Research Report

Curcumin upregulates transcription factor Nrf2, HO-1 expression and protects rat brains against focal ischemia

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ABSTRACT

Background: Oxidative and cytotoxic damage plays an important role in cerebral ischemic pathogenesis and may represent a target for treatment. Curcumin is proved to elicit a vanity of biological effects through its antioxidant and anti-inflammatory properties. But the mechanisms underlying are poorly understood. The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) coordinates expression of genes required for free radical scavenging, detoxification of xenobiotics, and maintenance of redox potential. This study evaluated the time course expression regularity of Nrf2, HO-1 and the curcumin’s role in cerebral ischemia and its potential mechanism. Methods: Male, Sprague-Dawley rats were subjected to permanent focal cerebral ischemia by right MCA occlusion. Experiment 1 was used to evaluate the expression of Nrf2 and HO-1 in the cerebral ischemia, 6 time points was included. Experiment 2 was used to detect curcumin’s neuroprotection in cerebral ischemia. At 24 h neurological deficit was evaluated using a modified six point scale; brain water content was measured; infarct size was analysed with 2, 3, 5-triphenyltetrazolium chloride (TTC). Immunohistochemistry, RT-PCR, Western blot, and confocal microscope were used to analyse the expression of Nrf2 and HO-1. Results: Compared with sham-operated, Nrf2 and HO-1 were upregulated at gene and protein level in ischemic brain, beginning at 3 h and peaking at 24 h after MCAO (P < 0.05). Curcumin high dose (100 mg/kg) upregulated Nrf2 and HO-1 in MCAO-affected brain tissue and reduced infarct volume (P < 0.05), brain water content (P < 0.05) and behavioral deficits (P < 0.05) caused by MCAO. Conclusions: Nrf2 and HO-1 were induced at the early stage after MCAO. Curcumin protected the brain from damage caused by MCAO, this effect may be through upregulation of the transcription factor Nrf2 expression. Nrf2 may be one of the strategic targets for cerebral ischemic therapies.

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1. Introduction

Oxidative stress leading to ischemic cell death involves the formation of reactive oxygen species/reactive nitrogen species (ROS/RNS) through multiple injury mechanisms, such as mitochondrial inhibition, Ca2+ overload, and inflammation (Coyle and Puttfarcken, 1993; Lipton, 1999; Lewen et al., 2000). The close relationship between oxidative stress and cerebral
ischemia has generated considerable interest in developing antioxidant therapies to combat ischemia-induced damage (Ikeda et al., 2003; Simonyi et al., 2005). Nuclear factor erythroid 2-related factor 2 (Nrf2), a cap ‘n’ Collar (CNC) transcription factor, regulates an expansive set of antioxidant/detoxification genes acting in synergy to remove ROS/RNS through sequential enzymatic reactions (Ishii et al., 2000; Shih et al., 2003).

Nrf2 controls the coordinated expression of important antioxidant and detoxification genes (Phase II genes) through a promoter sequence termed the antioxidant response element (ARE) (Ishii et al., 2000; Kobayashi and Yamamoto, 2005). Phase II genes, including heme oxygenase-1 (HO-1), glutathione S-transferases (GSTs) and NAD(P)H quinone oxidoreductase, work in synergy to constitute a pleiotropic cellular defense that scavenges reactive oxygen/nitrogen species (ROS/RNS), detoxifies electrophiles and xenobiotics, and maintains intracellular reducing potential (Satoh et al., 2006; Lee et al., 2003). HO-1 is a ubiquitous and redox-sensitive inducible stress protein that degrades heme to CO, iron and biliverdin (Motterlini et al., 2002). The importance of this protein in physiological and pathological states is underlined by the versatility of HO-1 inducers and the protective effects attributed to heme oxygenase products in conditions that are associated with moderate or severe cellular stress.

Curcumin (CUR), the yellow, bioactive component of turmeric, the powdered rhizome of Curcuma longa Linn, has been shown to elicit a variety of biological effects through its antioxidant and anti-inflammatory properties (Sreepriya and Bali, 2006; Al-Omar et al., 2006; Kim et al., 2008). But the mechanisms underlying are poorly understood. Here, we analysed whether the CUR had the neuroprotection and its potential mechanism in the rat MCAO model.

