 min) of drug-treated sample over that of drug free sample(s).

Protein kinase(s) activity assay

Leukocytes 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 PMSF, 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 seconds at 4°C five times and the cell lysate separated into the cytosol (as cytosolic fraction) and pellet fractions by centrifugation at 100,000×g for 60 min at 4°C. The pellet was solubilized by resuspension in extraction buffer with 0.1% Triton X-100 vortexed and incubated at 4°C for 60 min. The solubilized extract as particulate fraction was separated by centrifugation at 10,000×g for 5 min. Both cytosolic and particulate fractions were stored at 4°C and assayed for kinase activity within 3 days. Protein concentration was determined by a protein assay reagent (Bio-Red, USA). Protein kinase C (PKC) or adenosine-3',5'-cyclic monophosphate (cAMP)-dependent protein kinase (PKA) activity was measured by a non-radioactive protein kinase assay kit (Calbiochem®, Germany). This assay kit was based on an enzyme-linked immunosorbent assay that utilized a synthetic PKC/PKA pseudosubtrate and a monoclonal antibody that recognized the phosphorylated peptide. PKC or PKA phosphorylates pseudosubstrate (peptide) on serine through Ca^{++}/phosphatidylserine (PS)-dependent- or cAMP-dependent-mechanism, respectively. Ca^{++}/phospholipid-dependent PKC was assayed in the presence of Ca^{++} and PS, and the negative control was measured in the presence of 20 mM EGTA. In some experiments, PMA (100 ng/ml) were added to live leukocytes 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 cytolic PKC was translocated to the particulate fraction. In the PKA activity assay, the negative control was measured in the absence of cAMP. Data were expressed as percentages of control.

Estimation of myeloperoxidase (MPO) activities

MPO activity was evaluated according to Bani's [13] with some modifications. Briefly, MPO activity was determined in the presence or absence of HK by mixing 0.1 ml of cytosolic fractions (from 2×10^7cells/ml 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 MPO. Oxidized *o*-dianisidine formed a soluble chromophore and the absorbent (OD_{460}) was determined by spectrophotometry over 2 min. MPO activity (MPOA) was expressed as ΔMPOA by subtracting the value of OD_{460}x100 at time 0 min from that at 2 min for each sample. Data were expressed as percentages (%) of control by calculating the ratio of ΔMPOA resulting from HK treatments over that of drug-free alone.

Glutathione peroxidase (GPX) activity assay

Leukocytes were resuspended to a concentration of 2×10^7/ml in ice-cold extraction buffer as stated above. Cells were sonicated for 10 second at 4°C three times and the cytosolic fraction were separated by centrifugation at 100,00×*g* for 20 min at 4°C. Protein concentration was determined by protein assay reagent (Bio-Red, USA). GPX activity [14] 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 rate of reaction was measured by the decrease in NADPH at 340 nm using H_2O_2 (Se-dependent activity) as substrate against the reference that did not contain substrates. An additional blank containing all components except glutathione reductase was determined to correct non-enzymatic oxidation of GSH and NADPH by substrates (H_2O_2). One unit was determined as 1 mmol of GSH conjugated/min/mg protein. Data were expressed as percentages of control (drug free).

Cyclooxygenase (COX) activity assay

Cytosolic fraction of leukocytes was prepared as described above. COX activity was measured by a chemiluminescent-based COX assay kit (Calbiochem®, Germany). This assay kit utilized a specific chemiluminescent substrate to detect the peroxidative activity of COX enzymes. Briefly, after pretreatment with HK (0.1-10 μM), 50 μM NS-398 (COX-2 inhibitor) or 100 μM resveratrol (COX-1 inhibitor), the direct residual activity of COX was measured by addition of a proprietary luminescent substrate and arachidonic acid (AA). Light emission began immediately and was directly proportional to the COX activity in the sample. The chemiluminescent signal was measured over five seconds by a microplate luminometer reader (Orion®, Germany) and represented as relative light units (RLU). Data were expressed as percentages of control (drug free).

