行政院國家科學委員會專題研究計畫　期中進度報告

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(2/3)

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□成果報告　■期中進度報告

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共同主持人：

計畫參與人員： 劉國同；沈郁強；黃相碩；池春蓮

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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中文摘要及關鍵詞：

在本三年計劃的第一年計劃中，實驗研究顯示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，在本第二年計劃中，研究 honokiol 對於大腦局部缺血及再灌注傷害的神經保護作用的機轉，計劃中我們評估 honokiol 對於大花鼠大腦組織受到局部缺血及再灌注傷害時 myeloperoxidase（MPO）的活性，以探討 honokiol 的神經保護作用是否與抑制 leukocyte 的浸潤有關？此外，我們也評估一些離體 leukocyte 活性的指標，例如 leukocyte 黏著（adhesion）以及活性氧屬（reactive oxygen species（ROS））的產生。研究顯示由靜脈投與 honokiol（0.1 或 1.0 μg/ kg），不論是在中大腦動脈結紮前十五分鐘投與的預防組，或是在中大腦動脈結紮六十分鐘後投與的治療組，皆能夠有意義的減少浸潤的總體積。而在離體實驗結果顯示，投與 honokiol（1 至 10 μM），可以有意義的抑制以 phorbol-12-myristate-13-acetate（PMA）或 *N*-formyl-methionyl-leucyl-phenylalanine（fMLP）活化的 leukocyte 對於表面所包覆的 fibrinogen 的黏著，最大可達 50%，而對於調節 leukocytes 黏著的 Mac-1 表現，honokiol 則可以輕微的抑制 5%。預先投與 honokiol，可以減少以 PMA 或 fMLP 所引起 leukocyte 細胞內以及細胞外的活性氧屬的產生達 40%。我們認為 honokiol 改善大花鼠受到大腦局部缺血及再灌注的傷害的機轉是藉由抑制 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。

關鍵字：黏著；中樞缺血及再灌注傷害；leukocyte 浸潤；活性氧屬；honokiol

英文摘要及關鍵詞：

We have previously shown that honokiol (HK), an active component of *Magnolia officinalis*, displayed neuroprotective effect against focal cerebral ischaemia-reperfusion (FCI) injury in rats. To examine whether inhibition of leukocyte infiltration may confer on HK the ability to prevent FCI injury, myeloperoxidase (MPO) activity, a marker for leukocyte infiltration, in the rat brain tissue subjected to FCI injury and *in vitro* leukocyte activities such as leukocyte adhesion and reactive oxygen species (ROS) production were examined. Intravenous administration of HK (0.1 or 1.0 μg/ kg) 15 minutes prior to right middle cerebral artery (RMCA) occlusion (pretreatment) or 60 minutes after RMCA occlusion (treatment) both significantly reduced the total volume of infarction maximally by 70% and inhibited leukocytes infiltration by 100%. In vitro, HK (1-10 μM) significantly inhibited leukocytes adhesion to fibrinogen-coated surface induced by phorbol-12-myristate-13-acetate (PMA)- or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) maximally by 50%. Up-expression of Mac-1 (macrophage adhesion molecule-1 and CD11b/CD18), a beta 2 integrin mediating the firm adhesion of leukocytes, were slightly (5%) inhibited by HK. Pretreatment with HK diminished PMA- or fMLP-induced intracellular and extracellular reactive oxygen species (ROS) production by 40%. The mechanisms of the amelioration of FCI injury by HK may be due to inhibition of leukocyte infiltration resulting from inhibition of their adhesion and decreased ROS production intracellularlly and extracelluarly.

Key words: adhesion; cerebral ischaemia-reperfusion injury; leukocyte infiltration; reactive oxygen species; honokiol

目　　錄

報告內容：

前言：

Honokiol (HK, $C_{18}H_{18}O_2$, MW =266.33) is an active component isolated from the herb 'Houpo' (*Magnolia officinalis*) used in the treatment of a number of vascular diseases in traditional Chinese medicine. HK had been demonstrated to be an effective anti-oxidant [1], protects animal tissues against lipid peroxidation [2,3], serves as an anti-platelet drug [4], and displays anti-inflammatory effect in activated macrophages [5,6]. Recently we also reported that in animal models, HK can reduce myocardial infarct size and exhibit anti-arrhythmic effect in rats subjected to coronary artery occlusion [7,8] and ameliorate focal cerebral ischaemic reperfusion (I/R)-induced brain infarction [9].