2. Results

2.1. Nrf2 and HO-1 were upregulated in cerebral ischemia

Immunohistochemistry, RT-PCR, and Western blot were used to detect the expression of Nrf2 and HO-1 in brain tissue from 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h after MCAO, confocal microscope for Nrf2 at 24 h. We found that Nrf2 and HO-1 were upregulated compared with normal-control (P<0.05 Figs. 4a, c; Figs. 5a, c; Figs. 6a, c) and sham-operation (P<0.05), beginning at 3 h, and peaking at 24 h after MCAO. The result of immunohistochemistry was coincident with RT-PCR and Western blot.

2.2. CUR reduced neurologic deficits after MCAO in rats

This model for MCAO causes measurable NDS (Longa et al., 1989). Neurological deficit was examined and scored on a 6 point scale at 24 h after MCAO and Mann–Whitney U test analysis was conducted. In CUR-H group, neurologic deficit scores were significantly reduced (P<0.05) compared with vehicle control group. CUR-L reduced the scores, but did not reach a significance level (Fig. 1).

2.3. CUR reduced the infarct volume

We observed infarct size at 24 h after stroke using vital staining with 2, 3, 5-triphenyltetrazolium chloride (TTC). No infarction was observed in sham-operated group, while extensive lesion was developed in both striatum and lateral cortex in vehicle control group. In CUR-H group, infarct size (% HLH) was significantly reduced from 44.88±3.36 to 28.01±6.10 (P<0.05). But CUR-L did not display this significant neuroprotective effect compared with CUR-H group (P<0.05) (Fig. 2).

2.4. CUR reduced the brain water content

We observed brain water content at 24 h after stroke using the standard wet–dry method (Hatashita et al., 1988). CUR could reduce the brain water content of ipsilateral hemispheres. In the sham-operated group, water content was 77.60%±0.37%. In CUR-H group, the brain water content reduced (81.00%±0.53%, P<0.05) compared with vehicle controls (83.68%±0.90%). But no statistical significance was observed in CUR-L group (Fig. 3).
The results showed that systemic administration of CUR to cerebral ischemic rats significantly increased the expression of Nrf2 and HO-1 at gene and protein level (Figs. 4, 5, 6). Immunohistochemistry showed that the expression of Nrf2 and HO-1 was upregulated at 24 h after ischemia treated with CUR. In sham-operated group, few cells stained by Nrf2 and HO-1 were seen in the cortex. In vehicle control group, the number of cells stained by Nrf2 and HO-1 increased in the ischemic cortex, and Nrf2 expressed both at cytoplasm and nucleus. The number of cells labeled with Nrf2 and HO-1 in CUR-H group was significantly increased compared with vehicle control group (P<0.05), and a lot of cells labeled with Nrf2 in the nucleus increased. But CUR-L did not reach a significance level.

Western blotting analysis of protein level of Nrf2 in nuclear and HO-1 in cytosolic extracted from rat cortex. Nrf2 in nuclear and HO-1 in cytosolic were upregulated at 24 h after MCAO. After treatment with curcumin high dose, the protein expression of Nrf2 in nuclear and HO-1 in cytosolic was significantly increased vs vehicle control (P<0.05) (Figs. 5b, d; Figs. 6b, d).

In agreement with the results of immunohistochemistry and Western blot, the mRNA expression of Nrf2 and HO-1 were upregulated in vehicle control and significantly induced by treatment with curcumin high dose (P<0.05). But curcumin low dose did not display this effect (Figs. 4b, d).

We next examined which cell types and location of Nrf2 expression at 24 h after MCAO using confocal microscope. Quantification of immunofluorescent intensity showed a significant increase in Nrf2 immunoreactivity in CUR-H treated animals as compared to vehicle control (Figs. 7 and 8). And enhanced Nrf2 were localized in nucleus and cytoplasm of both neurons and astrocytes (Figs. 7 and 8).
Fig. 5 – Representative photographs of Western blot of dynamic expression (a) and CUR induction (b) of Nrf2 protein and actin control in the rat brain after MCAO (n=3). (c) Bar graph illustrating the protein dynamic expression of Nrf2. *P<0.05 vs normal-control. ×P<0.05 vs 3 h, 6 h, 12 h, 48 h, 72 h groups. (d) Bar graph illustrating the CUR induction to protein of Nrf2. *P<0.05 vs vehicle control and CUR-L.

