Biochanin A Provides Neuroprotection Against Cerebral Ischemia/Reperfusion Injury by Nrf2-Mediated Inhibition of Oxidative Stress and Inflammation Signaling Pathway in Rats

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Background:
Oxidative stress and neuroinflammation are 2 pivotal mechanisms in the progression of cerebral ischemia/reperfusion injury. Biochanin A, a natural phytoestrogen, has been reported to protect against ischemic brain injury in animal experiments, but the possible pharmacological mechanisms of its neuroprotection remain elusive. In this research, we sought to investigate the neuroprotective effects of biochanin A in experimental stroke rats and the probable mechanisms underlying oxidative stress and inflammation signaling pathways.

Material/Methods:
An ischemic stroke model was induced by inserting thread into the middle cerebral artery. Rats were pre-administered intraperitoneally with a vehicle solution or biochanin A (10, 20, or 40 mg·kg·d$^{-1}$) for 14 days prior to ischemic stroke. Neurological score, infarct volume, and cerebral edema were assessed after 2 h of ischemia and 24 h of reperfusion. The activities of SOD and GSH-Px and MDA content were measured. The expressions of Nrf2, HO-1, and NF-$\kappa$B and the activity of phosphor-$\kappa$Ba were detected by Western blotting.

Results:
Biochanin A pretreatment significantly improved neurological deficit and decreased infarct size and brain edema. Biochanin A also enhanced SOD and GSH-Px activities and suppressed the production of MDA. Additionally, biochanin A promoted Nrf2 nuclear translocation, promoted the expression of HO-1, and inhibited NF-$\kappa$B activation in ischemic brain injury.

Conclusions:
The results indicated that biochanin A protected the brain against ischemic injury in rats by anti-oxidative and anti-inflammatory actions. The activation of the Nrf2 pathway and the inhibition of the NF-$\kappa$B pathway may contribute to the neuroprotective effects of biochanin A.

MeSH Keywords:
NF-E2-Related Factor 2 • Oxidative Stress • Phytoestrogens • Stroke
Background

Stroke, including ischemic and hemorrhagic stroke, remains the main cause of death and disability in developed and developing countries [1]. Ischemic stroke is the most prevalent type of stroke and occurs due to decreased or blocked blood flow to brain tissues. Currently, thrombolytic agents and intravascular techniques are the 2 main treatment strategies for acute ischemic stroke [2]. However, there are limitations in the clinical use of thrombolytic agents, such as tissue plasminogen activator (tPA). Intravenous administration of tPA must be restricted to a strict 4.5-h therapeutic time window, and complications from bleeding and fatal edema are the main barriers [3]. Although restoration of blood supply is the goal of acute stroke treatment, it can further aggravate neuronal destruction due to ischemia/reperfusion (I/R) injury [4]. The pathophysiological injury mechanisms of cerebral I/R involve energy failure, the release of excitatory neurotransmitters, damage of the blood-brain barrier, intracellular Ca2+ accumulation, oxidative stress, inflammation, and apoptosis [5]. Therefore, developing neuroprotective agents as another promising approach may be an effective strategy for treatment after ischemic stroke [6].

Many recent studies have shown that phytoestrogens provide neuroprotective effects in animal models of cerebral I/R [7–10]. Because of its structural similarity to estrogen, phytoestrogens can selectively bind to estrogen receptors, thus regulating expression of related genes and producing estrogenic or anti-estrogenic effects [11]. Biochanin A, a natural O-methylated isoflavonoid phytoestrogen derived from red clover or chickpea, exhibits broad pharmacological functions such as anti-tumor, anti-oxidation, and hypoglycemic activity [12–15]. Our previous studies have also shown that biochanin A reduced the inflammatory injury of cerebral ischemia/reperfusion by inhibiting the P38MAPK signaling pathway [16].

A growing number of basic and clinical experiments have suggested that cerebral ischemia/reperfusion can cause cellular injury resulting from activation of multiple oxidative stress and inflammatory pathways [17, 18]. Nuclear factor E2-related factor-2 (Nrf2) is an important regulator of the antioxidant cell defense system. Once activated, Nrf2 translocates into the nucleus and activates its target genes through an antioxidant-response element (ARE) [19]. Among the target genes of Nrf2, heme oxygenase-1 (HO-1) is one of the anti-oxidative stress representatives. Currently, HO-1 is considered to protect tissues by repairing redox homeostasis and reducing inflammation. Research has shown that HO-1 expression is controlled by numerous signaling pathways and transcription factors, including Nrf2 and NF-kB [20]. Nevertheless, the detailed function of HO-1 and its role in cerebral I/R injury need to be further investigated.