When tissue suffers from ischaemia and reperfusion, infiltration of leukocytes to the foci plays a crucial role in the pathogenesis of I/R injury [10]. Furthermore, expression of adhesion molecules on leukocyte surfaces that promotes their adhesion to endothelial cells and generation of ROS by leukocytes are key factors that contribute to leukocyte infiltration. Evidences have shown that inhibiting leukocytes adhesion [11] or ROS production [12] can reduce the infiltration and recruitment of leukocytes in cerebral I/R injury.

研究目的：

The aims of this study were therefore to examine the mechanism(s) of HK in the protection of rat brain from I/R injury, especially how leukocyte infiltration in brain tissue is inhibited. To achieve this, we measured myeloperoxidase (MPO) activities (as an index of leukocyte infiltration) in rat brain tissue, and leukocyte adhesion, adhesion molecule (Mac-1) expression and ROS production with different *in vitro* cellular models.

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研究方法：

Animal preparation

The present investigation conforms 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 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. To study the protective effect by honokilol (HK) on cerebral infarction in rats subjected to focal cerebral ischaemia (FCI) injury, rats were prepared as in our previous report [9]. In brief, each rat was anesthetized with halothane (1-3.5 % in a mixture of 70% N_2O and 30% O_2) with the use of a mask. Then, rat was fixed on a table lined by an automatic heating pad

connected to a sensor at the tip of rectal thermometer to keep body temperature of the rat around 37 ±0.5℃. The right femoral artery of the rat was cannulated with PE-50 polyethylene catheters for continuous monitoring of heart rate and blood pressure by Statham P23 XL transducer displayed on a Gould RS-3400 physiological Recorder (Gould, Cleveland, OH, USA). After anterior nuchal midline incision, bilateral common carotid arteries (CCAs) were carefully separated without hurting vagus nerves. A 1.5 cm midline incision was then made on the scalp of right side temporal area and connective tissue over the temporal bone was dissected. Using a dental drill under a Wild M32 type-S microscope (Heerbrugg, Switzerland), a 2.5 mm burr hole was made on the temporal bone 5 mm lateral to the sagittal suture and 7 mm caudal to the coronal suture, over cerebral cortex supplied by right middle cerebral artery (RMCA). The drill pit was constantly cooled by normal saline to keep the temperature of operation field at that of the ambient and avoid any possible physical damages of the cerebral cortex created by the surgical procedures. RMCA became visible after the thin bone layer and dura within the burr hole were carefully removed. It was ligated as proximal as possible with an ophthalmic 10-0 Dermalon surgical nylon thread (Davis-Geck, Gosport, Hamshire, U.K.) to interrupt blood flow supplying the RMCA territory. Immediately after ligation of the RMCA, both CCAs were cilpped with Diffenbach's micro-clip (ST-M06, 6 mm, Klappenecker Co, Germany) for 1 hour to produce cortical infarction. The wound of temporal area was closed gently 60 minutes later and both clips in CCAs were removed to restore cerebral blood flow. Twenty-four hours after the surgery, rats were sacrificed under anesthesia. During the entire experiment, the blood gas data, including pH, PO_2, PCO_2, and physiological data like blood pressure and heart rates of the animals were not interfered by these surgical procedures as in our previous report [9].

Experimental design

Two protocols for honokiol (HK; Nacalai Tesque, Kyoto, Japan) treatment were established. In the pretreatment experiment, HK solution was given intravenously via right femoral vein 15 minutes before RMCA ligation. HK solutions of 0.3 ml were administered at three different doses (0.01, 0.1 and 1.0 μg/kg). While in the treatment experiment, the same dosage of HK solution was given 60 minutes after RMCA ligation followed by immediate release of clips in bilateral common carotid arteries. HK was dissolved in propylene glycol then diluted with normal saline to the desired concentrations. Final concentration of propylene glycol in the injected HK solution was 0.004 % (v/v). At this concentration, propylene glycol has no effect on the infarct size of focal cerebral ischaemia (FCI). Rats injected with 0.3 ml normal saline containing 0.004 % (v/v) of propylene glycol were used as control. Animals were randomly allocated to each drug treatment and control groups.