Determination of intracellular calcium concentration ($[Ca^{++}]_i$)

Prior to drug treatment, leukocytes were preloaded with 5 μM 1-[2-(5-carboxyoxal-2-yl)-6-amino-benzofuran-5-oxyl]-2-(2'-amino-5'-methylphenoxy-ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (fura 2-AM, Molecular Probes, Eugene, OR) at 37°C for 45 min, washed twice and resuspended at 2×10^6/ml in calcium-free HBSS containing HK or control vehicle. After pretreatments for 5 min, 1 ml cell suspensions from each sample and 1 ml HBSS containing 2 mM Ca^{++} were transferred to individual cuvettes and gently mixed with a micromagnetic stirrer at 37°C for 5 min before the addition of fMLP (1 μM) or AlF_4^- (10 mM NaF plus 30 μM $AlCl_3$) or thapsigargin (1 μM). The fluorescence of fura-2-loaded cells was measured by a spectrofluorometer (Hitachi F-4500) with excitations at 340 and 380 nm and emission at 510 nm. Intracellular calcium concentration for each sample was calculated from the ratio of emission versus excitation as previously described [15]:

$$[Ca^{++}]_i = K\cdot(R-R_{min})(S_{f380})/(R_{max}-R)(S_{b380})$$

Where: K=224 nM (Fura-2 at 37°C), R_{min} = ratio value in minimal Ca^{++} conditions, R_{max} = ratio value at a maximal Ca^{++} concentration, S_{f380} = 380 nm reading in minimal Ca^{++} conditions (corrected for background), S_{b380} = 380 nm reading in maximal Ca^{++} conditions (corrected for background). R_{max} and S_{b380} were obtained at the end of a measurement by permeabilizing the cells with 1.0% triton X-100, where R_{min} and S_{f380} were determined by adding 20 mM EGTA after digitonin lysis.

Statistical analysis

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

結果：

Effect of HK on the assembly or the activity of assembled NADPH oxidase

To examine whether assembling process of NADPH oxidase or its activity was modulated by HK, the oxidase activity was determined in particulate fractions prepared from cells pretreated with HK followed by PMA-activation for the study of NADPH oxidase 'assembly' or adding HK in PMA-preactivated particulate fraction for the study of the activity of assembled- NADPH oxidase. In PMA alone-treated groups, oxidase activities was enhanced to 203±16% or 159±9% for 'drug before PMA' or 'drug after PMA' group, respectively. Our results demonstrated that HK did not interfere with the assembly of NADPH oxidase (207±28 % or 195±35% for 1 μM or 10μM of HK, respectively) but inhibited the activity of assembled NADPH oxidase (159±9%) to produce superoxide by 101±14 % or 89.5±12% for 1 μM or 10μM of HK, respectively (Table 1). Staurosporine (0.1 μM), a PKC inhibitor, which was used to contrast the PKC-dependent assembly of NADPH oxidase, showed a significant inhibition on this process.

	[a]NADPH oxidase activities (% of control)	
	[b]Drug before PMA-activated	[c]Drug after PMA-activated
Control (Vehicle)	100	100
PMA alone	203±16	159±9
+ HK (1.0 μM)	207±28	101±14[†]
+ HK (10 μM)	195±35	89.5±12[†]
+ STAU (0.1 μM)	115±20[*]	ND

Effect of HK on the protein kinase(s) activity

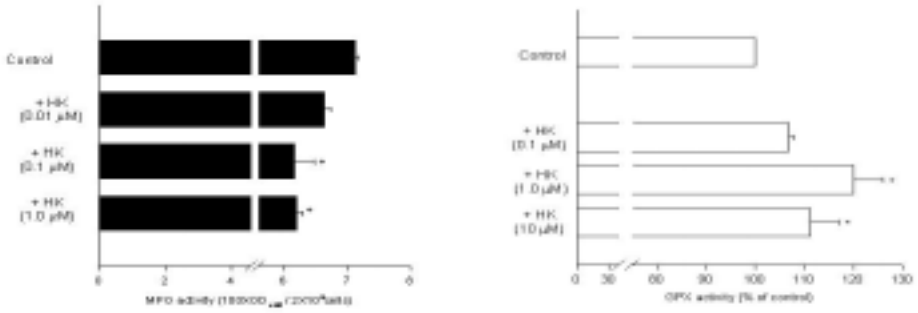
To examine whether modulation of protein kinase(s) activity mediating the inhibitory effect on NADPH oxidase activity by HK, we measured the activity of PKC and PKA by an enzyme-linked immunosorbent assay (ELISA). Cytosolic PKC activity was not inhibited by HK (Table 2). In the negative control groups, PKC activity was significantly reduced to 76.0±4.8 % in the presence of 20 mM EGTA (for negation of calcium effects) and reduced to 45.0±3.0 % in the presence of 100 ng/ml PMA (in which most of the cytosolic PKC was translocated to the particulate fraction). On the other hand, PKA activity was enhanced to 138.0±2.5% in the presence of

cyclic AMP (20 μM). HK also did not modulate PKA activity (Table 2).