In this research, we hypothesized that the administration of biochanin A would attenuate oxidative stress and neuroinflammation, and that the potential protective roles of biochanin A are mediated through the Nrf2-ARE pathway.

Material and Methods

Animals and reagents

Male Sprague-Dawley rats (45–50 days old, Grade II, 225–285 g) were purchased from the Experimental Animal Center, Guilin Medical University. All rats were raised in a 12-h light/dark cycle at 24±2°C and were given free access to water and food. The experimental protocols were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All animal studies were approved by the Animal Ethics Committee of Guilin Medical University.

Biochanin A ([C6H2O6 hydrogen molecular weight 284.27, purity more than 98%, and verified by high-performance liquid chromatography) was obtained from Sigma–Aldrich (Saint Louis, MO, USA). Biochanin A powder reagent was dissolved in dimethyl sulfoxide (DMSO) to prepare a mother solution of 200 mM and diluted with saline to a final concentration. The mother solution was stored at 4°C until further use.

Focal cerebral I/R model and neurological scoring

The rats used in this study were randomly assigned to the sham group (saline-treated), the I/R group (saline-treated), or the biochanin A pretreatment I/R groups. The biochanin A pretreatment I/R groups were injected intraperitoneally with biochanin A at low-dose (10 mg·kg·d\(^{-1}\)), middle-dose (20 mg·kg·d\(^{-1}\)), or high-dose (40 mg·kg·d\(^{-1}\)) for 14 days. A model of focal cerebral ischemia was established by middle cerebral artery occlusion (MCAO) as described in previous reports [21,22]. Briefly, the rats were anesthetized with 1.5% pentobarbital (30 mg/kg) at 1 h after the last dose. Then, the right common carotid artery was exposed over a midline neck incision. A monofilament coated with silicon tip (Jialing Biotechnology Co., Guangzhou, China) was inserted into the internal carotid artery through the common carotid artery until a slight resistance was felt, indicating it occluded the blood flow into the middle cerebral artery (MCA). After 2 h of ischemia, the monofilament was gently removed to induce MCA reperfusion, and the animals gradually recovered from anesthesia (Figure 1). In our experiment, the changes in cerebral blood flow during surgery were monitored using laser Doppler equipment (Periflux 5000, Sweden) with a probe positioned at the ipsilateral intact skull overlying the MCA area (2 mm posterior and 4 mm lateral to the bregma). The model rats whose cerebral blood flow was decreased by at least 70% by MCAO and who had quick restoration of blood flow were included.

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flow after reperfusion were included in the follow-up experiment. The rats in the sham group were subjected to a similar surgical treatment, but monofilament thread was not inserted into the internal carotid artery. During the operation, the body temperature of the rats was maintained at 38±0.5°C with a heating pad.

Neurological function was evaluated at 24 h after reperfusion using the 5-point scoring system described by Longa et al. [23]. The specific scoring method was: 0=no neurologic deficit (normal walk), 1=a mild focal neurologic deficit (failure to extend opposite forepaw fully), 2=a moderate focal neurologic deficit (circling to the contralateral side), 3=a severe focal neurologic deficit (falling to the contralateral side), and 4=no spontaneous walking and a depressed consciousness level. Neurological deficit scoring was carried out by a researcher blinded to the experimental groups.

Infarct size analysis

We used 2,3,5-triphenyltetrazolium chloride (TTC) staining to assess the cerebral infarct volume. The rats were anesthetized and decapitated at 24 h after reperfusion, and the brain samples were removed quickly and kept in a freezer at –20°C for 30 min at 37°C. The normal brain tissue was stained deep red, but the infarcted area remained unstained. The stained brain slices were photographed, and the infracted sizes were quantified using ImageJ software (Image J, Version 1.50, National Institutes of Health, Bethesda, MD, USA). The area of the lesion was calculated by the following formula: percentage of infarct volume=(Vf–Vn)/Vc×100.

Vf is the volume of normal tissue in the lesioned hemisphere and Vn is the volume of normal structure in the control hemisphere. The volume was estimated by summing each area times the distance between sections.

Assessment of the brain edema

Brain water content was determined by wet-dry method. After reperfusion for 24 h, the whole brain of each rat was taken out rapidly and the wet weight was obtained with an electronic analytical balance. The dry weight was obtained again after drying in an oven for 24 h at 100°C. Brain water content was calculated by the following formula: percentage of brain water=[(wet weight–dry weight)/wet weight] ×100%.