Evaluation of leukocyte infiltration in the brain tissue

Myeloperoxidase (MPO) activity was used for quantitative assessment of leukocyte infiltration into ischaemic tissue [13]. We measured MPO activity based upon Bani’s method [14]. Briefly, frozen samples of brain tissue weighing approximately 100 mg were homogenized in 1.5 ml of potassium phosphate buffer (PPB, 50 mM, pH 6). One ml of the homogenate was centrifuged at 10,000 × g for 10 min, and the pellet was suspended in 1 ml of PPB containing 0.5% hexadecyltrimethylammonium bromide (Sigma) to negate the pseudoperoxidase activity of hemoglobulin and to solubilize membrane-bound MPO. The suspensions were treated with three cycles of freezing and thawing, sonicated on ice for 10

seconds, and centrifuged at 12,000 × g for 10 min. MPO activity was determined in the supernatants. Briefly, 0.1 ml of the supernatant was mixed with 2.9 ml of PPB containing 0.19 mg/ml of *o*-dianisidine chloride and 0.0005% hydrogen peroxide (H_2O_2) as a substrate for MPO. Oxidized *o*-dianisidine forms a soluble chromophore absorbing at wavelength of 460 nm. The absorbance (OD_{460}) was then determined by spectrophotometry over 2 min. The values of tissue MPO activity (MPOA) were expressed as OD_{460}×100/mg of protein. Protein concentration was determined with a BCA kit (Pierce, Rockford, USA). Data are expressed as percentages (%) of control (FCI) by calculating the MPOA of HK-treated group over that of control.

Leukocyte preparation

Peripheral whole blood was collected from Long-Evans rats (250 to 350 grams) with 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. Leukocyte-rich (upper) layer 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) [15]. The leukocytes were mixed with various concentrations of HK or solvent control (vehicle, 0.05% DMSO) in Hank´s balanced saline solution (HBSS) for 10 min at 37 °C.

Measurement of leukocyte adhesion in response to PMA or fMLP stimulation

Isolated leukocytes were preloaded with 1 μM of 2',7'-bis-(2-carboxyethyl)-5,6- carboxyfluo-rescencein acetoxymethyl ester (BCECF-AM; Molecular Probes) and incubated for 30 min at 37°C, following which the cells were pelleted and washed twice with cold PBS, then resuspended in HBSS before use. Adhesion of leukocyte was performed in flat-bottom 24-well tissue culture plates (Costar, Cambridge, MA, USA) pre-coated with fibrinogen as described previously [16]. Two hundred μl per well of BCECF-labeled leukocytes (5×10^5 cells/ml in HBSS) were plated into individual wells. After stimulation with PMA (0.1 μg/ml) or fMLP (1 μM) for 30 min at 37°C, non-adherent cells were removed by aspiration and the wells were gently washed twice with warm PBS. Adherent leukocytes were determined by measuring the fluorescence using the fluorescent plate reader (Cytofluor 2300, Millipore®) with excitation at 485 nm and emission at 530 nm. Data are expressed as fluorescence intensity.

Determination of Mac-1 upregulation of leukocytes by flowcytometry

Upregulation of Mac-1 was analyzed as in our previous study [16]. Briefly, HK-pretreated leukocytes (2×10^6/ml in HBSS) were stimulated with fMLP (1 μM) or PMA (0.1 μg/ml) for 20 min. The cells were then pelleted and resuspended in 1 ml ice-cold PBS containing 10% heat-inactivated fetal bovine serum (FBS) and 10 mM of sodium azide (NaN_3). For staining of Mac-1, all subsequent steps were carried out in an ice bath. Cells were incubated in the dark for 60 min with a proper aliquot of fluorescein isothiocyanate (FITC)-conjugated anti-Mac-1 antibody (mouse anti-human CD11b, class IgG_1; BD Biosciences Pharmingen) or a non-specific mouse antibody (class IgG_1, Sigma) as a negative control. After two washes with PBS containing 5% FBS, stained cells were resuspended in flow cytometer sheath fluid (Becton Dickinson) containing 1% of paraformaldehyde and analyzed by a flow

cytometer for Mac-1 expression. Data were expressed as mean channel fluorescence for each sample as calculated by the CellQuest® software (Becton Dickinson) on a Power Macintosh 7300/200 computer.

