Mechanisms of lumbrokinase in protection of cerebral ischemia

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ARTICLE INFO

ABSTRACT

The present study was designed to explore the mechanisms involved in the anti-ischemic action of lumbrokinase (LK) in brain. The enzyme immunoassay, spectrofluorimeter and flow cytometry were used to detect the level of adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP), the Ca^{2+} mobilization, and human platelet surface antigen expression in order to elucidate the anti-platelet action involved in LK cerebroprotection. RT-PCR and western blot were used to identify the role of Intercellular adhesion molecule-1 (ICAM-1) and Janus Kinase1/Signal Transducers and Activators of Transcription1 (JAK1/STAT1) pathway in protecting brain against ischemic injury by anti-thrombosis and anti-apoptosis. Results showed that LK significantly potentiated the activity of adenylate cyclase (AC), increased the cAMP level in vivo, remarkably inhibited the rise of rat platelet intracellular Ca^{2+} ($[Ca^{2+}]_i$), and attenuated the expression of Glycoprotein IIB/IIIA (GPIIB/IIIA) and P-selectin in human platelet stimulated by thrombin in vitro. Furthermore, the expressions of ICAM-1 and JAK1/STAT1 were remarkably regulated by LK in Human Umbilical Vein Endothelial Cell (HUVEC) and ischemic cerebral tissues. These data indicated that the anti-ischemic activity of LK was due to its anti-platelet activity by elevating cAMP level and attenuating the calcium release from calcium stores, the anti-thrombosis action due to inhibiting of ICAM-1 expression, and the anti-apoptotic effect due to the activation of JAK1/STAT1 pathway.

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1. Introduction and background

Ischemic cerebrovascular disease and clinical manifestation of acute arterial thrombosis are the most common causes of mortality and morbidity (Strong et al., 2007). After injury of focal cerebral ischemia, many events happened such as platelet activation and aggregation, activation of Janus protein tyrosine kinases (JAK)/Signal Transducers and Activators of Transcription (STAT) signaling pathway, Ca^{2+} overload and high expression of adhesion molecule in endothelial cells (Mark, 2005; Bhatt and Topol, 2003). It is concluded that multiple mechanisms might attribute to cerebral ischemia, so agents that can affect these events have been extensively researched and developed as potential therapies for both the treatment and prevention of cerebral ischemia.

Lumbrokinase (LK) extracted from the earthworm has been used to treat stroke and cardiovascular diseases (Jin et al., 2000). It was firstly extracted and named lumbrokinase by Mihara et al (Mihara et al. (1989). LK is a group of proteolytic enzymes with molecular weight from 25 to 32 kDa (Cho, 2004), which includes plasminogen activator and plasmin (Matsuba, 2004). Recent studies (Dong et al., 2004; Jin et al., 2000) have shown that the fibrinolytic enzymes could dissolve blood fibrin clots and inhibit the platelets activation and aggregation. However, most of studies

(Hu et al., 2004; Yuan et al., 2006) mainly focused on the purification, physical/chemical properties, and clinical application of earthworm fibrinolytic enzyme from Lumbicus rubellus, Eisenia foetida or Lumburcus bimastus. There were fewer studies reported on the pharmacological mechanisms of LK till now. So the purpose of this study was to determine the effect and mechanism of LK on cerebral ischemia in rats.

LK was prepared from the fresh earthworms after abstraction and depuration. Based on enzymatic characteristics in fresh earthworms, crude products were made through the following method that enzymes material in earthworm homogenate was fully extracted with the solvent of alkalescent salt solution followed by salt fractionation and dislysis-dialysis. Refined products were made through further abstraction, depuration and removal of inactive protein. Following freeze drying, LK was obtained. LK used in this research was provided by Harbin Jiuxin Science & Technology Industrial Co., Ltd., which batch number was 20050818. As an injectable power, it is in the process of clinical trial.

Previous study (Justicia et al., 2000) has shown that JAK/STAT signaling pathway is involved in the neuronal response to focal cerebral ischemia. Furthermore, the adenosine 3′, 5′-cyclic monophosphate (cAMP) and guanosine 3′, 5′-cyclic monophosphate (cGMP) levels are critical factors of platelet activation. The increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) is a characteristic feature of platelet aggregation and is believed to play an important role as a second messenger for platelet activation (Katoh et al., 2000). Intercellular adhesion molecule-1 (ICAM-1) expression in human umbilical vein endothelial cell (HUVEC) might be an important indicator of

atherosclerosis (Lee et al., 2004). Meanwhile, P-selectin expression on platelets may result in monocyte expression of tissue factor, the major initiator of the coagulation cascade, Glycoprotein IIB/IIIA (GPIIB/IIIA) expression on platelets also affects the endothelial function (Steinhubla et al., 2007). Therefore, our experiments were also designed to study the mechanisms of LK in the protection of cerebral ischemia from above aspects in vitro and in vivo.

2. Materials and methods

2.1. Chemicals and reagents

LK (Harbin Jiuxin Science & Technology Industrial Co., Ltd., Batch number was 20050818); cAMP and cGMP ELISA kit (Cayman Chemical Co.); Trizol, RT-PCR kit, Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, Fetal Bovine Serum (FBS), Platinum *Taq* DNA Polymerse (Invitrigen); PageRuler™ Prestained Protein Ladder Plus (#SM0671) (Fermentas); thrombin, ethylenediamine tetraacetic acid (EDTA), medium-131, endothelial cell growth supplement, heparin, 2,3,7-triphenyltetrazolium chloride (TTC) (Sigma Chemical Co.); Polyvinylidene fluoride (PVDF) membrane (Millipore); Fura-2/AM (Beyotime Institute of Biotechnology), collagenase II (Gibco); 25 cm^2 flasks (Corning Incorporated, Corning, N.Y.); ICAM-1 mouse monoclonal antibody, mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Inc); Rabbit anti-Factor VIII (ZSGB-BIO); Apyrase (BioLabs. InC); Ethyleneglycol bis (2-aminoethyl ether) tetraacetic acid (EGTA), Hepes, Trypsin (1:250), Bovine Serum Albumin (BSA), Diethyl pyrocarbonate (DEPC) (Amresco); Dimethyl sulfoxide (DMSO), Dithiothreitol (DTT) (Solarbio); Proteaseinhibitor (Shanghai Biocolor BioScience & Technolgy company); P-section, GPIIB and GPIIIA mouse monoclonal antibody (Haematology Research Institute of Jiangsu Province); Urokinase (UK) (Tiane Pharmaceutical of Harbin High Tech (Group) Co. Ltd); Other reagents were of analytical grade.

