Pharmacology, Biochemistry and Behavior 96 (2010) 469–475

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Baicalein is neuroprotective in rat MCAO model: Role of 12/15-lipoxygenase, mitogen-activated protein kinase and cytosolic phospholipase A2

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ARTICLE INFO

Article history:
Received 20 February 2010
Received in revised form 22 June 2010
Accepted 7 July 2010
Available online 14 July 2010

Keywords:
Baicalein
Cerebral ischemia
Neuroprotection
Lipoxygenase
Mitogen-activated protein kinase
Phospholipase A2

ABSTRACT

Inflammatory damage and oxidative stress play an important role in cerebral ischemic pathogenesis and may represent a target for treatment. Baicalein, isolated from the traditional Chinese herbal medicine Huangqin, is an antioxidant and anti-inflammatory agent on one hand and a lipoxygenase inhibitor on the other hand. However, little is known regarding the mechanism of baicalein's neuroprotection in ischemic stroke. We therefore investigated the potential neuroprotective effects of baicalein and explored the underlying mechanisms. Male, Sprague–Dawley rats were subjected to permanent middle cerebral artery occlusion (MCAO) and baicalein was administered intravenously immediately after cerebral ischemia. At 24 h after MCAO neurological deficit, brain water content and infarct sizes were measured. Immunohistochemistry, western blot and reverse transcription-polymerase chain reaction (RT-PCR) were used to analyse the expression of 12/15-lipoxygenase (12/15-LOX), p38 mitogen-activated protein kinase (p38 MAPK) and cytosolic phospholipase A2 (cPLA2) at gene and protein levels in ischemic brain cortex. The results showed that baicalein improved neurological deficit, reduced brain water content and infarct sizes, and downregulated the overexpression of 12/15-LOX, p38 MAPK and cPLA2 typically seen with MCAO. The results indicated that baicalein protected the brain from damage caused by MCAO, and this effect may be through downregulation of 12/15-LOX, p38 MAPK and cPLA2 expression.

1. Introduction

Contributing factors to the pathology following cerebral infarction include oxidative stress, neurotoxicity and excessive inflammatory response (Lakhan et al., 2009; Lo et al., 2005). Previous studies have demonstrated that oxidative stress and inflammation play an important role in the pathogenesis of cerebral infarction (Chan, 2001; De Simoni et al., 2002; Huang et al., 2006). Cerebral ischemia induces formation of reactive oxygen species (ROS) in brain tissue (Dirnagl et al., 1995). ROS can activate diverse signaling pathways, and regulate the expression of genes encoding a variety of proinflammatory proteins such as cyclooxygenases (COXs), interleukin-1β(IL-1β), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), and lipoxygenases (LOXs). Oxidative stress and inflammation-related factors including LOXs, phospholipases (PLAs) and mitogen-activated protein kinases (MAPKs) are upregulated in brain tissue during ischemia (Bernaudin et al., 2002; Stephenson et al., 1999), and preventing the overexpression of these factors may protect neuronal tissue from ischemic injury.

Baicalein, isolated from the traditional Chinese herbal medicine *Huangqin* (*Scutellaria baicalensis Georgi*), has been shown to have multiple biological activities, such as anti-inflammation (Shen et al., 2003; Hsieh et al., 2007), antivirus (Wu et al., 2001) and antitumor (Ikemoto et al., 2000; Parajuli et al., 2009). Baicalein is also a potent antioxidant through free radical scavenging and lipid peroxidase inhibiting (Hara et al., 1992; Shieh et al., 2000). The protective effects of baicalein on the central nervous system have been well documented. It has been reported that baicalein decreases the production of inflammatory cytokines (Chen et al., 2008), attenuates cerebral cortex apoptosis (Lebeau et al., 2001) and prevents neurotoxicity induced by both glutamate and glucose deprivation (Lee et al., 2003). Furthermore, baicalein delays the progression of arteriosclerosis and hypertension by protecting endothelial and vascular cells (Machha and Mustafa, 2005; Huang et al., 2005). All of these properties suggest that baicalein may be a potential agent for prevention and treatment of nervous system diseases.

It has long been known that free fatty acids increase rapidly during cerebral ischemia, with arachidonic acid showing the most prominent relative increase (Yoshida et al., 1980). Arachidonic acid itself can cause blood–brain barrier (BBB) dysfunction and subsequent brain edema (Chan and Fishman, 1978; Papadopoulos et al., 1989). In addition, arachidonic acid can also be converted to lipid mediators through COX and LOX pathways (Smith et al., 1996; Wolfe et al., 1985), which play an important role in ischemic brain injury. Cytosolic phospholipase A2 (cPLA2) is the only PLA2 with a high preference for arachidonic acid esterified at the 2-position of

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doi:10.1016/j.pbb.2010.07.007

phospholipids, and contributes to this early increase in arachidonic acid and other free fatty acids. Several reports have shown that 12/15-lipoxygenase (12/15-LOX) and cPLA2 increased rapidly in the penumbra and tissue surrounding the infarct area following middle cerebral artery occlusion (MCAO), suggesting that 12/15-LOX and cPLA2 might be involved in ischemia-induced brain injury (Saluja et al., 1999; Van Leyen et al., 2006; Jin et al., 2008). 12/15-LOX and cPLA2 knockout have been proved to improve neurological deficit and reduce infarction and edema after ischemic brain injury in mice (Van Leyen et al., 2006; Tabuchi et al., 2003). Several studies have demonstrated that p38 MAPK is linked to activation and phosphorylation of cPLA2 and arachidonic acid release (Coulon et al., 2003; Nito et al., 2008). Besides, arachidonic acid metabolite of 12/15-LOX, 12 (S) -hydroxyeicosatetraenoic acid (12 (S)-HETE), also has direct effect on the activation of p38 MAPK and stimulates phosphorylation of p38 MAPK (Reddy et al., 2002). Thus inhibition of arachidonic acid metabolism could attenuate brain injury and provide therapeutic strategy for cerebral ischemia (Adibhatla and Hatcher, 2003; Van Leyen et al., 2006). A recent study has shown that baicalein protects tissue against inflammatory injury by inhibiting the expression of 12/15-LOX (Van Leyen et al., 2006). However, the relationship between baicalein and p38 MAPK and cPLA2 expression in cerebral ischemia has not been investigated so far. We therefore investigated the potential neuroprotective effects of baicalein and explored the underlying mechanisms during ischemic stroke.