Detection of SOD, GSH-Px, and MDA

The brain tissues were washed, weighed, and homogenized with an external ice-cold saline bath. Then, the protein content was determined using a BCA (bicinchoninic acid) protein assay kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). The levels of SOD, GSH-Px, and MDA in the ischemic boundary zone were detected with the corresponding assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the product manual. The test results were normalized to the protein content and are expressed as U or nmol/mg protein.

Quantitative real-time PCR (qRT-PCR) of HO-1

Total RNA was extracted from the ischemic penumbras zone of rat brain tissues using Trizol reagent (Invitrogen, NY, USA). The cDNA was subsequently synthesized from the total RNA with a Revert Aid First Strand cDNA Synthesis Kit (TIANGEN, Beijing, China) following the product manual. The expression levels of HO-1 mRNA were determined by qRT-PCR with the ABI PRISM 7500 Sequence Detector System (Applied Biosystems,
Carlsbad, CA, USA). The relative amount of OH-1 mRNA was calculated by the 2^{-ΔΔCt} method and normalized to an endogenous reference gene (β-actin) in all samples. The primer pair used for HO-1 detection was 5'-ACTCAGTTTCTGTTGGCGA-3' (forward) and 5'-GGGGCCACACTGATTTC-3' (reverse). The primer pair used for β-actin detection was 5’-GCAGGAGTACGATGGCTCG-3’ (forward) and 5’-ACGCAACCTAGTAACTGCC-3’ (reverse).

Results

Biochanin A improved neurological deficits, decreased infarct sizes, and alleviated brain edema in cerebral I/R rats

To determine whether biochanin A pretreatment improves neurological function after I/R injury, we observed the neurological deficit scores at 24 h after reperfusion in rats. As displayed in Figure 2A, no neurological deficits were discovered in the sham group, while severe neurological deficits were found in the I/R group, indicating that the MCAO model was successfully established. Biochanin A treatment markedly decreased the neurological deficit scores dose-dependently compared with that in the I/R group, suggesting that biochanin A improved brain function.

In addition, the infarct volume and water content of brain tissues were assessed in the different groups. TTC staining of brain slices showed that no infarct damage was observed in the sham group, but there was a significant rise of infarct volume in the I/R group. Compared to the I/R group, biochanin A pretreatment markedly reduced the infarct volume (Figure 2B). Accordingly, the brain water content was markedly increased in the I/R group, and was decreased dose-dependently by biochanin A treatment (Figure 2C). These results show that biochanin A had a neuroprotective effect in cerebral I/R rats.

Biochanin A improved antioxidant defenses and reduced lipid peroxidation in cerebral I/R rats

To reveal the mechanisms underlying the neuroprotective effect of biochanin A, we measured the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), as well as the contents of malondialdehyde (MDA), in brain tissues. Figure 3 shows that SOD and GSH-Px activity decreased and MDA level markedly increased in the I/R group. Compared to the I/R group, biochanin A pretreatment significantly increased SOD and GSH-Px activities and reduced MDA level in the brain tissues. These findings suggest that biochanin A suppressed brain oxidative stress after ischemia.

Biochanin A promoted Nrf2 nuclear translocation and increased the expression of HO-1 in cerebral I/R rats

To investigate the biochanin A-induced anti-oxidative effects, we next examined the expressions of Nrf2 and HO-1 in the brain tissues. Western blot analysis showed that Nrf2 expression was significantly increased in the nucleus but was markedly decreased in the cytoplasm with biochanin A treatment compared with that in the I/R group (Figure 4). The results demonstrated that biochanin A pretreatment induced Nrf2 nuclear localization. In addition,
HO-1, which is a key antioxidant enzyme, was evaluated in the ischemic penumbras zone of rat brain tissues. There was an increase in expression levels of HO-1 mRNA and protein following ischemia reperfusion, and biochanin A pretreatment augmented the MCAO-induced expression of HO-1 in a dose-dependent manner (Figure 5).

Biochanin A inhibited NF-κB activation in cerebral I/R rats

To further elucidate the potential mechanism of biochanin A in cerebral ischemia protection, we next investigated whether biochanin A affects the NF-κB signaling pathway in cerebral ischemia. The expression of NF-κB p65 and the activity of phosphor-IkBα were assessed by Western blotting. As shown in Figure 6A, phosphorylation of IkBα was significantly higher in the I/R group than in the sham group. In the biochanin A-treated groups, phosphorylation of IkBα was dose-dependently reduced compared with the I/R group. In addition, NF-κB p65 was mainly expressed in cytoplasm in the sham group and NF-κB p65 was predominately expressed in the nucleus in the I/R group, indicating the nuclear translocation of NF-κB p65 in MCAO-induced ischemia. Biochanin A pretreatment significantly prevented nuclear translocation in a dose-dependent manner (Figure 6B). These results indicate that biochanin A inhibited NF-κB activation and translocation in ischemic brain tissues.