2.2. Animals

Adult Male Wistar rats (300 ± 20 g) were purchased from Laboratory animal center (The Second Affiliated Hospital of Harbin Medical University, China). The animals had free access to a commercial pellet diet and drinking water before experiments. All procedures performed in this experiment have previously been approved by the School of Medical Science, Harbin Medical University and all efforts were made to minimize suffering and the number of rats used.

2.3. Middle cerebral artery (MCA) occlusion

The procedure for MCA occlusion was described in detail previously (Kusaka et al., 2004). Animals were fasted overnight with access to water before surgery. After the rats were anesthetized with chloral hydrate (0.35 mg/kg, i.p.), a paraffin wax-coated fishing thread (overall diameter 0.28 mm) was aseptically inserted from the right common carotid artery 2×10^{-3} m distal to the carotid bifurcation. The distal end of the fishing thread was placed in the internal carotid 17.5 ± 0.5 mm above the bifurcation to block the blood flow to the right middle cerebral artery. The wound was closed and the animal returned to its cage. Rectal temperature was maintained at 37 ± 0.5 °C with a heating pad until recovery from anesthesia. Control rats were sham operated by omitting only the occlusion. After occluding the MCA for 2 h, the operator carefully removed the fishing thread to restore blood flow and then sutured the skin and allowed the rat to wake up.

2.4. Identification of cerebral infarction and evaluation of neurological deficit after MCA occlusion in rats

The neurological deficits were tested by single blind method after 24 h of reperfusion. Menzies et al. (1992) developed the method for testing behavioral deficits on a cumulative scale from 0 to 4. This examination was used to evaluate ischemic injury: 0, no visible neurological deficits; 1, forelimb flexion; 2, contralateral forelimb grips weakly; 3, circling to the paretic side only when pulled by the tail (the animal was allowed to move about freely); and 4, spontaneous circling. In this experiment, all rats after MCA occlusion scaled 3 were chosen. Rats of MCA occlusion model were decapitated on hour 24 after operation. The coronal sections were obtained by cutting the brain at a distance of 2, 4, 6, 8, and 10 mm from the extremity of the frontal cortex, followed by immersed in a 1% solution of TTC. The white-colored areas represented the infarction regions in these sections. The stained slices were then fixed by immersion in phosphate-buffered 4% paraformaldehyde.

2.5. Experimental design

Rats, weighting 300–320 g, were divided into six experimental groups. Group I: sham-operated group, neither MCA occlusion nor reperfusion, and decapitation on 24 h after operation; Group II: model group, MCA occlusion for 2 h followed by reperfusion for 24 h, and decapitation on 24 h after reperfusion; Group III–V: LK treatment groups, MCA occlusion for 2 h followed by reperfusion, then treatment with LK by the dose of 300 μg/kg/d, 600 μg/kg/d, 1200 μg/kg/d respectively, once daily and for 3 days. The first treatment was within 4 h after reperfusion. Rats were decapitated on the 6 h after the last treatment; Group VI: UK treatment group, used as a positive control group. MCA occlusion for 2 h followed by reperfusion and treatment with UK of 4×10^4 U/kg/d. The treatment time and interval dose were alike to those of LK treatment groups. All treatment drugs were dissolved in physiologic saline and administered from tail vein. Sham-operated rats were given saline only.

2.6. Preparation of rat washed platelet

Rats of different groups described above were fixed and blood anti-coagulated with sodium citrate (3.8%, 1:9 v/v) was collected from common carotid artery. Rat citrated blood was immediately centrifuged at 58.7 g for 5 min to obtain platelet-rich plasma. Washed platelet was prepared from the platelet-rich plasma by washing procedures. The platelet pellets were finally suspended in Tyrode's solution containing BSA (0.35%), of the following composition (mM): CaCl$_2$ (1), NaCl (136.8), KCl (2.7), NaHCO$_3$ (11.9), MgCl$_2$ (2.1) and glucose (10). The platelet concentration was adjusted to 3.0×10^8 platelets/ml.

2.7. Measurement of rat platelet cAMP and cGMP

Washed platelet described above was lysed by a lysis reagent, which was obtained from the supplier of the cAMP and cGMP ELISA kits. The amount of cAMP and cGMP in supernatants was measured with a cAMP and cGMP ELISA system, following acetylation of the samples as described by the manufacturer.

2.8. Measurement of rat platelet adenylate cyclase (AC) and guanylate cyclase (GC)

Washed platelet after ultrasonication was centrifuged at 11,000 g for 15 min at 4 °C. AC was into the supernatant and the GC into precipitation. The concentrations of AC and GC were measured and adjusted to 1 mg/ml. Reaction system of 200 μl included the following composition (mmol/L): GTP 0.2 or ATP 0.5, Tris–HCl 50 (pH 8.0), KCl 200, ATP 0.1, MgCl$_2$ 1, DTT 2, PMSF 1, GC or AC 50 μl. Reaction started from the mixing of reaction system at 37 °C for 15 min and concluded by 10% perchloric acid of 300 μl, following the neutralization of 1.01 mol/L KOH of 500 μl and centrifugation at 825 g for 10 min. cAMP and cGMP in supernatants was were measured with a cAMP and cGMP

EIA system. Activities of AC or GC were calculated by the following equation (Leoncini et al., 2004).

$$\text{Activity of AC} = \text{cAMP (pmol)}/\text{Content of AC (mg)} \cdot 15 \text{ min}$$

$$\text{Activity of GC} = \text{cGMP (pmol)}/\text{Content of GC (mg)} \cdot 15 \text{ min}.$$

2.9. Expression of JAK1/STAT1mRNA in brain of cerebral ischemia–reperfusion rats by RT-PCR

Total RNA was extracted from impaired cerebral cortex of different groups described above by the trizol reagent following the manufacturer's instructions. Purity of RNA was estimated by measuring OD at a ratio of 260 to 280 nm and a RT-PCR kit was used for the RT synthesis of cDNA. PCR amplification was performed with 35 cycles of 40 s at 95 °C, 40 s at 55 °C, and 1 min at 72 °C. Oligonucleotide pairs were 5′-GGCAGCCTGTCTACTCTA-3′ and 5′-TCATCGTCCTTGTAATCC-3′ for rat JAK1. A 136-bp product was expected in the reaction. Oligonucleotide pairs were 5′-CGAAGAGCGACCAGAAAC-3′ and 5′-CTGATCCAGG-CAGGCATT-3′ for rat STAT1. A 212-bp product was expected in the reaction. The primers used to amplify β-actin were 5′-GAGACCTT-CAACACCCCAGCC-3′ and 5′-AATGTCACGCACGATTTCCC-3′. A 263-bp product was expected in the reaction. PCR products (5 µl) were electrophoresed through 1.5% agarose gels and viewed under UV illumination and photographed. Data was analyzed by Bio-RAD using AutoGel software.