2. Materials and methods

2.1. Animals and ischemia protocol

Male Sprague–Dawley rats (250–300 g) were provided by Hebei Medical University. The protocol was approved by the institutional animal care and use committee and the local experimental ethics committee. All rats were kept on a 12-h light/12-h dark regime, with free access to food and water. General anesthesia was conducted with intraperitoneal injection of chloral hydrate (10%). A standard model of intraluminal MCAO was used to make permanent focal ischemia as described previously (Longa et al., 1989). Sham-operated control rats received the same procedure except filament insertion. At 24 h after MCAO, the neurological function was assessed, and the brains were collected by decapitation.

2.2. Drugs, groups and drug administration

Baicalein is a flavonoid originally isolated from the roots of *S. baicalensis Georgi* and was provided and purified by Cayman Chemical (Ann Arbor, MI, USA). The purity of baicalein is more than 95%. Rats were randomly assigned to five groups (24 rats in each group), and drug or solvent was administrated intravenously after MCAO.

Sham-operated group (Sham): animals received sham operation and equal volume of normal sodium; Sham–vehicle group (SH-DM): animals received sham operation and equal volume of DMSO; MCAO group (MCAO): animals received MCAO and equal volume of normal sodium; MCAO-vehicle group (MC-DM): animals received MCAO and equal volume of DMSO; and Baicalein group (Bai): animals received MCAO and 30 mg/kg of baicalein (dissolved in DMSO). Rats were reanesthetized and killed at 24 h after MCAO.

2.3. Neurological function assessment

A neurological test was administered by the same examiner blinded to the experimental groups at 24 h postischemia. A modified five point scale system was used (n = 10 in each group), 0: normal spontaneous movements; 1: left front leg was flexed but no circling clockwise; 2: circling clockwise; 3 spin clockwise longitudinally; and 4: unconsciousness and no response to noxious stimulus.

2.4. Determination of brain water content

Rats were killed by decapitation at 24 h after MCAO. After dissecting free 4 mm of frontal pole, the brains were cut coronally in 2-mm, and each slice was divided into ipsilateral and contralateral hemispheres. The two hemispheres slices, packaged with tin foils, were weighted and dried for 24 h at 100 °C to calculate dry weights. Brain water content was calculated as (wet weight − dry weight) / wet weight × 100%.

2.5. Determination of infarct volume

At 24 h after MCAO, rats were reanesthetized with chloral hydrate. The fresh brains were dissected and cut into 5 coronal slices, 2-mm each starting 2 mm from the frontal pole, the slices were incubated in 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) for 15 min at 37 °C (Bederson et al., 1986), and followed by immersion-fixation in 4% paraformaldehyde. TTC-stained sections were photographed and the digital images were analyzed using image analysis software (Image-Pro Plus 5.1). Infarct areas were first measured using image analysis software and then compiled to obtain the infarct volume (mm^3) per brain. The lesion volumes were calculated as a percentage of the contralateral hemisphere volume to compensate for the effect of brain edema using following formula (Tatlisumak et al., 1998): {[total infarct volume − (the volume of intact ipsilateral hemisphere − the volume of intact contralateral hemisphere)] / contralateral hemisphere volume} × 100%.

2.6. Immunohistochemistry

Paraffin-embedded sections were used to assess the expression of 12/15-LOX, phospho-p38 MAPK and cPLA2 according to standard histological procedures. We chose 12-Lipoxygenase (murine leukocyte) polyclonal antiserum (Cayman Chemical, Ann Arbor, MI), phospho-p38 MAPK (Tyr180 / Tyr182) rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA) and cPLA2 rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA) to detect expression. The immunoreactive cells were counted under a 400× light microscope in five visual fields of the ischemic cortex region of the infarct.

2.7. Western blot

Protein extraction for 12/15-LOX, phospho-p38 MAPK and cPLA2 of ischemic brain cortex was obtained using a Total Protein Extraction Kit and Neuclear-Cytosol Extraction Kit (Applygen Technologies Inc., Beijing) following the manufacturer's protocols. The protein concentration of the supernatant was measured using a BCA Protein Assay reagent kit (Novagen, Madison, WI, USA). Equal amounts of protein samples (n = 3 in each group) was separated by SDS/PAGE and transferred on to PVDF membranes. After blocked with tris buffered saline, 0.1% Tween 20 with 5% w/v nonfat dry milk, membranes loaded with protein of interest were incubated with primary antibodies at 4 °C overnight. Fluorescently labeled secondary antibodies (goat anti-rabbit, 1:8000 dilution, Rockland, Gilbertsville, PA) was used to identify primary antibodies. Relative density of each band was analyzed by an imaging densitometer (LI-COR Bioscience). The densitometric values were normalized to actin immunoreactivity.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (n = 3 in each group) of ischemic brain cortex was extracted using Trizol reagent (Invitrogen, Carlsbad, CA), cDNA was transcribed with RevertAid first Strand cDNA Synthesis Kit (Fermentas Internatioal Inc, Canada). GoTaq®Green Master Mix (Promega, Madison, WI) was used for PCR amplification. The RT-PCR products

were separated on 2% agarose gel and the intensity of each band was quantified using SynGene software and expressed in arbitrary units. The primer sequences are showed in Table 1.

3. Statistical analysis

Neurological deficit assessment was tested with One-way ANOVA–Tukey's multiple comparison test and each value represented mean ± S.E.M. Other data were analyzed with ANOVA and followed by Student–Newman–Keuls test, and presented as means ± S.D. The significance level was set at $P<0.05$.

4. Results

4.1. Baicalein improved neurological deficit

Neurological deficit was examined and scored on a 5-point scale and One-way ANOVA–Tukey's multiple comparison test was conducted. Neurological deficit scores were significantly higher in MCAO group compared with Sham control. Baicalein decreased these scores in Bai group ($P<0.05$ vs. MCAO and MC-DM, Table 2), while DMSO had no effect on neurological deficit scores ($P>0.05$ vs. MCAO).

4.2. Baicalein reduced brain edema

Wet–dry method was used to measure brain water content. In Sham and SH-DM groups, the water content in ipsilateral hemispheres were 77.5% ± 2.28% and 77.6% ± 2.35% respectively, while in MCAO and MC-DM groups, the water content was increased to 85.94% ± 1.17% and 85.93% ± 1.09% respectively ($P<0.05$, compared with Sham group). Baicalein significantly decreased water content in Bai group rats (Bai vs. MCAO: 82.93% ± 0.67% vs. 85.94% ± 1.17%, $P<0.05$; Bai vs. MC-DM: 82.93% ± 0.67% vs. 85.93% ± 1.09%, $P<0.05$). However, DMSO has no effect on water content (MC-DM vs. MCAO: 85.93% ± 1.09% vs. 85.94% ± 1.17%, $P>0.05$).