Measurement of extracellular $O_2^{\cdot J}$ (superoxide anion) generation

Extracellular $O_2^{\cdot -}$ generation was evaluated according to Korchak's method [17]. PMA (2.5 µg/ml)- or fMLP (1µM)-induced production of $O_2^{\cdot -}$ by leukocytes was determined in the presence or absence of 1-10 µM honokiol (HK) as superoxide dismutase-(SOD) inhibitable cytochrome *c* (80 µM) reduction by measuring the changes of absorbance at 550 nm ($\ddot{A}OD_{550}$) in the presence or absence of SOD (133 U/ml) at 37°C for 1 hour. Staurosporine (STAU), a protein kinase C (PKC) inhibitor, was included as positive control for the inhibition of extracellular $O_2^{\cdot -}$ production. Data were expressed as $O_2^{\cdot -}$ nmol/2×10^5cells/hour using the molar extinction coefficient of 2.1×10^4 L/mole/cm for cytochrome *c*, with a path length of 8 mm.

Flow cytometric analysis of intracellular ROS production

Intracellular accumulation of ROS by leukocytes was measured by a flow cytometer (FACSCalibur™; Becton Dickinson) according to our previous report [18]. Briefly, leukocytes (2×10^6/ml) were incubated at 37℃ for 20 min with 20 ì M of 2',7'-dichlorofluorescin diacetate (DCFH-DA). The acetate moieties of DCFH-DA were cleaved off intracellularly by esterases, liberating the membrane impermeable 2',7'-dichlorofluorescin (DCFH), which fluoresced when oxidized to 2',7'-dichlorofluorescein (DCF) by H_2O_2. After labeling, cells were treated with 1 or 10 ì M of honokilol (HK) or 0.2 ì M staurosporine (STAU, a PKC inhibitor) for 20 min, then stimulated with fMLP (1ì M), PMA (100 ng/ml). Production of intracellular H_2O_2 was then determined 30 min later by a flow cytometer (FACSCalibur™; Becton Dickinson) for measurements with emission at 525 nm (FL1) for DCF. Data are expressed as mean channel fluorescence for each sample as calculated by the CellQuest® software (Becton Dickinson) on a Power Macintosh 7300/200 computer.

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 FCI-induced leukocyte infiltration in the brain

HK (0.01-1.0 µg/kg) dose-dependently ameliorated FCI-induced infarction in the brain by 20-70% with comparable potency as in our previous report [9]. The infarction was correlated with an increase in leukocyte infiltration (measured as increased MPO activity) in the brain tissue. MPO activity in FCI group was five fold (509.8±95%) that of in the control group. However, pretreatment or treatment of HK at concentrations of 0.1 or 1.0 µg/kg both significantly decreased the leukocyte infiltration in the brain (Fig. 1) (Dunnett's test, n=6, $P<0.05$).

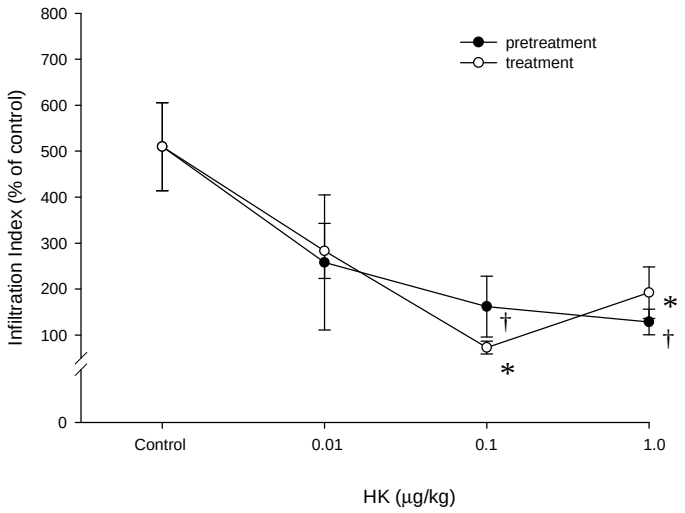

Fig. 1. Effect of honokiol (HK) on FCI-induced leukocyte infiltration in the rat brain. Long Evans rats were subjected to focal cerebral ischaemia (FCI) injury in the absence or presence of HK (0.01 to 1.0 µg/kg) by pretreatment ( ) or treatment ( ). Leukocyte infiltration (Infiltration Index) in the brain was determined by MPO activities. Data are expressed as percentage (%) of control (FCI only). In FCI rats, MPO activity ($100\times OD_{460}$/mg protein), was 509.8±95.6. $^{*,\dagger}P<0.05$ as compared with the FCI only group (HK free) by Dunnett's test.