2.10. Measurement of intracellular Ca²⁺ ($[Ca^{2+}]_i$) in platelets induced by thrombin

Platelet-rich plasma containing apyrase (0.94 mmol/ml) of normal rats without any drugs were was prepared as described above, then washed platelet was suspended and washed in Ca^{2+}-free pH 7.4 Hepes buffer (145 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l MgSO₄, 10 mmol/l Hepes, 0.5 mmol/l Na₂HPO₄ and 10 mmol/l glucose) at a concentration of 2×10^9 platelets/ml. After addition of BSA (1.5 g/L), washed platelet was incubated for 40 min at 37 °C with fura-2/AM (5 µmol/l) dissolved in DMSO, then by centrifugation and resuspension in a Hepes buffer or Ca–Hepes buffer (120 mmol/l CaCl₂, 145 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l MgSO₄, 10 mmol/l Hepes, 0.5 mmol/l Na₂HPO₄ and 10 mmol/l glucose) at a concentration of 2×10^6 platelets/ml. Washed platelet in a Hepes buffer was respectively treated as followed: 1) incubated with thrombin of 0.5 U/ml for 4 min; 2) incubated with LK of 25 µg/ml, 50 µg/ml and 100 µg/ml for 10 min following thrombin of 0.5 U/ml for 4 min. After centrifugation, washing and suspension, $[Ca^{2+}]_i$ of different treatments were measured using a Shimazu RF-510 spectrofluorimeter. Fluorescence was measured at the emission wavelength of 510 nm, with the excitation wavelength between 340 and 380 nm. The ration of the fluorescence intensities at the two excitation wavelengths was used to determine $[Ca^{2+}]_i$ by the following equation. $[Ca^{2+}]_i = k_d (F-F_{min})/(F_{max}-F) \cdot (k_d = 224 \text{ nmol/L in physiological condition})$. At the end of the experiment the cells were treated with 0.1% Triton X-100 (v/v) followed by the addition of 10 mM of EGTA (pH 9.0) to obtain the maximal and minimal fluorescence respectively. The procedures of treating and measurement of $[Ca^{2+}]_i$ of washed platelet in a Ca–Hepes buffer was were similar to those of washed platelet in a Hepes buffer.

2.11. Culture of human umbilical vein endothelial cells

HUVECs were isolated from fresh-term umbilical cords as previously described (Lee et al., 2004). In brief, both ends of the umbilical cord were cannulated with one-way stopcocks and the lumen was perfused with PBS. The lumen was filled with PBS containing 0.1% collagenase and incubated for 15 min. The collagenase solution was flushed into conical tubes by using an equal volume of Hanks' balanced salt solution, and the endothelial cells were pelleted by centrifugation at 206 g for 5 min. The endothelial cells were resuspended in medium-131 containing 20% FBS, 20 µg/ml endothelial cell growth supplement, 90 µg/ml heparin, antibiotic solution (100 U/ml penicillin, 100 µg/ml streptomycin) and plated into 0.1% gelatin-coated 25 cm² flasks. Cells were used from passages 2 to 5 in all experiments. Isolated cells were characterized as endothelial cells by morphology and Factor VIII staining.

2.12. Expression of Icam-1mRNA of HUVEC by RT-PCR

HUVEC were grown in 25 cm² flasks and were serum-starved 24 h before experimentation. Flasks were incubated with 1) thrombin (2 U/ml), 2) LK (100 µg/ml) and thrombin (2 U/ml), 3) LK (50 µg/ml) and thrombin (2 U/ml), 4) LK (25 µg/ml) and thrombin (2 U/ml) for 1 h. Total RNA from the cells was extracted by using trizol following the manufacturer's instructions. Purity of RNA was estimated by measuring OD at a ratio of 260 to 280 nm. Total RNA (1 µg) was subjected to first-strand cDNA synthesis. PCR was carried out under the following conditions: denaturation for 30 s at 94 °C, primer annealing for 30 s at 56 °C, and primer extension for 30 s at 72 °C for 35 cycles. Oligonucleotide primers used for ICAM-1 were 5′-TATGGCAAC-GACTCCTTCT-3′ (sense) and 5′-CATTCAGCGTCACCTTGG-3′ (anti-sense) to amplify a 238-bp PCR product. Primers for β-actin were 5′-TTCAGAGGGGATCGTTGTAGAAGTC-3′ (sense) and 5′-CAAGCTTGTCGG-CATACTGTTTCAG-3′ (anti-sense) to amplify a 268-bp product. PCR products (5 µl) were electrophoresed through 2% agarose gels and viewed under UV illumination and photographed. Data was analyzed by Bio-RAD using AutoGel software.

2.13. Western blotting for ICAM-1 of HUVECS

HUVECs were grown to confluence, which were incubated with 1) thrombin (2 U/ml), 2) LK (100 µg/ml) and thrombin (2 U/ml), 3) LK (50 µg/ml) and thrombin (2 U/ml), 4) LK (25 µg/ml) and thrombin (2 U/ml) for 1 h. Treated cells were then lysed with RIPA lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% Sodium dodecyl sulphate (SDS), and 50 mM Tris, pH8.0) containing protease inhibitor followed by ultrasonic processing for 5 s twice. Proteins (40 µg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to PVDF membranes. The transferred membranes were blocked with 5% BSA in 0.1 M PBS (pH 7.40) at room temperature for 2 h. The membranes of 72–130 kDa or 30–50 kDa were immunoblotted respectively with anti-human ICAM-1 monoclonal antibody diluted 1:100 or with β-actin mouse monoclonal antibody diluted 1:1000 overnight and then washed in PBS Tween (PBST) washing buffer one time for 15 min followed by two washes for 5 min, following immunoblotting with goat anti-mouse IgG-FITC diluted 1:2000 for 1.5 h and then washed in PBST once for 15 min followed by two washes for 5 min. The sheet was then soaked into PBS, and scanned by Odyssey LI-COR (Gene Company. Limited) followed by analyzing using Odyssey Image Analysis Software.