4.3. Baicalein reduced infarct sizes

Infarct sizes were observed at 24 h after stroke using staining with TTC. No infarction was observed in Sham and SH-DM groups. An extensive lesion was found in both striatum and cortex in MCAO and MC-DM groups (Fig. 1). In MCAO group, the infarct volume was 52.51% ± 2.10%. Baicalein reduced the infarct volume from 52.51% ± 2.10% (MCAO) and 51.44% ± 0.86% (MC-DM) to 43.10% ± 2.81% (Bai), $P<0.05$. However, there weren't significant differences in infarct volume between MCAO group and MC-DM group (MC-DM vs. MCAO: 51.44% ± 0.86% vs. 52.51% ± 2.10%, $P>0.05$).

4.4. Baicalein reduced positive immunoreactive cells of 12/15-LOX, phospho-p38 MAPK and cPLA2

We observed the expression of 12/15-LOX, phospho-p38 MAPK and cPLA2 in the rat ischemic cortex before and after treatment. In Sham group there were some positive cells of 12/15-LOX, phospho-p38 MAPK and cPLA2 in rat cortex (Fig. 2). In MCAO and MC-DM groups, the expression of 12/15-LOX, phospho-p38 MAPK and cPLA2 were significant increased ($P<0.05$ vs. Sham group). Compared with MCAO and MC-DM groups, Baicalein dramatically decreased the positive cells of 12/15-LOX, phospho-p38 MAPK and cPLA2 in the ischemic cortex ($P<0.05$ vs. MCAO and MC-DM groups).

Table 1
Summary of the RT-PCR primer sequences.

Gene	Primers	Sequences
12/15-LOX	Forward	5′-TGGGTTCAGGGCAGAAGCAT-3′
	Reverse	5′-GCGGGCAGGAAGACAAGTAGAG-3′
p38MAPK	Forward	5′-TCCAAGGGCTACACCAAATC-3′
	Reverse	5′-TGTTCCAGGTAAGGGTGAGC-3′
cPLA2	Forward	5′-GCAAACCGAACAAAGGGAGAACC-3′
	Reverse	5′-GGAGACACCTTGACCTAAATACGAGACC-3′
β-actin	Forward	5′-GCCATGTACGTAGCCATCCA-3′
	Reverse	5′-GAACCGCTCATTGCCGATAG-3′

Table 2
Neurological deficit scores.

Group	Score					Average score
	0	1	2	3	4	
Sham/SH-DM, n = 10	10	–	–	–	–	–
MCAO, n = 10	–	3	5	2	–	1.90 ± 0.23
MCAO + DMSO, n = 10	–	3	4	3	–	2.00 ± 0.25
MCAO + Baicalein, n = 10	–	8	2	–	–	1.20 ± 0.13*

One-way ANOVA–Tukey's multiple comparision test was used Each value represents mean ± S.E.M. Compared with MCAO group, neurological deficit scores were reduced in Bai group. *$P<0.05$ vs. MCAO group and MC-DM group. No differences were observed between MCAO group and MC-DM group.

4.5. Baicalein decreased 12/15-LOX at protein and mRNA levels

Western blot and RT-PCR analysis of the 12/15-LOX in total protein and cytosolic extracts from ischemic cortex were shown in Fig. 3. The results showed that the expression of 12/15-LOX in MCAO group was increased at protein and mRNA levels (Fig. 3A–F;), and the 12/15-LOX content was significantly increased in both total protein (Fig. 3 A, B; T-12/15-LOX, MCAO vs. Sham: 1.66 ± 0.10 vs. 1.07 ± 0.09, $P<0.05$) and cytosolic extracts (Fig. 3 C, D; C-12/15-LOX, MCAO vs. Sham: 1.62 ± 0.21 vs. 0.86 ± 0.07, $P<0.05$). Compared with Sham group and SH-DM group, total protein of 12/15-LOX in MCAO and MC-DM groups were respectively increased by 46% (MCAO vs. Sham) and 39% (MC-DM vs. SH-DM), while the expression of 12/15-LOX in cytosolic extracts in MCAO and MC-DM groups were respectively increased by 88% (MCAO vs. Sham) and 60% (MC-DM vs. SH-DM). Baicalein significantly decreased 12/15-LOX expression both in total and cytosolic extracts (total level in Bai group: 1.07 ± 0.13, $P<0.05$ vs. MCAO group; cytosolic level in Bai group: 1.17 ± 0.05, $P<0.05$ vs. MCAO group). But, these decreases were not in the MC-DM group ($P>0.05$). Moreover, there were still significant differences in the expression of both total and cytosolic 12/15-LOX between Bai group and MC-DM group ($P<0.05$). Consistent with the result of western blot, the mRNA level of 12/15-LOX were increased after ischemia and suppressed by baicalein (Fig. 3 E, F).

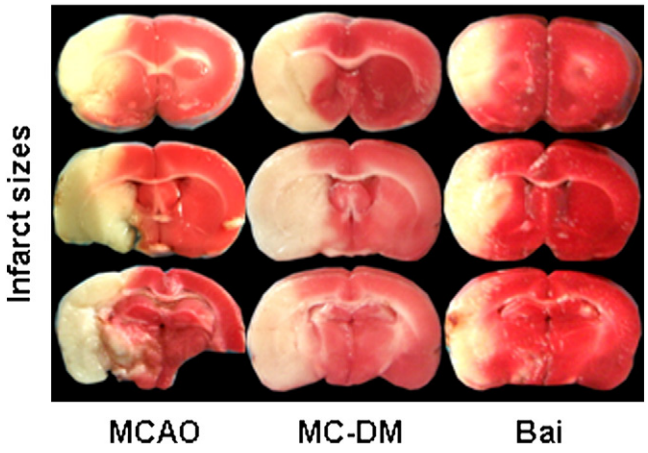

Fig. 1. Effect of baicalein on infarct sizes. An extensive lesion was found in both striatum and cortex of ischemic hemisphere in MCAO and MC-DM rats. The infarct size was reduced by baicalein after MCAO (6 animals in each group).