Effect of HK on fMLP- or PMA-induced adhesion by peripheral leukocytes

Leukocyte firm adhesion, a function closely related to leukocyte recruitment and infiltration, was examined by an *in vitro* assay system using fMLP (1 ì M) or PMA (0.1 ì g/ml) as leukocyte adhesion stimulants. FMLP or PMA caused up to 250% enhancement in leukocyte adhesion in comparison with background levels (Fig. 2). Pretreatment of leukocytes with 0.1 to 10 µM of HK significantly inhibited leukocyte adhesion induced by PMA, therefore 1 and 10 µM of HK significantly inhibited leukocyte adhesion induced by fMLP (Fig. 2).

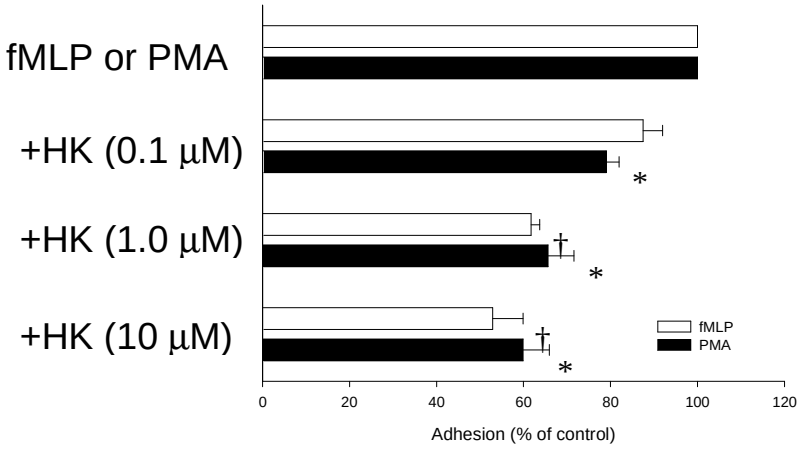

Fig. 2. Effect of HK on fMLP- or PMA-induced adhesion of peripheral leukocytes. Leukocytes were isolated and loaded with BCECF-AM (1 µM). BCECF-labelled neutrophils (5×10^5 cells /ml) were plated on fibrinogen-coated 24-well plates and stimulated with fMLP (1 µM) or PMA (0.1 µg/ml) for 30 min. Non-adherent cells were removed and adherent leukocytes were determined by measuring the fluorescence on a fluorescent plate reader. Data are expressed as percentages (%) of control (PMA or fMLP). $^{*,\dagger}P<0.05$ as compared to PMA or fMLP, respectively.

Effect of HK on fMLP- or PMA- induced Mac-1 expression by peripheral leukocytes

To examine whether HK could modulate adhesion molecules involved in the firm adhesion of leukocytes, Mac-1 expression levels on leukocytes surfaces was measured after fMLP- or PMA-stimulation via flow cytometric analysis. FMLP or PMA caused a marked increase in Mac-1 expression to 1.5 or 1.9 fold more than that of resting cells, respectively. Pretreatments with 1 or 10 ì M of HK slightly (5%) decreased the Mac-1 up-expression induced by PMA but not that of fMLP (Fig. 3).