2.14. Flow cytometric analysis of human platelet surface antigen expression

Blood was obtained by venipuncture from healthy adult volunteers who did not take any drugs for the previous 10 d. Washed platelet was isolated as previously described. Membrane expression of GPIIb/IIIa and P-selectin were analysed in washed platelet during the 3 h following the blood collection. Following fixing in 1% paraformalde-hyde at a concentration of 1×10^9 platelets for 10 min, washed platelet in Hepes buffer were respectively treated as followed: 1) incubated with thrombin of 2 U/ml for 5 min; and 2) incubated with LK of 25 µg/ml, 50 µg/ml and 100 µg/ml for 30 min followed by thrombin of 2 U/ml

Table 1
Effect of LK on cAMP, cGMP, AC and GC in washed platelet of cerebral ischemia–reperfusion rats ($\overline{X}$ ±S.D.)

Group	Dose	cAMP (×10⁻²) (pmol/10⁹plt)	cGMP (×10⁻²) (pmol/10⁹plt)	AC (pmol/mg·min)	GC (pmol/mg·min)
Sham	0	54.7±7.82[b]	48.4±5.94[b]	2.97±0.233[b]	3.10±0.252[b]
Model	0	27.2±7.29	17.7±2.99	1.78±0.515	2.05±0.244
UK	4×10⁴ U/kg	44.8±5.54[b]	28.7±7.34[b]	2.48±0.330[a]	2.46±0.400[b]
LK	1.2 mg/kg	49.8±5.60[b]	21.7±4.48	2.59±0.458[b]	2.15±0.317
LK	0.6 mg/kg	37.7±4.75[a]	22.0±3.58	2.46±0.446[a]	2.12±0.296
LK	0.3 mg/kg	31.8±5.16	21.3±3.15	1.98±0.440	2.07±0.167

Data are expression as the means±S.D. ($n=8$). Results of ANOVA showed significant difference between groups, cAMP: $F=24.167$ and $df=5$; cGMP: $F=43.077$ and $df=5$; AC: $F=8.674$ and $df=5$; GC: $F=16.126$ and $df=5$. [a]$P<0.05$ and [b]$P<0.01$ vs model group.

2.15. Statistical analysis

The experimental results are presented as mean±S.D. of at least 5 separate experiments. Statistical analysis was performed with analysis of variance (ANOVA) followed by post-hoc Student–Newman–Keuls test. The level of significance was set at $P<0.05$ or $P<0.01$.

3. Results

3.1. Verification of cerebral infarction and evaluation of neurological deficit after MCA occlusion in rats

MCA occlusion was used to study focal cerebral ischemia in rats. After MCA occlusion, the behavioral deficits were observed as shown

Fig. 1. Results of the neurological deficit in MCA Occlusion. The neurological deficits were tested after 24 h of reperfusion. Menzies developed the method for testing behavioral deficits on a cumulative scale from 0 to 4. This examination was used to evaluate ischemic injury: 0, no visible neurological deficits; 1, forelimb flexion; 2, contralateral forelimb grips weakly; 3, circling to the paretic side only when pulled by the tail (the animal was allowed to move about freely); and 4, spontaneous circling. In this experiment, all rats after MCA occlusion scaled 3 were chosen. Rats of MCA occlusion were decapitated on hour 24 after operation. The coronal sections were obtained by cutting the brain at a distance of 2, 4, 6, 8, and 10 mm from the extremity of the frontal cortex, followed by immersion in a 1% solution of TTC. The white-colored areas represented the infarction regions in these sections. The stained slices were then fixed by immersion in phosphate-buffered 4% paraformaldehyde. A. Circling to the paretic side and scaled 3; B. Contralateral forelimb gripped weakly and scaled 1. C. Infarction size of the brain in LK treatment groups. From left to right model group, sham operation group, LK (1.2 mg/kg) group, LK (0.6 mg/kg) group, LK (0.3 mg/kg) group can be seen respectively.

for 5 min. For each condition, a 50 µl sample of final solution was then labelled with 12 µl of monoclonal antibodies (mAbs) for 30 min. The mAbs were P-section, GPIIB and GPIIIA mouse monoclonal antibody. The samples were then incubated for 30 min on ice with FITC-conjugated goat anti-mouse IgG. Finally, samples were washed twice and diluted in Hepes buffer for flow cytometric analysis. A flow cytometer (FACScan, Becton Dickinson) was used to measure immunostain fluorescence with excitation via argon laser light at 488 nm, and emissions were collected at 530 nm for FITC.

Fig. 2. Effect of LK on Janus Kinase1/Signal Transducers and Activators of Transcription1 (JAK1/STAT1) mRNA expression and β-actin in brain of cerebral ischemia–reperfusion rats by RT-PCR. A. Expression of JAK1 mRNA and β-actin mRNA. lane1: LK (1.2 mg/kg), lane2: LK (0.6 mg/kg), lane3: LK (0.3 mg/kg), lane4: UK group, lane5: model group, lane6: sham operation group. B. Expression of STAT1 mRNA and β-actin mRNA. lane1: LK (1.2 mg/kg), lane2: LK (0.6 mg/kg), lane3: LK (0.3 mg/kg), lane4: UK (4×10⁴ U/kg) group, lane5: model group, lane6: sham operation group. C represented quantification of RT-PCR corrected with β-actin and analyzed by Bio-RAD using AutoGel software. $n=6$, * $P<0.05$ and ** $P<0.01$ vs with the model group.
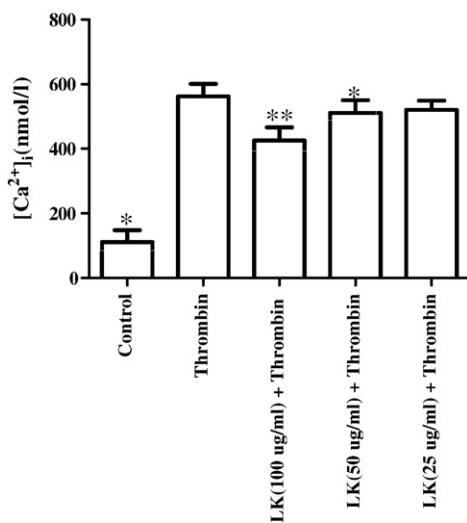
Fig. 3. Effect of LK on Ca^{2+} mobilization in rat platelet induced by thrombin. Fura-2-loaded platelets were preincubated with LK for 40 min and then stimulated with thrombin (0.5 U/ml) in the presence of $CaCl_2$ (1 mM), and the change in $[Ca^{2+}]_i$ was calculated from the fluorescence values obtained at 4 min after the addition of thrombin. Data is expression as the means±S.D. $n=6$, * $P<0.05$ and ** $P<0.01$ vs with the model group.