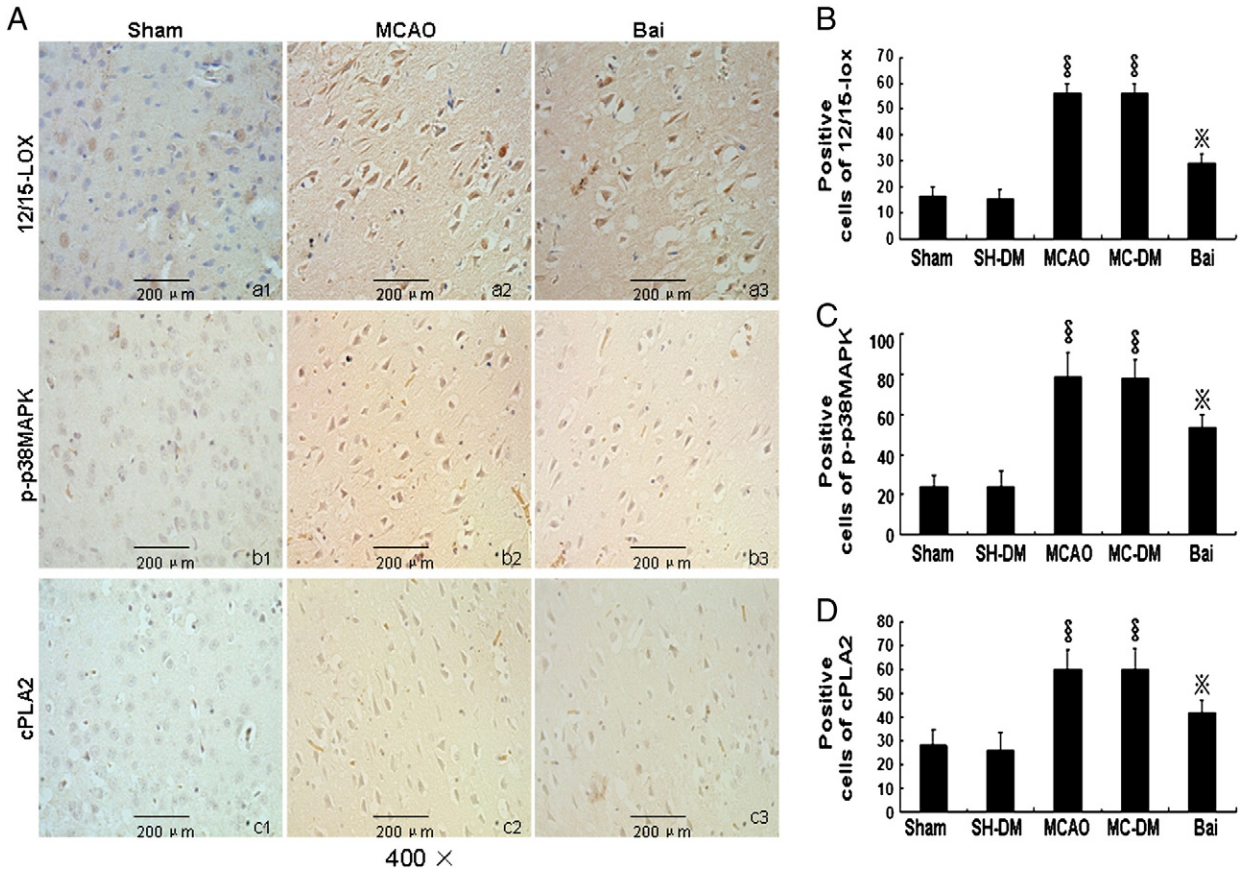

Fig. 2. Graph A showing the results of immunohistochemistry for 12/15-LOX (a_1, a_2, and a_3), phospho-p38 MAPK (b_1, b_2, and b_3) and cPLA2 (c_1, c_2, and c_3). Sham group (a_1, b_1, and c_1), MCAO group (a_2, b_2, and c_2) and Baicalein group (a_3, b_3, and c_3). Bar graph B showing the number of positive cells of 12/15-LOX. Bar graph C showing the number of positive cells of phospho-p38 MAPK. Bar graph D showing the number of positive cells of cPLA2. $^{§}P<0.05$ vs. Sham group, $^{※}P<0.05$ vs. MCAO group and MC-DM group.

4.6. Baicalein decreased the expression of p38 MAPK, phospho-p38 MAPK and cPLA2

Western blot analysis demonstrated a significant increase of phospho-p38 MAPK (MCAO vs. Sham: 1.95 ± 0.19 vs. 0.89 ± 0.20, $P<0.05$) and cPLA2 (MCAO vs. Sham: 0.16 ± 0.01 vs. 0.12 ± 0.01, $P<0.05$) in ischemic cortex after MCAO (Fig. 4 A–D). After systemic administration of baicalein the expression of cPLA2 and phospho-p38 MAPK protein was decreased (cPLA2 in Bai group: 0.12 ± 0.11, $P<0.05$ vs. MCAO and MC-DM groups; phospho-p38 MAPK in Bai group: 1.21 ± 0.09, $P<0.05$ vs. MCAO and MC-DM groups) in MCAO. DMSO has no role in the expression of phospho-p38 MAPK and cPLA2 (phospho-p38 MAPK, MC-DM vs. MCAO: 1.90 ± 0.16 vs. 1.95 ± 0.19, $P>0.05$; cPLA2, MC-DM vs. MCAO: 0.162 ± 0.02 vs. 0.164 ± 0.01, $P>0.05$). mRNA transcriptions of p38 MAPK and cPLA2 were also increased after ischemia and suppressed by baicalein (Fig. 4 E - H).

5. Discussion and conclusion

Cerebral ischemia induces a complex cascade of biochemical and molecular changes, in which inflammation and oxidative stress contribute to stroke-related brain injury. In ischemic brain injury cytokines, such as LOXs, TNF-α, IL-1β, IL-6, and iNOS, are produced by a variety of activated cell types such as monocytes, macrophages, endothelial cells, microglia, and neurons (Huang et al., 2006). Inflammation extends ischemic injury to adverse outcome and provides new therapeutic targets for patients outside of thrombolysis window to save the hypoperfused, nonfunctional, but still viable brain tissue surrounding the irreversible infarct core. For example, in our recent studies it was shown that the systemic administration of oxymatrine and curcumin during cerebral infarction, which have been proved to be antioxidants and anti-inflammatory agents, could improve neurological deficit, reduce brain edema and infarct sizes, and regulate cytokines expression in cortex, such as downregulating transcription factor nuclear factor kappa B, Toll-like receptor-4, toll-like receptor-2 and myeloid differentiation factor 88, and upregulating nuclear factor erythroid 2-related factor 2 and heme oxygenase-1 (HO-1) expression (Liu et al., 2009; Yang et al., 2009; Fan et al., 2009). We also demonstrated that the drug Tanshinone II A could suppress the activation of high-mobility group box 1, improve neurological status and lead to a reduction in BBB disruption, edema, and infarct volume (Wang et al., 2010).