Discussion

Previous reports have demonstrated that estradiol and phytoestrogen mitigate ischemic stroke-induced damages by binding to the estrogen receptors [25–27]. However, the potential neuroprotective mechanisms of these compounds need to be studied further. In the present study, biochanin A, a typical phytoestrogen, was shown to improve neurological effect and to reduce infarct volume and brain edema in cerebral I/R rats. Moreover, biochanin A treatment suppressed brain oxidative stress levels, promoted Nrf2 nuclear translocation, increased the expression of HO-1, and inhibited activation of the NF-κB pathway in ischemic brain tissues. These findings suggest that biochanin A provides neuroprotection through regulation of oxidative stress and inflammation pathways.
Figure 3. Biochanin A attenuated oxidative stress in cerebral I/R rats. After 2 h of ischemia and 24 h of reperfusion, the activities of SOD (A), GSH-Px (B), and MDA (C) contents in the ischemic boundary zone were measured. The levels of SOD, GSH-Px, and MDA were compared between the sham group and the I/R group (**P<0.01). Differences were significant when compared with the I/R group (*P<0.05, **P<0.01), n=8.

Figure 4. Biochanin A induced Nrf2 nuclear translocation in cerebral I/R rats. Protein extractions in the cytosolic and nuclear fractions from the ischemic penumbras zone of rat brain tissues were isolated, and the expression of Nrf2 was determined by Western blotting at 24 h after reperfusion. Representative Western blots and protein expression of Nrf2 in the cytosolic fractions (A) and nuclear fractions (B) were analyzed. α-Tubulin and PCNA were used as a loading control in cytosolic protein and in nuclear protein. The protein expression of Nrf2 were compared between the sham group and the I/R group (**P<0.01). Differences were significant when compared with the I/R group (*P<0.05, **P<0.01), n=3.
Figure 5. The effects of biochanin A on expression levels of HO-1 mRNA (A) and protein (B) in cerebral I/R rats. The mRNA and protein extracted from the ischemic penumbra cortex was analyzed by Western blotting at 24 h after reperfusion. The mRNA expression of HO-1 was normalized to β-actin, and the protein expression of HO-1 was normalized to α-Tubulin. The mRNA and protein expression of HO-1 were compared between the sham group and the I/R group (* P<0.05, ** P<0.01); Differences were significant when compared with the I/R group (* P<0.05, ** P<0.01), n=3.

Figure 6. The effects of biochanin A on the activity of IkBα (A) and NF-κB p65 translocation (B) in I/R rats. Protein were extracted from the ischemic penumbra cortex at 24 h after reperfusion. The nucleus and cytoplasm fractions were isolated and used for examining the nuclear translocation of NF-κB p65. The activity of IkBα and the protein expression level of NF-κB p65 were determined by Western blotting. The ratio of phosphor-IkBα to total IkBα was used to evaluate protein activity, and nucleus/cytoplasm ratio of NF-κB p65 was used for quantitative analysis of nuclear translocation. PCNA was the internal control of nuclear fraction, and GAPDH was the internal control of cytoplasm fraction. The nucleus/cytoplasm ratio was normalized to their own internal control. The activity of IkBα and NF-κB p65 translocation were compared between the sham group and the I/R group (## P<0.01). Differences were significant when compared with the I/R group (* P<0.05, ** P<0.01), n=3.
Oxidative stress is mainly caused by excess production of reactive oxygen species (ROS) and is crucial in cerebral I/R injury. In organisms, antioxidant enzymes such as SOD, GSH-Px, and catalase can inhibit oxygen free radical production and protect brain tissue against ROS cytotoxicity [28]. Additionally, excessive ROS cause lipid peroxidation and produce active aldehydes, including malondialdehyde (MDA), 4-hydroxynonenal (HNE), and acrolein [29]. MDA, which is an important marker of oxidative stress, induces more serious oxidative stress damage by destroying lipids, enzymes, and nucleic acids in biological membranes or organelles. In the present study, we found that biochanin A significantly enhanced the activity of SOD and GSH-Px antioxidant enzymes, and reduced MDA levels. These findings revealed that biochanin A effectively prevents ischemic brain injury caused by ROS-mediated oxidative damage.