in Fig. 1A and B and the rats scaled 3 were selected in our experiment. Meanwhile, the images of brain sections of different treatment groups were shown in Fig. 1C with 1% TTC. White-colored areas represented the infarction regions in these sections.

3.2. Effect of LK on cAMP, cGMP, AC and GC of washed rat platelet

To determine whether LK inhibited platelet activation by increasing the intracellular level of cAMP or cGMP, the generation of cAMP or cGMP of different groups were was assessed. Results of ANOVA showed significant difference between groups (cAMP: $F=24.167$, degree of freedom $(df)=5$; cGMP: $F=43.077$, $df=5$). As shown in Table 1, the cAMP and cGMP levels of washed platelet in model rats were significantly lower than those in sham group ($P<0.01$). LK (0.6 and 1.2 mg/kg) and UK resulted in remarkable increase of the cAMP levels compared with model group. And there is no significant difference between UK group and LK (1.2 mg/kg) group ($P>0.05$). However, there was no remarkable difference of the cGMP levels treated with LK (0.3, 0.6 and 1.2 mg/kg) compared with model group.

The cAMP and cGMP levels were regulated by AC or GC, so AC and GC in washed platelets were also measured. Results of ANOVA showed significant difference between groups (AC: $F=8.674$, $df=5$; GC: $F=16.126$, $df=5$). As shown in Table 1, the activities of AC and GC in model group were lower compared with that of sham group ($P<0.05$), LK (0.6 and 1.2 mg/kg) and UK resulted in significant increase of AC levels ($P<0.05$) compared with model group. And there is no significant difference between UK group and LK (1.2 mg/kg) group ($P>0.05$). However, LK (0.3, 0.6 and 1.2 mg/kg) had no significant effect on the activity of GC compared with that of the model group.

3.3. mRNA expressions of JAK1/STAT1 of brain in cerebral ischemia reperfusion rats

RT-PCR was performed using specific primers, as described above to confirm the effect of LK on JAK1/STAT1 mRNA expression in brain of cerebral ischemia reperfusion rats. JAK1/STAT1 mRNA expressions were clearly detected as shown in Fig. 2A and B. The mRNA expression levels (Fig. 2C) of JAK1 and STAT1 were represented as the ratio of (JAK1 or STAT1)/β-actin. Results of ANOVA showed significant difference between groups (JAK1: $F=15.768$, $df=5$; STAT1: $F=6.675$,

$df=5$). The ratio of (JAK1 or STAT1)/β-actin were was significantly higher in model group (0.554±0.121, 0.941±0.0986) than those in sham group (0.299±0.0600, 0.569±0.151) ($P<0.01$, $P<0.05$). The ratios of JAK1/β-actin in LK (0.6 and 1.2 mg/kg) (0.700±0.148, 0.878±0.106, respectively) groups also resulted in remarkable increase compared with that of model group ($P<0.05$, $P<0.01$) and there is no significant difference between UK group and LK (1.2 mg/kg) group ($P>0.05$). However, the mRNA expression levels of STAT1 in LK (1.2 mg/kg) group (0.622±0.241) decreased obviously than that of model group (0.914±0.0986) ($P<0.05$) as shown in Fig. 2C.
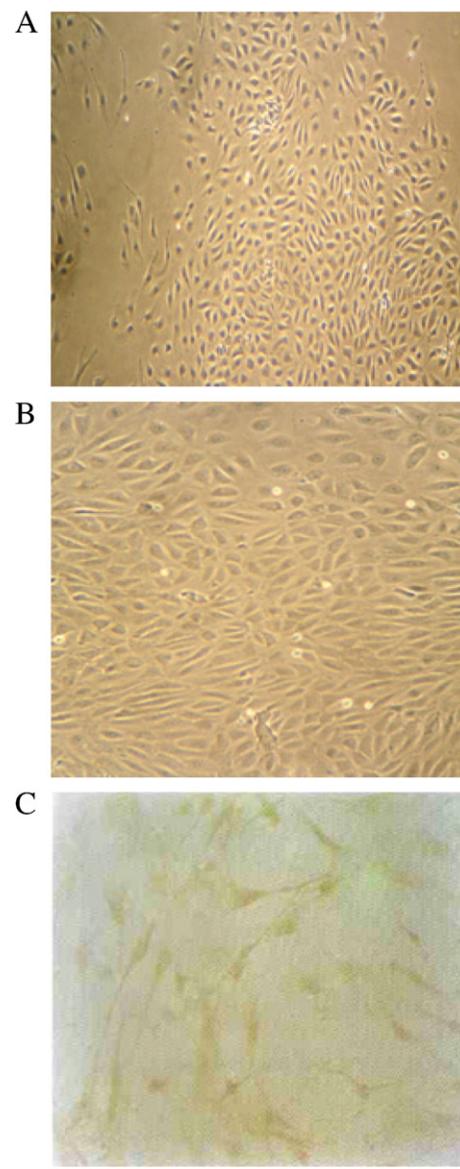
Fig. 4. Morphological appearance and identification of HUVECs. HUVECs were isolated from fresh-term umbilical cords. Both ends of the umbilical cord were cannulated with one-way stopcocks and the lumen was perfused with PBS. The lumen was filled with PBS containing 0.1% collagenase and incubated for 15 min. The collagenase solution was flushed into conical tubes by using an equal volume of Hanks' balanced salt solution, and the endothelial cells were pelleted by centrifugation at 206 g for 5 min. The endothelial cells were resuspended in medium-131 containing 20% FBS, 20 μg/ml endothelial cell growth supplement, 90 μg/ml heparin, antibiotic solution (100 U/ml penicillin, 100 μg/ml streptomycin) and plated into 0.1% gelatin-coated 25 cm^2 flasks. A. Cell proliferation began on 5th day after primary culture when endothelial cell colonies were evident (×10). B. Cell exhibited "cobblestone" appearance on 9th day after primary culture (×20). C. Monolayer of culture cells exhibited cytoplasmic localization of factor VIII antigen (×150).
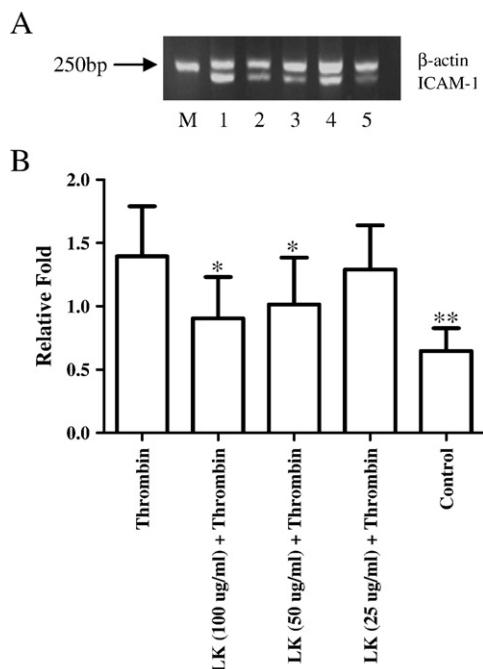
Fig. 5. Effect of LK on ICAM-1mRNA in HUVECs by RT-PCR. A. HUVECs were incubated with LK or LK+thrombin at various concentrations as indicated for 1 h. RNAs from treated cells were subjected to RT-PCR reactions using specific primer sets for human ICAM-1 or β-actin. The reaction products were separated on 2% agarose gels and photographed. lane1: thrombin (2 U/ml), lane2: LK (100 μg/ml)+thrombin (2 U/ml), lane3: LK (50 μg/ml)+thrombin (2 U/ml), lane4: LK (25 μg/ml)+thrombin (2 U/ml), lane5: control. B represented quantification of RT-PCR corrected with β-actin and analyzed by Bio-RAD using AutoGel software. Data are expression as the means±S.D. (n=7). * $P < 0.05$ and ** $P < 0.01$ vs thrombin group.