Baicalein, one of the major flavonoids, has been demonstrated to be an anti-inflammatory, antioxidant and free radical scavenging agent (Shen et al., 2003; Hara et al., 1992; Shieh et al., 2000), more than those, baicalein has been regarded as a specific 12/15-LOX inhibitor. Increasing evidences have shown the neuroprotective effects of baicalein in stroke-induced brain injury. Baicalein could attenuate behavioral deficits associated with multiple infarct ischemic events in rabbits following small clot embolic strokes (Lapchak et al, 2007). In addition, baicalein (2 or 4 mg/kg/day) significantly improved cognitive deficits and neuropathological changes induced by permanent occlusion of bilateral common carotid arteries (Liu et al., 2007). Researchers also revealed that this effect may be through reducing activities of superoxide dismutase and malondialdehyde, and increasing activities of glutathione peroxidase and catalase (Liu et al., 2007). In a recent study, it was shown that baicalein (300 mg/kg) reduced infarct volume and brain water content, and improved neurological deficit in mice model of acute ischemic stroke (Van Leyen et al., 2006). Liu et al (2010) also demonstrated that baicalein (20 mg/

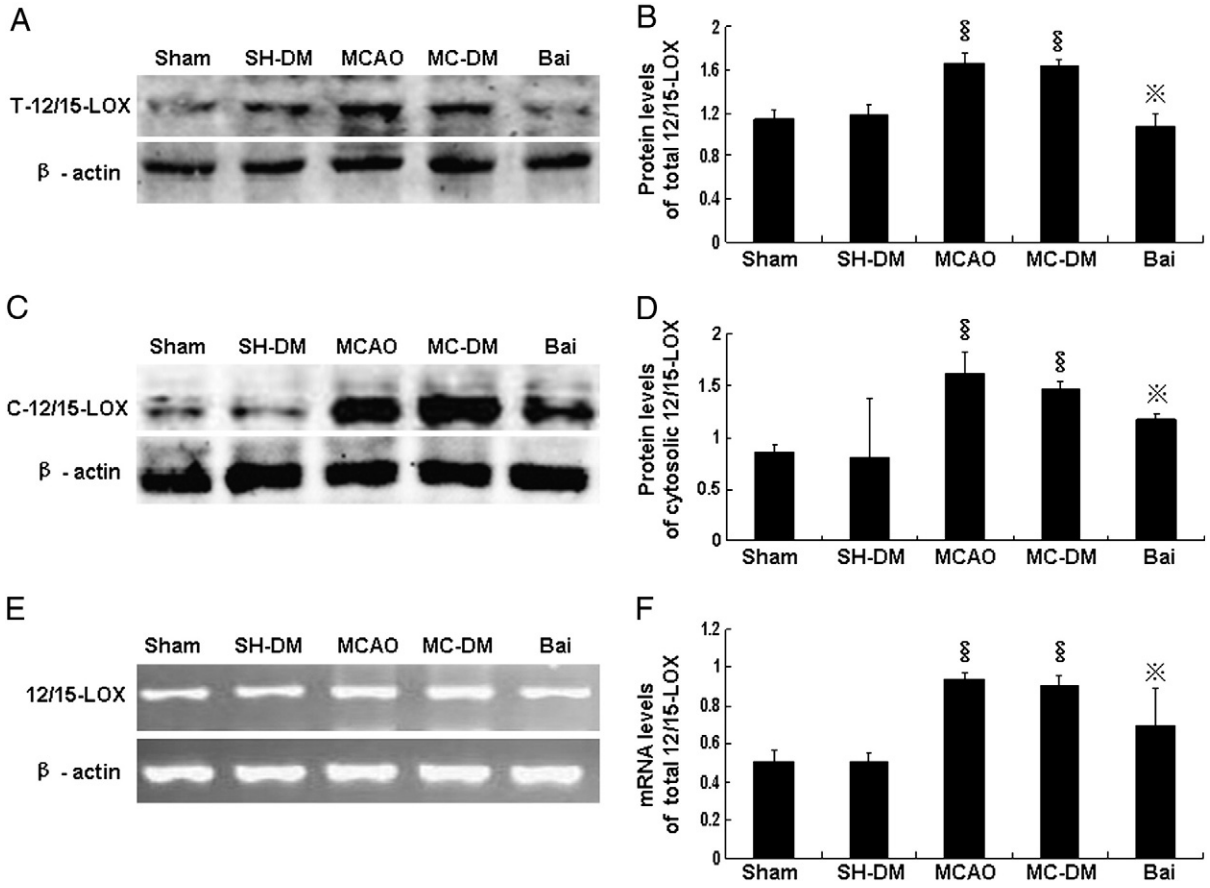

Fig. 3. Graphs A and B showing the expression of total protein of 12/15-LOX. Graphs C and D showing the expression of cytosolic protein of 12/15-LOX. Graphs E and F showing the expression of 12/15-LOX mRNA. $^{§}P<0.05$ vs. Sham group, $^{※}P<0.05$ vs. MCAO group and MC-DM group.

kg/day) significantly reduced infarct volume in transient MCAO, but this reduction is not in permanent MCAO. However, in our study we found that intravenous injection of baicalein (30 mg/kg) could effectively attenuate neurological deficit scores, and reduce brain edema and infarct volume at 24 h after MCAO. Maybe the effect of baicalein on infarct volume in permanent cerebral ischemia will be well elucidated in larger number of animals or possibly in bigger animals as well as humans.