	[a]Protein kinase(s) activity (% control)	
	PKC	[c]PKA
Control (Vehicle)	100	100
+ HK (1.0 μM)	97.6±1.7	99.3±2.6
+ HK (10 μM)	95.8±3.5	102.5±5.4
+ EGTA (20 mM)	76.0±4.8*	ND
[b]PMA-treated	45.0±3.0*	ND
cAMP (20 μM)	ND	138.0±2.5†

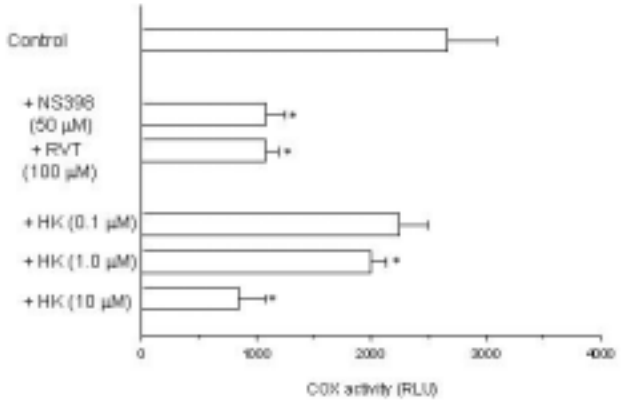
Effect of HK on myeloperoxidase (MPO) activity or glutathione peroxidae (GPX) activity

To study whether modulation of MPO or GPX activity contributed to the anti-oxidant effect by HK, we examined the HK's effect on cytosolic MPO or GPX. The MPO activity in the control (vehicle only) sample was 7.12±0.05 ($100\times OD_{460}/2\times10^6$cells). HK significantly inhibited MPO activity in leukocytes at concentrations of 0.1 and 1.0 μM (Fig. 1). On the other hand, we observed that HK (1μM) significantly enhanced the GPX activity by 20% (Fig. 2). These data illustrate that multiple mechanisms are responsible for the ROS scavenging effect by HK.



Effect of HK on the cyclooxygenase (COX)

COX is an important enzymatic source of ROS [16,17]. Our results demonstrated that HK concentration (0.1-10 μM)-dependently inhibited COX activity (Fig. 3). Resveratrol (100 μM), a selective COX-1 inhibitor [18], and NS-398 (100 μM), a selective COX-2 inhibitor, both significantly inhibited the COX-activity (Fig. 3).



Effect of HK on fMLP-, AlF_4^- or thapsigargin induced $[Ca^{++}]_i$ mobilization

Mobilization of intracellular calcium mediates the ligand-initiated signaling for activation and the ROS production by leukocytes [19]. Therefore, effects of HK on fMLP (a receptor agonist)-, AlF_4^-- (a G-protein activator) or thapsigargin-induced calcium influx or mobilization were studied. FMLP (1μM), AlF_4^- and thapsigargin (1μM) triggered rapid increases in intracellular calcium ($[Ca^{++}]_i$). HK (0.1-10 μM) impeded the calcium influx induced by fMLP, AlF_4^- or thapsigargin (Table 3).

	$\Delta[Ca^{++}]_i$ (% of agonist)		
	fMLP (1 μM)	AlF_4^-	Thapsigargin (1 μM)
Agonist only	100	100	100
+ HK (0.1 μM)	65.7±12.1*	58.9±14.0†	68.8±11.2‡
+ HK (1.0 μM)	64.8±9.1*	58.6±14.2†	67.7±3.9‡
+ HK (10 μM)	46.4±8.4*	53.7±13.3†	63.8±16.5‡

討論：

Production of ROS by leukocyte predominantly comes from the activation of NADPH oxidase to generate $O_2^{\cdot -}$. This process involves the assembly of cytosolic components (e.g., p47-phox, p67-phox) and membrane-associated components (gp9l-phox and p22-phox) to form an active NADPH oxidase enzyme complex [11, 20-21]. To further understand whether the assembling process of NADPH oxidase or the activity of assembled NADPH oxidase was modulated by HK, its effects on the oxidase activity in particulate fractions were studied. Particulate fraction isolated from cells pretreated with PMA completed NADPH oxidase assembly and generated $O_2^{\cdot -}$ 1.8 fold compared with that of resting cells (vehicle only; non PMA-treated) in the presence of NADPH (400 μM). HK treatment before PMA-activation did not inhibit the NADPH oxidase activity in the particulate fraction. In the sample pretreated with staurosporine or cromolyn (data not shown), an inhibitor for the assembly of NADPH

oxidase [22], the oxidase activity was significantly suppressed.