Fig. 6. Effect of LK on ICAM-1 protein in HUVECs by Western blot analysis. A. HUVECs were incubated with LK or LK LK+thrombin at various concentrations as indicated for 1 h. Total cell lysates were isolated, and ICAM-1 protein expression levels were monitored by Western blotting. lane1: thrombin (2 U/ml), lane2: control, lane3: LK (100 μg/ml)+thrombin (2 U/ml), lane4: LK (50 μg/ml)+thrombin (2 U/ml), lane5: LK (25 μg/ml)+thrombin (2 U/ml). B. Data are expressed as the means±S.D. (n=4). * $P < 0.05$ and ** $P < 0.01$ vs thrombin group.

3.4. Effect of LK on $[Ca^{2+}]_i$ mobilization induced by thrombin

$[Ca^{2+}]_i$ mobilization is a critical step in various aspects of platelet activation. The aim was to determine whether LK would inhibit thrombin-induced Ca^{2+} mobilization in rat platelet. Change of $[Ca^{2+}]_i$ in rat platelets were was measured by using the Fura-2/AM loading method by Shimazu RF-510 fluorospectro-photometer. Results of ANOVA showed significant difference between groups (F=147.35, df=4). In the presence of $CaCl_2$ of 1 mM, thrombin group (562±38.5) increased $[Ca^{2+}]_i$ significantly compared with control (111±36.4) ($P < 0.01$). Pretreatment of platelets with LK (50 and 100 μg/ml) for 10 min prior to the addition of thrombin remarkably attenuated the rise of $[Ca^{2+}]_i$ (511±38.8 and 425±40.9) ($P < 0.05$, $P < 0.01$) as shown in Fig. 3. In addition, in the absence of extracellular Ca^{2+}, the inhibition of LK on the rise of $[Ca^{2+}]_i$ induced by thrombin was also observed, which was similar to that observed in the presence of 1 mM extracellular Ca^{2+}.

3.5. Culture and identification of HUVEC

The primary cultured HUVECs started to grow attaching to flask about 1 h after inoculated. During 3–4 days, HUVEC grew the fastest, in a pattern of strict monolayer growth and contact inhibition, showing a cobblestone or pitching stone-like appearance under light microscopy, as shown in Fig. 4A and B. Factor VIII in HUVEC showed positive reaction by immunohistochemistry as shown in Fig. 4C.

3.6. Effect of LK on the mRNA expression of ICAM-1 induced by thrombin on HUVEC by RT-PCR

The mRNA expressions of ICAM-1 induced by thrombin and then treated by LK were showed in Fig. 5A. The mRNA expression level (Fig. 5B) of ICAM-1 was represented as the ratio of ICAM-1/β-actin.

Results of ANOVA showed significant difference between groups (F=5.692 and df=4). The ratio of ICAM-1/β-actin was significantly higher in thrombin group (1.39±0.396) than those of control group (0.648±0.179, $P < 0.01$). LK (50 and 100 μg/ml) resulted in the remarkable decrease of the mRNA expressions of ICAM-1 (1.01± 0.371 and 0.941±0.0986) compared with that of thrombin group ($P < 0.05$, $P < 0.05$) as shown in Fig. 5B.

3.7. Effect of LK on the protein expression ICAM-1 induced by thrombin on HUVEC by western blotting

Because mRNA levels stimulated by thrombin were decreased by LK, we further investigated whether the decreased mRNA levels of ICAM-1 were related with protein expression levels. The total ICAM-1 protein levels in LK-treated cells were detected by western blotting as showed in Fig. 6A. Results of ANOVA showed significant difference between groups (F=11.294, df=4). Proteins of thrombin group

Table 2
Effect of LK on the expression of P-selectin, GPIIB and GPIIIA in human platelet by flow cytometry ($\overline{X}$ ±S.D.)