It has long been known that 12/15-LOX is detrimental to brain tissue subjected to oxidative stress. Neuronal 12-LOX leads to the influx of Ca^{2+}, the production of peroxides, and ultimately to cell death (Li et al., 1997). It has been reported that the gene expression of 12/15-LOX could be induced by hypoxia/ischemia. Furthermore, large amounts of 12/15-LOX metabolites also have been found in ischemic brains. Recently, Van Leyen et al (2006) found that the expression of 12/15-LOX was increased in both neurons and endothelial cells in the peri-infarct region after ischemia in mice, suggesting that 12/15-LOX may contribute to the damage in both cell types. They also found that knocking down ALOX15 gene or inhibiting 12/15-LOX by baicalein attenuated the ischemic injury and protected the cerebral vascular endothelial cell function (Jin et al., 2008). The present study revealed that 12/15-LOX was significantly upregulated in ischemic region. The cytosolic 12/15-LOX protein in MCAO groups was 60%–88% higher than that in Sham group. Baicalein effectively reduced 12/15-LOX expression in both mRNA and protein level in cytoplasm. These results further confirmed the neuroprotective effects of baicalein in cerebral ischemia.

cPLA2 is one of the superfamilies of esterases that specifically hydrolyze the acyl ester bonding at the sn-2 position of membrane phospholipids including arachidonic acid (Farooqui and Horrocks, 2006). Release of arachidonic acid by cPLA2 is the rate-limiting step in the LOX pathway. Accumulative evidences suggested that increased cPLA2 activity generated proinflammatory lipid mediators, such as leukotrienes, eicosanoids, prostaglandins, and platelet-activating factor, and these factors played an important role in acute inflammatory responses and oxidative stress associated with neurological diseases (Stephenson et al., 1999; Farias et al., 2008). It has been reported that p38 MAPK is linked to the activation and phosphorylation of cPLA2 and arachidonic acid release. The drug SB203580, a p38 MAPK inhibitor, suppressed activation and phosphorylation of cPLA2, and this pharmacological inhibition of p38 MAPK led to a reduction in edema, infarct volume and BBB disruption (Nito et al., 2008). Thus, inhibition of p38 MAPK and cPLA2 activity provides a potential approach for the treatment of inflammation and oxidative stress associated with acute neural lesion (Nito et al., 2008; Pilitsis et al., 2002). The present study showed that baicalein decreased the positive staining cells of phospho-p38 MAPK and cPLA2, and p38 MAPK and cPLA2 mRNA were also reduced by baicalein. These results suggested that baicalein not only downregulated the expression of 12/15-LOX, but also downregulated the expression and phosphorylation of p38 MAPK and the activation of cPLA2 in the brain tissue after ischemia.

MAPKs have crucial roles in regulating cell death and survival through signal translocation pathways. p38 MAPK and c-Jun N-terminal kinases (JNKs) are induced and activated by a variety of cellular stresses, such as hypoxia and inflammatory cytokines. In contrast, extracellular signal-regulated kinases (ERKs) are often activated by intracellular calcium increase, glutamate receptor stimulation, and several kinds of growth factors. These three kinds of signals are all involved in oxidative stress. In a recent study, it was shown that baicalein protected against H_2O_2-induced apoptosis via suppression of ERK activation in glioma C6 cells, and interestingly baicalein also induced HO-1 expression via stimulation of ERK phosphorylation in the absence of H_2O_2 (Chen et al., 2006). Besides,

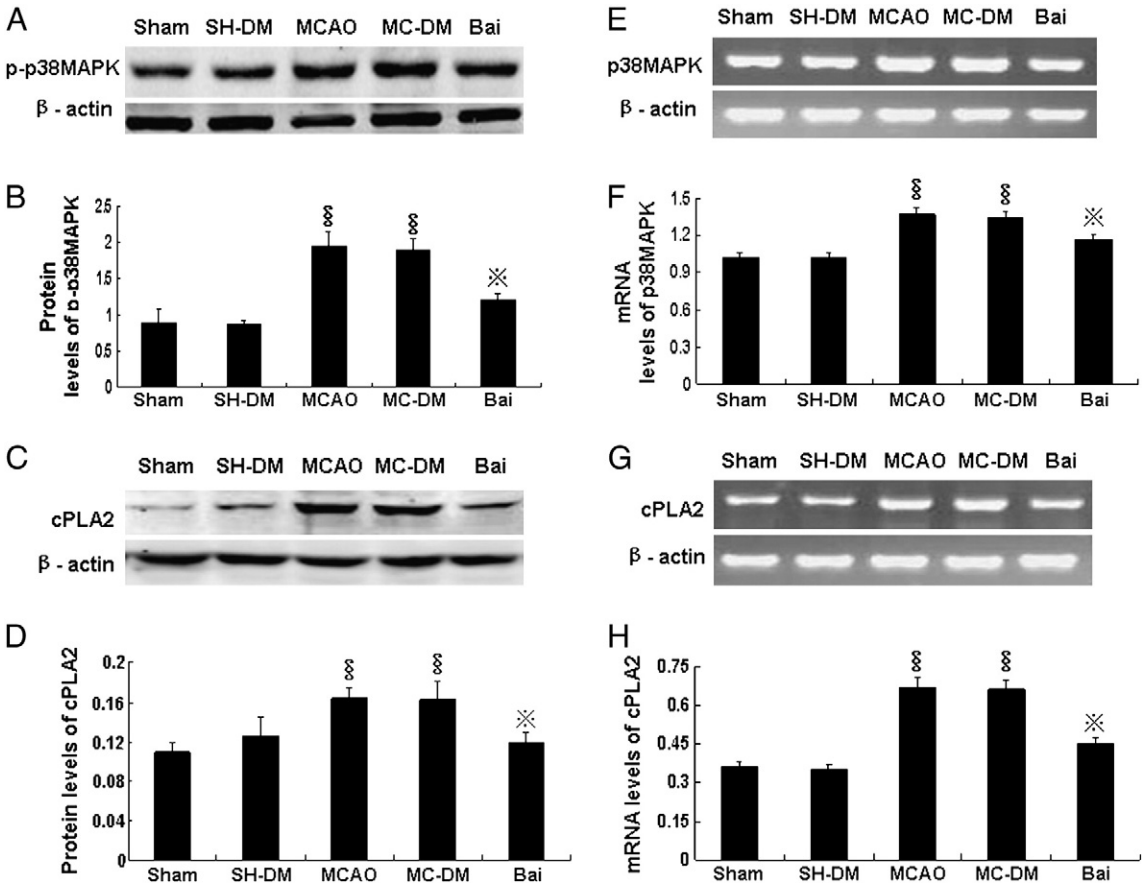

Fig. 4. Graphs A and B showing the protein level of phospho-p38 MAPK. Graphs C and D showing the protein level of cPLA2. Graphs E and F showing the mRNA level of p38 MAPK. Graphs G and H showing the mRNA level of cPLA2. $^{§}P<0.05$ vs. Sham group, $^{※}P<0.05$ vs. MCAO group and MC-DM group.