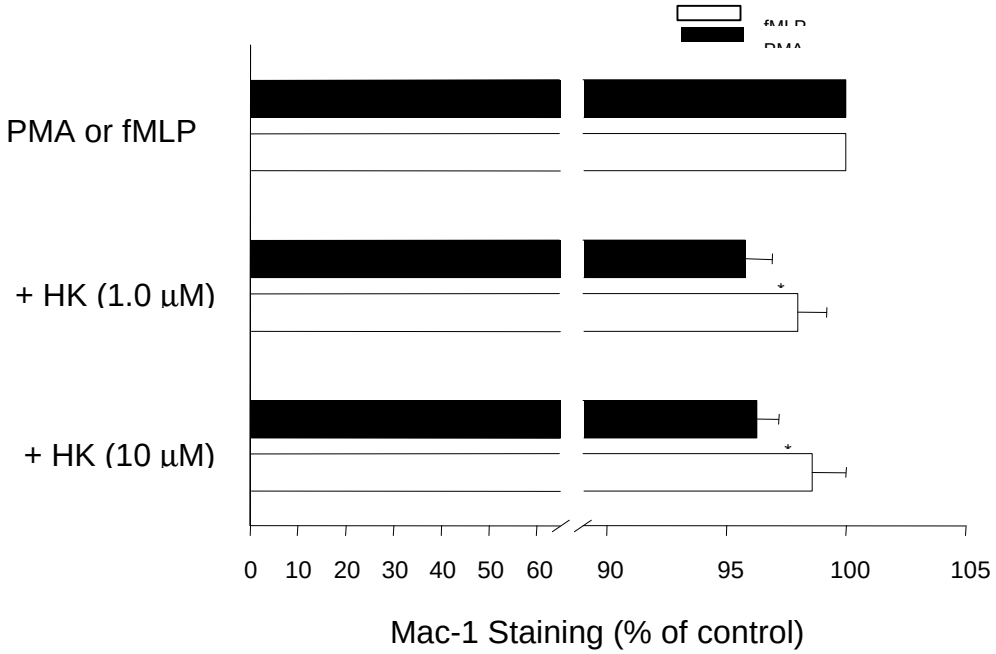

Fig. 3. Effect of HK on leukocyte surface Mac-1 upregulation induced by fMLP or PMA. Surface levels of Mac-1 were measured by staining with FITC-conjugated anti-rat CD11b and analysed on a flow cytometer (FACSort®) by gating neutrophils from total leucocytes. Data are expressed as percentages (%) of control (PMA or fMLP) by calculating the mean channel fluorescence intensity of HK-treated group over that of control. $^{*}P<0.05$ as compared to PMA.

Effect of HK on fMLP- or PMA-induced extracellular $O_2^{\cdot J}$ (superoxide anion) production

Both fMLP- and PMA-induced extracellular $O_2^{\cdot -}$ accumulation were concentration -dependently inhibited by HK as shown in Table 1. Staurosporin, a protein kinase C (PKC) inhibitor, used in this test as the positive control, also significantly inhibited PMA- or fMLP-induced extracellular accumulation of $O_2^{\cdot -}$ (Table 1).

Table 1. Effect of HK on fMLP- or PMA-induced extracellular $O_2^{\cdot -}$ production

	Extra-cellular ROS production (% of control)	
Control (Vehicle)	100% (fMLP)	100% (PMA)

+ HK (1.0 μM)	83.0±2.4%†	87.1±0.89%*
+ HK (10 μM)	65.0±1.2%†	68.2±0.74%*
+ STAU (0.5 μM)	23.4±0.32%†	16.6±0.65%*

Data are expressed as % of control (fMLP or PMA alone). Values represent the means±S.E.M. of six experiments. Production of $O_2^{\cdot -}$ (nmol/2×10^5 cells/hour) in fMLP or PMA was 0.8±0.3 or 1.2±0.3 , respectively. $^{*,\dagger}P < 0.05$ as compared to samples treated with fMLP or PMA alone, respectively.

Effect of HK on fMLP- or PMA-induced intracellular ROS production by leukocytes

FMLP- or PMA-triggered prompt accumulation of intracellular H_2O_2 (measured as increased DCF fluorescence) by 4.8 or 4.3 fold more than that of resting, respectively (Table 2). HK concentration-dependently decreased the production of intracellular H_2O_2 induced by fMLP or PMA. Staurosporin (0.2 μM) completely abolish the intracellular ROS production by fMLP or PMA.

Table 2. Effect of HK on intracellular ROS production

	Intracellular ROS production (Mean channel fluorescence/2x10^6 cells /30 min)	
	fMLP-activated	PMA-activated
Control (Vehicle)	10.0±0.57*	9.9±0.18†
Agonist alone	48.3±4.0	43.5±3.4
+ HK (1 μM)	38.3±3.7*	42.2±4.1
+ HK (10 μM)	30.6±4.0*	31.5±2.6†
+ STAU (0.2 μM)	13.0±1.0*	12.9±0.48†

Values represent the means±S.E.M. of six experiments. $^{*,\dagger}P < 0.05$ as compared to samples treated with fMLP or PMA alone, respectively.