Group	Dose	Fluorescence intensity(Ig)		
		P-selectin	GPIIB (CD41)	GPIIIA (CD61)
Control	0	29.6±2.61[b]	46.6±3.18[b]	15.8±2.31[b]
Thrombin	2 U/ml	48.2±3.66	80.1±2.88	32.9±3.20
LK+Thrombin	(100 μg+2 U)/ml	43.7±3.32[a]	67.1±3.95[a]	29.8±2.15[a]
LK+Thrombin	(50 μg+2 U)/ml	44.3±3.89[a]	78.6±2.21	30.3±1.11[a]
LK+Thrombin	(25 μg+2 U)/ml	48.6±2.73	79.1±2.57	30.7±1.31

Data are expression as the means±S.D. (n=6). Results of ANOVA showed significant difference between groups, P-selectin: F=33.455 and df=4; GPIIB (CD41): F=134.492 and df=4; GPIIIA (CD61): F=61.631 and df=4. [a] $P < 0.05$ and [b] $P < 0.01$ vs model group.
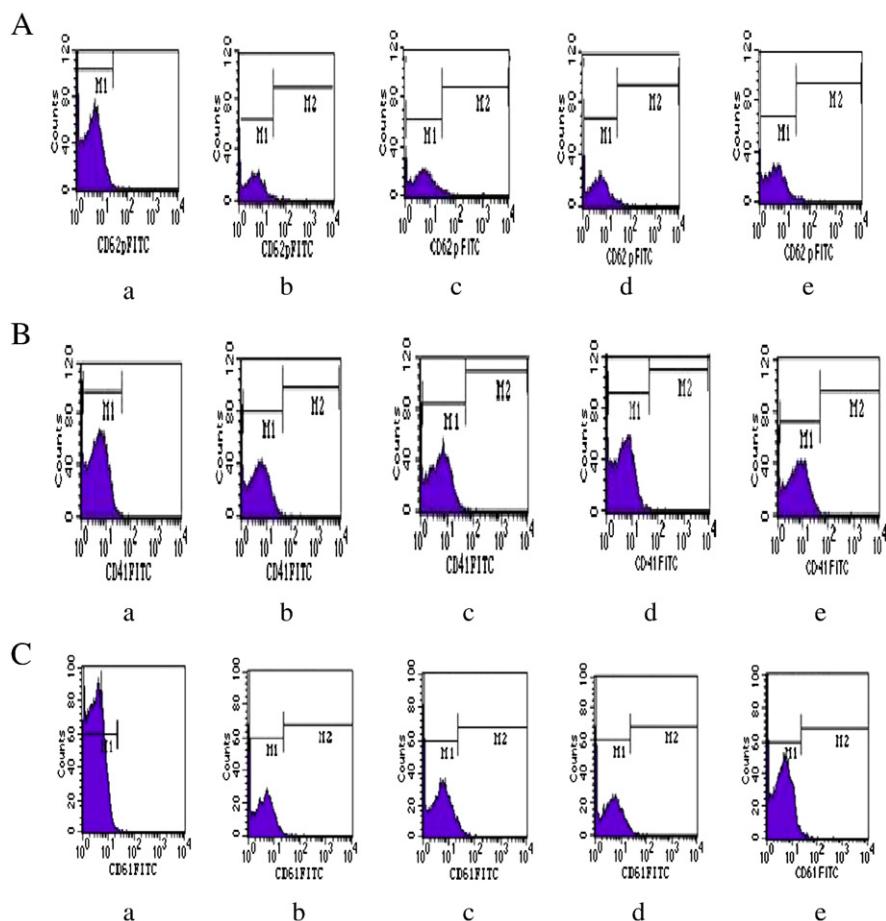
Fig. 7. Membrane expression of glycoproteins GPIIb/IIIa and P-selectin were was analysed in human washed platelet during the 3 h following the blood collection. Following fixing in 1% paraformaldehyde at a concentration of 1×10^9 platelets for 10 min, washed platelets in Hepes buffer were respectively treated as followed: 1) incubated with thrombin of 2 U/ml for 5 min; and 2) incubated with LK of 25 µg/ml, 50 µg/ml and 100 µg/ml at for 30 min followed by thrombin of 2 U/ml for 5 min. For each condition, a 50 µl sample of final solution was then labelled with 12 µl of monoclonal antibodies (mAbs) for 30 min. The mAbs were P-section, GPIIB and GPIIIA mouse monoclonal antibody. The samples were then incubated for 30 min on ice with FITC-conjugated goat anti-mouse IgG. Finally, samples were washed twice and diluted in Hepes buffer for flow cytometric analysis. A flow cytometer (FACScan, Becton Dickinson) was used to measure immunostain fluorescence with excitation via argon laser light at 488 nm, and emissions were collected at 530 nm for FITC. A. Expression of P-selectin on the surface of platelets were was determined by FCM. a, control; b, Thrombin 2 U/ml; c, d, e (Thrombin 2 U/ml + LK 25, 50 and 100 µg/ml). B. Expression of GPIIB (CD41) on the surface of platelets was determined by FCM. a, control; b, Thrombin 2 U/ml; c, d, e (Thrombin 2 U/ml + LK 25, 50 and 100 µg/ml). C. Expression of GPIIIA (CD61) on the surface of platelets was determined by FCM. a, control; b, Thrombin 2 U/ml; c, d, e (Thrombin 2 U/ml + LK 25, 50 and 100 µg/ml).

increased significantly (0.709±0.0444) compared with that of the control group (0.385±0.0919) ($P < 0.01$). LK (100 µg/ml) resulted in remarkable decrease (0.595±0.0866) compared with that of the thrombin thrombin-induced group ($P < 0.05$) as shown in Fig. 6B. Result was consistent with that of RT-PCR. There are no significant difference between the LK (25 and 50 µg/ml) group and the thrombin group.

3.8. Effect of LK on the expression of P-selectin, GPIIB and GPIIIA evoked by thrombin in human platelet by flow cytometry

The purpose was to determine whether LK would inhibit thrombin-induced platelet GPIIB/IIIA, P-selectin expression. Results of ANOVA showed significant difference between groups (P-selectin: $F = 33.455$, $df = 4$; GPIIB (CD41): $F = 134.492$, $df = 4$; GPIIIA (CD61): $F = 61.631$, $df = 4$). Their expressions on human platelet surface increased remarkably after incubating with thrombin for 30 min, as showed in Table 2 and Fig. 7A–C, whereas decreased significantly after pretreatment with LK for 30 min. LK (50 and 100 µg/ml) resulted in significant decrease on the expressions of P-selectin and GPIIIA and LK (50 µg/ml) on GPIIB compared with that of the thrombin group. It is suggested that LK inhibited expression of P-selectin, GPIIB and GPIIIA in human washed platelets evoked by thrombin.