it proposed that induction of ERKs, but not p38 MAPK or JNKs protein phosphorylation is involved in baicalein's induction of cellular protective genes such as HO-1 protein expression in glioma C6 cells (Chen et al., 2006). Natarajan et al demonstrated that 12-LOX inhibitor, baicalein, blocked ANG II-induced p38 MAPK activation in H295R (Natarajan et al., 2002). In the following study, Kalyankrishna et al. also showed that p38 MAPK activation elicited by norepinephrine was decreased significantly by baicalein in vascular smooth muscle cells (Kalyankrishna and Malik, 2003). In our study, immunohistochemistry and Western blot analysis showed that phosphorylation of p38 MAPK increased at 24 h after ischemia. Moreover, we also found that both phosphorylated p38 MAPK protein and p38 MAPK mRNA levels were decreased by LOX inhibitor baicalein during cerebral ischemia.

In summary, our study has confirmed that baicalein ameliorated the neurological deficit, decreased the infarct size and improved the brain edema caused by MCAO. Those effects may be through downregulation of 12/15-LOX, p38 MAPK and cPLA2 activity. The elevated expression of 12/15-LOX, p38 MAPK and cPLA2 is thought to be consistent with ischemia evoked neuron injury and death through inflammatory mechanism. LOX and cPLA2 pathway of arachidonic acid metabolism may be one of the strategic targets for cerebral ischemic therapies and baicalein maybe an effective therapeutic drug for the treatment of ischemic brain injury.

Acknowledgments

This work was funded by Hebei Province, No: C2010000564 and No: 10276104D; and we thank Ruichun Liu and Hongran Wu for their technical assistance.

References

Adibhatla RM, Hatcher JF. Citicoline decreases phospholipase A2 stimulation and hydroxyl radical generation in transient cerebral ischemia. J Neurosci Res 2003;73:308–15.

Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM. Evaluation of 2, 3, 5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke 1986;17:1304–8.

Bernaudin M, Tang Y, Reilly M, Petit E, Sharp FR. Brain genomic response following hypoxia and re-oxygenation in the neonatal rat. Identification of genes that might contribute to hypoxia-induced ischemic tolerance. J Biol Chem 2002;277:39728–38.

Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. J Cereb Blood Flow Metab 2001;21:2-14.

Chan PH, Fishman RA. Brain edema: induction in cortical slices by polyunsaturated fatty acids. Science 1978;201:358–60.

Chen SF, Hsu CW, Huang WH, Wang JY. Post-injury baicalein improves histological and functional outcomes and reduces inflammatory cytokines after experimental traumatic brain injury. Br J Pharmacol 2008;155:1279–96.

Chen YC, Chow JM, Lin CW, Wu CY, Shen SC. Baicalein inhibition of oxidative-stress-induced apoptosis via modulation of ERKs activation and induction of HO-1 gene expression in rat glioma cells C6. Toxicol Appl Pharmacol 2006;216:263–73.

Coulon L, Calzada C, Moulin P, Véricel E, Lagarde M. Activation of p38 mitogen-activated protein kinase/cytosolic phospholipase A2 cascade in hydroperoxide-stressed platelets. Free Radic Biol Med 2003;35:616–25.

De Simoni MG, Milia P, Barba M, De Luigi A, Parnetti L, Gallai V. The inflammatory response in cerebral ischemia: focus on cytokines in stroke patients. Clin Exp Hypertens 2002;24:535–42.

Dirnagl U, Lindauer U, Them A, Schreiber S, Pfister HW, Koedel U, et al. Global cerebral ischemia in the rat: online monitoring of oxygen free radical production using chemiluminescence in vivo. J Cereb Blood Flow Metab 1995;15:929–40.

Fan H, Li L, Zhang X, Liu Y, Yang C, Yang Y, et al. Oxymatrine downregulates TLR4, TLR2, MyD88, and NF-kappaB and protects rat brains against focal ischemia. Mediat Inflamm 2009;2009:704706.

Farias SE, Basselin M, Chang L, Heidenreich KA, Rapoport SI, Murphy RC. Formation of eicosanoids, E2/D2 isoprostanes, and docosanoids following decapitation-induced ischemia, measured in high-energy-microwaved rat brain. J Lipid Res 2008;49:1990–2000.

Farooqui AA, Horrocks LA. Brain cytosolic phospholipase A2-generated lipid mediators in the brain: the good, the bad, and the ugly. Neuroscientist 2006;12:245–60.

Hara H, Sukamoto T, Ohtaka H, Abe K, Tatumi Y, Sait Y, et al. Effects of baicalein and alpha-tocopherol on lipid peroxidation, free radical scavenging activity and 12-O-tetradecanoylphorbol acetate-induced ear edema. Eur J Pharmacol 1992;221: 193–8.

Hsieh CJ, Hall K, Ha T, Li C, Krishnaswamy G, Chi DS. Baicalein inhibits IL-1beta- and TNF-alpha-induced inflammatory cytokine production from human mast cells via regulation of the NF-kappaB pathway. Clin Mol Allergy 2007;5:5.

Huang J, Upadhyay UM, Tamargo RJ. Inflammation in stroke and focal cerebral ischemia. Surg Neurol 2006;66:232–45.

Huang Y, Tsang SY, Yao X, Chen ZY. Biological properties of baicalein in cardiovascular system. Curr Drug Targets Cardiovasc Haematol Disord 2005;5:177–84.

Ikemoto S, Sugimura K, Yoshida N, Yasumoto R, Wada S, Yamamoto K, et al. Antitumor effects of *Scutellariae radix* and its components baicalein, baicalin, and wogonin on bladder cancer cell lines. Urology 2000;55:951–5.

Jin G, Arai K, Murata Y, Wang S, Stins MF, Lo EH, et al. Protecting against cerebrovascular injury: contributions of 12/15-lipoxygenase to edema formation after transient focal ischemia. Stroke 2008;39:2538–43.

Kalyankrishna S, Malik KU. Norepinephrine-induced stimulation of p38 mitogen-activated protein kinase is mediated by arachidonic acid metabolites generated by activation of cytosolic phospholipase A(2) in vascular smooth muscle cells. J Pharmacol Exp Ther 2003;304:761–72.

Lakhan SE, Kirchgessner A, Hofer M. Inflammatory mechanisms in ischemic stroke: therapeutic approaches. J Transl Med 2009;7:97.