Activation/assembly of NADPH oxidase involves the phosphorylation and translocation of cytosolic component(s) (such as p47-phox) to surface membrane by PKC [23]. Furthermore, the PKC and cAMP-dependent PKA have been reported to regulate the activation of leukocytes [24,25]. In our study, staurosporine (a PKC inhibitor), but not HK, inhibited the PKC activity and prevented the activation of NADPH oxidase suggesting that HK did not interfere with the PKC-dependent phosphorylation and translocation of cytosolic component(s). A previous report by Orlic et al. suggested that activation of PKA could negatively regulate ROS production [26]. Here, we found that HK did not interfere with PKA activity. On the other hand, incubation of PMA-pretreated particulate fractions (NADPH oxidase well-assembled) with HK showed significant decrease on $O_2^{\cdot -}$ generation, indicating that HK could inhibit the activity of the assembled NADPH oxidase complex, probably through interfering with NADPH binding.

In addition to acting as pathological factors and immuno-modulating effectors, ROS also serve as signaling molecules in the activation of inflammatory cells [5], and promote leukocytes recruitment and infiltration by enhancing their adhesion to endothelial cells [27,28]. Besides, NADPH oxidase enzyme complex and protein kinases, there are several other possible targets could be modulated by HK to interfere ROS production. These possible targets include the following: (1) MPO activity, which generates potent oxidants HOCl from H_2O_2 ; (2) catalase (CAT) and/or glutathione peroxidase (GPX) activity, which convert $O_2^{\cdot -}$ to water and oxygen and (3) cyclooxygenase (COX), another enzymatic source of oxygen radical [17]. We found that GPX, but not CAT, activity was enhanced by HK. HK also significantly inhibited COX and MPO activities. Based on these findings, we propose that multiple mechanisms through inhibiting free radical production and enhancing free radical metabolism might be responsible for HK's anti-oxidative effects, which eventually lead to the amelioration of FCI injury through the inhibition of leukocyte adhesion/infiltration.

Drugs with anti-oxidative effect could have the potential for the prevention of leukocyte-mediated inflammatory responses such as I/R injury. This can be further illustrated by our studies that HK, an effective anti-oxidant, protects animals against myocardial and cerebral I/R injuries [2-4]. Similar observations in probucol, an antioxidant [29], can improve post-myocardial infarction by reducing both oxidative stress and expression of pro-inflammatory cytokines [30], or attenuate reperfusion-induced cardiac injury [31]. In addition to acting as free radical scavengers, there are several possible mechanisms for antioxidants to achieve their beneficial effects in I/R injury. These include increasing glutathione peroxidase (GPX) activity [32], modulating adhesion of leukocyte to endothelium cells [33], preventing leukocyte infiltration [34], and inhibiting NADPH oxidase [35] as well as myeloperoxidase (MPO) [36].

According to our previous study, modulation of PKC-dependent activation [37] or impedment to calcium influx [38] can account for the inhibition of Mac-1 dependent leukocyte adhesion. Since PKC activity was not inhibited by HK in the present study, we further elucidate the effect of HK on intracellular calcium ($[Ca^{++}]_i$) mobilization induced by fMLP, AlF_4^- (a G-protein activator) or thapsigargin (releaser of calcium from intracellular store). Our data established that HK significantly inhibited fMLP-, AlF_4^-- or thapsigargin- induced $[Ca^{++}]_i$ mobilization indicating that HK may interfere with G-protein activation as fMLP binds to its receptor. In this study, samples pretreated with pertussis toxin (a G-protein inhibitor) or BAPTA-AM (an intracellular

calcium chelator) were included to contrast the specificity of G-protein activation and intracellular calcium mobilization by AlF_4^- or thapsigargin, respectively (data not shown).

In conclusion, our data suggest that HK impaired ROS production, at least in part, through (1) inhibiting NADPH oxidase activity without interfering in PKC- or PKA-dependent signaling pathway, (2) inhibiting MPO and COX activities, (3) enhancing GPX activity, and/or (4) antagonizing G protein-mediated calcium influx. The proposed mechanism(s) for the inhibition of free radical production in leukocytes by HK are summarized as illustration 1.

計畫成果自評：

在計劃進行過程中，雖然有一些技術需要修正改善，經過努力，我們已經完成本三年計劃，並已經將研究成果攥寫成論文，且此論文已分別投稿在 Planta Medica 2003; 69: 130-134，Brain Research 2003; 992: 159-166 以及 European Journal of Pharmacology 2003; 475: 19-27。