Nrf2 is an important transcription factor inducing the expression of antioxidant proteins such as SOD, catalase (CAT), and HO-1 by binding to antioxidant-response elements (ARE) [30]. It is known that the upregulation of these enzymes due to Nrf2 transcription results in the cellular defense against oxidative stress triggered by hypoxia, inflammation, and other injury. HO-1 is an enzyme that catalyzes the breakdown of pro-oxidant heme into the antioxidant biliverdin, carbon monoxide, and iron. The gene expression of HO-1 is also regulated by transcription factor Nrf2. Under normal circumstances, Nrf2 is mainly located in the cytoplasm and binds to Keap1 (Kelch-like ECH associated protein 1, also termed inhibitor of Nrf2). When oxidative stress occurs, Nrf2 is dissociated from Nrf2-Keap1 complex and is translocated to the nucleus, activating the expression of its target gene, HO-1 [31]. It has been reported that the neuroprotective effect of Nrf2 activators is weakened when HO-1 expression is inhibited, and gene knockout of Nrf2 abolishes the upregulation of HO-1 [32]. In addition, researchers have reported that upregulation of HO-1 in brain tissue following ischemic preconditioning protects against cerebral ischemic injury [33], while the protective effects were weakened after treatment with the HO-1 inhibitor ZnPP [34]. Our data show that biochanin A pretreatment markedly increased Nrf2 nuclear translocation and upregulated expression of its downstream gene, HO-1. These results indicate that the activation of the Nrf2/HO-1 pathway can increase the activity of SOD and GSH-Px antioxidant enzymes induced by biochanin A.

Given the interaction between oxidative stress and neuroinflammation, the Nrf2/HO-1 signaling pathway may also regulate anti-inflammatory gene expression and inhibit progression of inflammation [35]. NF-κB is a central regulator of inflammatory response, and activation of the NF-κB signaling pathway is required for transcriptional induction of many inflammatory mediators in neuroinflammation. The most common form of NF-κB is a heterodimer of the p50 and p65 (RelA) subunits. The canonical NF-κB signaling pathway is generally activated in response to various stimuli, including cytokines, bacterial toxins, and oxygen-glucose deprivation. The initiation of this pathway depends on the phosphorylation of inhibitor of κB kinase (IKK) complex, which is composed of the kinases IKKα, IKKβ, and IKKγ. Activated IKK-complex phosphorylates the inhibitory subunit IκBα (Inhibitor κBα), promoting IκBα degradation by the proteasome and inducing the transformation of p105 into its mature form, p50. Then, p65/p50 heterodimers are released from the inhibitor IκBα and translocated into the nucleus, where they bind to the DNA κB sites to induce transcription of NF-κB-targeted genes [36]. Recent studies have shown that many upstream molecules in cerebral ischemia reperfusion can regulate the NF-κB signaling pathway, including silent information regulator 1 (SIRT1), signal transducers, and activators of transcription (STATs) [37,38]. In addition, there have been reports that the absence of Nrf2 can exacerbate NF-κB activity, leading to increased cytokine production [39,40], and NF-κB also can regulate Nrf2-mediated ARE expression, suggesting that the cross-talk between NF-κB activation and Nrf-2 inhibition results in neuroinflammation [41,42]. Therefore, the mutual promotion of neuroinflammation and oxidative stress can induce the further development of cerebral ischemia reperfusion injury. In our study, we found that biochanin A markedly inhibited the activation of IκBα and the nuclear translocation of NF-κB in ischemic brain tissue. These results indicate that the ameliorative effect of biochanin A in neuroinflammation and oxidative stress may also be partly related to inhibition of the NF-κB signaling pathway. Our study, however, has potential limitations. First, our pretreatment strategy is intended for prevention, not therapy, and it would have been of greater clinical value if the biochanin A treatment had been administered after establishment of the MCAO model in the experiment. Second, we merely observed changes of the Nrf2/HO-1 and NF-κB signaling pathway in ischemia/reperfusion brain injury. Finally, the details of the relationship between oxidative stress and neuroinflammation should be explored further.

Conclusions

Our findings suggest that biochanin A is a therapeutic candidate for ischemic stroke because of its anti-oxidative and anti-inflammatory properties. It provides promising targets for attenuating neurological impairment in stroke. However, whether biochanin A directly targets Nrf2 or NF-κB signaling molecules requires further investigation, and in vivo and in vitro experiments are needed to elucidate the specific mechanisms involved.

Conflict of interest

None.
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ANIMAL STUDY

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