Lapchak PA, Maher P, Schubert D, Zivin JA. Baicalein, an antioxidant 12/15-lipoxygenase inhibitor improves clinical rating scores following multiple infarct embolic strokes. Neuroscience 2007;150:585–91.

Lebeau A, Esclaire F, Rostene W, Pelaprat D. Baicalein protects cortical neurons from beta-amyloid (25–35) induced toxicity. NeuroReport 2001;12:2199–202.

Lee HH, Yang LL, Wang CC, Hu SY, Chang SF, Lee YH. Differential effects of natural polyphenols on neuronal survival in primary cultured central neurons against glutamate- and glucose deprivation-induced neuronal death. Brain Res 2003;986: 103–13.

Li Y, Maher P, Schubert D. A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. Neuron 1997;19:453–63.

Liu C, Wu J, Gu J, Xiong Z, Wang F, Wang J, et al. Baicalein improves cognitive deficits induced by chronic cerebral hypoperfusion in rats. Pharmacol Biochem Behav 2007;86:423–30.

Liu C, Wu J, Xu K, Cai F, Gu J, Ma L, et al. Neuroprotection by baicalein in ischemic brain injury involves PTEN/AKT pathway. J Neurochem 2010;112:1500–12.

Liu Y, Zhang XJ, Yang CH, Fan HG. Oxymatrine protects rat brains against permanent focal ischemia and downregulates NF-kappaB expression. Brain Res 2009;1268: 174–80.

Lo EH, Moskowitz MA, Jacobs TP. Exciting, radical, suicidal: how brain cells die after stroke. Stroke 2005;36:189–92.

Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 1989;20:84–91.

Machha A, Mustafa MR. Chronic treatment with flavonoids prevents endothelial dysfunction in spontaneously hypertensive rat aorta. J Cardiovasc Pharmacol 2005;46:36–40.

Natarajan R, Yang DC, Lanting L, Nadler JL. Key role of P38 mitogen-activated protein kinase and the lipoxygenase pathway in angiotensin II actions in H295R adrenocortical cells. Endocr 2002;18:295–301.

Nito C, Kamada H, Endo H, Niizuma K, Myer DJ, Chan PH. Role of the p38 mitogen-activated protein kinase/cytosolic phospholipase A2 signaling pathway in blood-brain barrier disruption after focal cerebral ischemia and reperfusion. J Cereb Blood Flow Metab 2008;28:1686–96.

Papadopoulos SM, Black KL, Hoff JT. Cerebral edema induced by arachidonic acid: role of leukocytes and 5-lipoxygenase products. Neurosurgery 1989;25:369–72.

Parajuli P, Joshee N, Rimando AM, Mittal S, Yadav AK. In vitro antitumor mechanisms of various *Scutellaria* extracts and constituent flavonoids. Planta Med 2009;75:41–8.

Pilitsis JE, Diaz FG, O'Regan MH, Phillis JW. Differential effects of phospholipase inhibitors on free fatty acid efflux in rat cerebral cortex during ischemia-reperfusion injury. Brain Res 2002;951:96-106.

Reddy MA, Thimmalapura PR, Lanting L, Nadler JL, Fatima S, Natarajan R. The oxidized lipid and lipoxygenase product 12(S)-hydroxyeicosatetraenoic acid induces hypertrophy and fibronectin transcription in vascular smooth muscle cells via p38 MAPK and cAMP response element-binding protein activation. Mediation of angiotensin II effects. J Biol Chem 2002;277:9920–8.

Saluja I, O'Regan MH, Song D, Phillis JW. Activation of cPLA2, PKC, and ERKs in the rat cerebral cortex during ischemia/reperfusion. Neurochem Res 1999;24:669–77.

Shen YC, Chiou WF, Chou YC, Chen CF. Mechanisms in mediating the anti-inflammatory effects of baicalin and baicalein in human leukocytes. Eur J Pharmacol 2003;465: 171–81.

Shieh DE, Liu LT, Lin CC. Antioxidant and freeradical scavenging effects of baicalein, baicalin and wogonin. Anticancer Res 2000;20:2861–6.

Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases) -1 and -2. J Biol Chem 1996;271:33157–60.

Stephenson D, Rash K, Smalstig B, Roberts E, Johnstone E, Sharp J, et al. Cytosolic phospholipase A2 is induced in reactive glia following different forms of neurodegeneration. Glia 1999;27:110–28.

Tabuchi S, Uozumi N, Ishii S, Shimizu Y, Watanabe T, Shimizu T. Mice deficient in cytosolic phospholipase A2 are less susceptible to cerebral ischemia/reperfusion injury. Acta Neurochir Suppl 2003;86:169–72.

Tatlisumak T, Carano RA, Takano K, Opgenorth TJ, Sotak CH, Fisher M. A novel endothelin antagonist, A-127722, attenuates ischemic lesion size in rats with temporary middle cerebral artery occlusion: a diffusion and perfusion MRI study. Stroke 1998;29:850–7.

Van Leyen K, Kim HY, Lee SR, Jin G, Arai K, Lo EH. Baicalein and 12/15-lipoxygenase in the ischemic brain. Stroke 2006;37:3014–8.

Wang L, Zhang X, Liu L, Cui L, Yang R, Li M, et al. Tanshinone II A down-regulates HMGB1, RAGE, TLR4, NF-kappaB expression, ameliorates BBB permeability and endothelial cell function, and protects rat brains against focal ischemia. Brain Res 2010;1321:143–51.

Wolfe LS, Pappius HM, Pokrupa R, Hakim A. Involvement of arachidonic acid metabolites in experimental brain injury. Identification of lipoxygenase products in brain. Clinical studies on prostacyclin infusion in acute cerebral ischemia. Adv Prostaglandin Thromboxane Leukot Res 1985;15:585–8.

Wu JA, Attele AS, Zhang L, Yuan CS. Anti-HIV activity of medicinal herbs: usage and potential development. Am J Chin Med 2001;29:69–81.

Yang C, Zhang X, Fan H, Liu Y. Curcumin upregulates transcription factor Nrf2, HO-1 expression and protects rat brains against focal ischemia. Brain Res 2009;1282: 133–41.

Yoshida S, Inoh S, Asano T, Sano K, Kubota M, Shimazaki H, et al. Effect of transient ischemia on free fatty acids and phospholipids in the gerbil brain. Lipid peroxidation as a possible cause of postischemic injury. J Neurosurg 1980;53: 323–31.