4. Discussion

Ischemic cerebrovascular diseases lead to about 6 million deaths every year all over the world (Strong et al., 2007). Scientists are now focusing more than ever on discovering an effective anti-ischemia drug. Lumbrokinases, a group of potential fibrinolytic enzymes, are being extensively investigated. The evidence (Jin et al., 2000) demonstrated that LK was able to protect brain and vessels against ischemic injury. However, the detail mechanism was incompletely clarified. The study was therefore designed to study the unknown mechanism of LK on ischemic cerebrovascular diseases.

There was increasing evidence that platelets played crucial roles in the initiation and development of ischemic vascular diseases, and anti-platelet therapy has become the useful means for preventing or treating ischemic cerebrovascular diseases. Recent studies (Zhu et al., 2000) showed that LK benefited the body to break up and dissolving the unhealthy coagulation of blood and had potent anti-platelet activity. The anti-ischemic activity of LK may be due, at least partly, to inhibiting platelet aggregation and facilitating platelet disaggregating. However, little information relating to detailed mechanisms of LK on anti-platelet activity has been reported. In this study, the principal objective was to further determine the detailed mechanisms that the LK inhibits platelet's activity. Platelet activation was a result of a complex signal transduction

cascade reaction mediated by various stimulants (Chou et al., 2000). One important mediator in regulating platelet activation was cAMP and cGMP. Increased intracellular cAMP and cGMP level resulted in inhibition of platelet activation, adhesion, and release of granule contents (Katoh et al., 2000). Many agents exerted their inhibitory effects by increasing the intracellular concentration of cAMP or cGMP. Therefore, we initially observed whether LK inhibited platelet activity by increasing the intracellular level of cAMP or cGMP. The results clearly demonstrated that LK markedly increased platelet cAMP level and activity of AC shown in Table 1, whereas it had no significant effect on cGMP level or activity of GC. It suggested that LK inhibited platelet aggregation by elevating the intracellular cAMP level directly or potentiating the activity of AC to increase the cAMP that leads to suppression of platelet function.

Many reports (Chou et al., 1999) presented that the cAMP and cGMP level was directly mediated by cytosolic free Ca^{2+}, so we further investigated the effects of LK on intracellular Ca^{2+}. Thrombin was used to induce a 10-fold increase in cytosolic free Ca^{2+}, which is thought to facilitate subsequent events, including platelet aggregation and secretion (Chou et al., 2000). The present study showed that LK significantly attenuated the rise of $[Ca^{2+}]_i$ induced by thrombin in the presence of external Ca^{2+}. Meanwhile, the inhibition of LK on the rise of $[Ca^{2+}]_i$ induced by thrombin was also observed in the absence of extracellular Ca^{2+}. These results implied that the inhibitory action of LK on $[Ca^{2+}]_i$ mobilization mainly or at least partially due to inhibition of calcium release from calcium stores resulted in the platelet disaggregating.

JAK/STAT pathway played a crucial role to promote cell proliferation, survival and cell fate determination (Seki et al., 2000; Rawlings et al., 2004; Silver et al., 2005; Mascareno et al., 1998). It was reported that JAK1 promoted the development of procerebrum and protected neuron from injury induced by nerve cells (Justicia et al., 2000). Whether JAK/STAT signaling was also involved in the onset of ischemic injury was not known at present. So, in this study, the effect of LK on JAK1 and STAT1mRNA after MCA occlusion was determined. As shown in Fig. 7C, LK resulted in a remarkable increase on JAK1mRNA and decrease on STAT1mRNA. It was presumed that LK, acting at a certain step of the signal transduction pathway, protected ischemic injury by enhancing JAK1mRNA to conflict apoptosis and decreasing STAT1mRNA to alleviate apoptosis of neurons induced by STAT1. Through this study, the effect of LK on JAK1/STAT1 pathway in the brain of MCA occlusion rat was firstly revealed.

A large body of evidence (Lee et al., 2004) revealed that the adhesion of platelet to the vascular endothelial cells was a critical step in the ischemic brain injury. Inhibiting the excessive activation of ICAM-1 on endothelial cells has been proved be an effective way to treat atherosclerosis and cerebrovascular diseases. This study showed that LK remarkably inhibited ICAM-1 expression at both the mRNA and the protein levels in HUVEC. The activation of platelet adhesion molecules was another important pathway to prevent platelets aggregation and thrombus formation (Podolnikova et al., 2003). As two key molecules, P-selectin and GPIIb/IIIa were studied in this study. P-selectin was released from platelet granules to the surface membrane during platelet activation, where it permits platelet binding to white blood cells and to the endothelium (Zhang et al., 2003). GPIIb/IIIa complex was constitutively expressed on the platelet surface membrane and, when activated, serves as an anchoring site for soluble fibrinogen and von Willebrand factor and takes on a new three-dimensional conformational state and in doing so reveals binding sites for fibrinogen (Erlandsen et al., 2001). Present results show that LK inhibited expression of P-selectin, GPIIB/IIIA in human washed platelets evoked by thrombin to some extent, which indicated that LK inhibit platelet aggregation meanwhile through inhibition of fibrinogen binding to P-selectin and GPIIb/IIIa receptors in human platelets.

5. Conclusions and suggestions for this study may be as follows

LK may be a promising candidate for a potential anti-ischemic agent providing effective prophylactic and/or therapeutic means of treating cerebral ischemia and atherosclerosis. The anti-platelet activity may involve three mechanisms, that is, elevating significantly the intracellular cAMP level and enhancing activity of AC by inhibiting calcium release from calcium stores in vivo; protecting ischemic injury by enhancing JAK1mRNA to conflict apoptosis and decreasing STAT1mRNA to inhibit apoptosis in vivo; remarkably inhibiting expression of ICAM-1 in HUVEC to protect HUVEC from injury induced by thrombin and interfering on the interaction of fibrinogen with its receptors on platelet surfaces in vitro.

These results indicate that a novel approach of anti-ischemic activity of LK could be taken in the design of a widely clinical application.

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