討論：

Infiltration of leukocytes to the infarct areas following FCI plays a crucial role in the development of cerebral infarction and neuronal damage [19, 20]. This begins with enhanced adhesion of peripheral leukocytes to the endothelium, as primed and activated by proinflammatory stimuli from the ischaemic core region, and subsequent transmigration of leukocytes to injury sites where they release ROS and proteolytic enzymes that mediate FCI injury [10,21,22]. In our study, the results demonstrated that rats subjected to RMCA and CCAs occlusion followed by subsequent reperfusion developed cerebral infarction marked with leukocyte infiltration to the infarct area. However, in rats pretreated or treated with intravenous 0.1 and 1.0 μg/kg HK, cerebral infarction was 20-70% reduced as in our previous study [9]. This ameliorating effect of HK was accompanied with significant impediment of leukocyte infiltration into the cerebral foci, as well as the decrease of MPO activity in brains administered with HK observed in this study.

When tissues suffer from ischaemia and reperfusion, inflammatory mediators,

such as cytokines (e.g., tissue necrosis factor), inflammatory factors (e.g., cyclooxygenase), vasoactive factors (e.g., interleukine-6), and ROS (e.g., superoxide anion, hydrogen peroxide) are produced within minutes to hours by the ischaemic tissue through which they alter the functional activities of local resident cells (e.g., microglia) as well as peripheral leukocytes by priming them for subsequent activation and infiltration in response to these inflammatory mediators[21]. Once primed and activated, these leukocytes up-express their surface adhesion molecules, such as Mac-1 (CD11b/CD18) [23], resulting in enhanced adhesion to endothelial cells [22]. Therefore, enhancement of leukocyte adhesion molecule expression and leukocyte endothelial adhesion play a pivotal role in mediating the recruitment and infiltration of leukocytes [24, 25]. In this study, leukocyte adhesion induced by PMA or fMLP was both significantly inhibited by HK indicating that the receptor is not the direct target site of HK. The anti-adhesive effect of HK was not due to cytotoxic effect because under these conditions there was no difference in viability between HK-treated leukocytes and control (solvent only) cells.

Activation and degranulation of leukocyte can up-regulate the expression of Mac-1, a beta 2 integrin predominantly mediating the firm adhesion of leukocytes to endothelium [26]. Since antioxidants abolish Mac-1 dependent leukocyte adhesion [18], we hypothesized that the *de novo* production of ROS by activated leukocyte may enhance the leukocyte adhesion through up-regulation of Mac-1, which could be diminished by HK. To further understand whether adhesion molecules were modulated by HK, especially up-expression of Mac-1 was examined. Although Mac-1 up-regulation was only slightly prevented by HK in our study, it is possible that HK can interfere with the expression of an activation-induced specific epitope on Mac-1 or block an unknown process that is permissive for Mac-1-dependent adherence, mechanisms that were proposed by Endemann et al. [27]. Besides, we also have previously demonstrated that modulation of PKC-dependent activation [28] or impediment to calcium influx [16] can account for the inhibition of Mac-1 dependent leukocyte adhesion. Whether these signaling pathways can be targeted by HK is currently under investigation by our laboratory.

Ischaemic reperfusion injury can generate chemotactic activity and subsequently activate leukocytes to produce inflammatory mediators such as ROS that not only damage tissue but also promote leukocyte adhesion and infiltration in injury sites [29]. In our study, PMA and fMLP both triggered marked ROS production extracellularly and intracellularly by leukocytes. We found that HK can prevent both the extracellular and intracellular ROS production induced by PMA or fMLP illustrating that a non receptor-related mechanism might mediate its anti-oxidative effect. It has been demonstrated that ROS acts as signaling molecules in modulating inflammatory response [18, 30], leukocyte Mac-1 expression and leukocyte endothelial adhesion [24, 25]. We postulate that the possible mechanisms of improving FCI injury by HK might rely on its anti-oxidative potential and its capacity to inhibit leukocyte activation/adhesion and subsequent infiltration into infarct areas.

In conclusion, our study showed that HK is effective in protecting rats from cerebral infarction through limiting the activation/infiltration of leukocytes to FCI injury sites by down-regulating their adhesion and ROS production, in turn, interfering with their transmigration and subsequent generation of deleterious products that eventually lead to permanent damage of the brain. Our study supports that HK may be clinically beneficial in the prevention or treatment of FCI related diseases.

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中的第二年計劃，並已經將研究成果攥寫成論文，目前正在投稿中，靜待結果。