Fig. 6 – Representative photographs Western blot of dynamic expression (a) and CUR induction (b) of HO-1 protein and actin control in the rat brain after MCAO (n=3). (c) Bar graph illustrating the protein dynamic expression of HO-1. *P<0.05 vs normal-control. ×P<0.05 vs 3 h, 6 h, 12 h, 48 h, 72 h groups. (d) Bar graph illustrating the CUR induction to protein of HO-1. *P<0.05 vs vehicle control and CUR-L.
3. Discussion

MCAO was a well-characterized and classical model inducing cerebral ischemia in rats (Longa et al., 1989; Cheatwood et al., 2008). Evidence of increased oxidative damage is observed in this model (Ikeda et al., 2003; Simonyi et al., 2005; Liu et al., 2004). The cytotoxic response occurs within minutes from the onset of cerebral ischemia and encompasses oxidative stress, proinflammatory responses, cell death, and neurologic damage (Lipton, 1999; Ikeda et al., 2003; Simonyi et al., 2005; Chan, 2001). This model allows us to investigate the role of Nrf2 specifically during oxidative stress/ischemia-mediated brain injury.

In recent research, the Nrf2/ARE signaling pathway has been gaining recognition as a key contributor to the cellular response to neuronal injury in vitro and in vivo (Calkins et al., 2004). The cytotoxic response occurs within minutes from the onset of cerebral ischemia and encompasses oxidative stress, proinflammatory responses, cell death, and neurologic damage (Lipton, 1999; Ikeda et al., 2003; Simonyi et al., 2005; Chan, 2001). This model allows us to investigate the role of Nrf2 specifically during oxidative stress/ischemia-mediated brain injury.

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4. Experimental procedures

4.1. Experiment 1: the time course expression of Nrf2 and HO-1 in the brain tissue after MCAO

Three groups were studied, including normal-control, sham-operated group, and the MCAO group. The last two groups included 3 h, 6 h, 12 h, 24 h, 48 h and 72 h sub-groups.
4.2. **Experiment 2: curcumin’s neuroprotection against damage from cerebral ischemia**

Curcumin was injected intraperitoneally 15 min after MCAO. Rats were reanesthetized and killed 24 h after MCAO. In this part, rats were divided into 4 groups randomly. Group 1: rats as sham-operated group that received equal volume PBS including 1% DMSO; group 2: vehicle controls that received equal volume PBS including 1% DMSO after MCAO; group 3: Curcumin low dose (CUR-L) rats that received CUR at 50 mg/kg after MCAO; and group 4: Curcumin high dose (CUR-H) rats that received CUR at 100 mg/kg after MCAO.

4.3. **Rat model of permanent focal cerebral ischemia**

Male Sprague–Dawley rats weighing 250 to 280 g were used in this study. They were housed under temperature-controlled conditions with a 12-h-light/12-h-dark cycle and ad libitum access to water and food. The right MCA was occluded by intraluminal placement a filament, as described previously (Longa et al., 1989; Cheatwood et al., 2008). In brief, rats were anesthetized with pentobarbital sodium (50 mg/kg) intraperitoneally. The CCAs were exposed and isolated. MCA was occluded by inserting a filament into the internal carotid artery, which was advanced further until it closed the origin of the MCA. Sham-operated rats underwent the same surgical procedures without inserting a filament.

4.4. **Administration of CUR**

CUR was from Sigma (St. Louis, MO, USA). CUR low dose at 50 mg/kg or high dose 100 mg/kg in PBS including 1% DMSO was injected intraperitoneally at 15 min after MCAO.

4.5. **Analysis of neurologic deficit scores**

A neurologic test was carried out by an examiner blinded to the experimental groups before they were killed. The deficits were scored on a modified scoring system based on that developed by Longa et al. (1989), as follows: 0, no deficits; 1, difficulty in fully extending the contralateral forelimb; 2, unable to extend the contralateral forelimb; 3, mild circling to the contralateral side; 4, severe circling; and 5, falling to the contralateral side.

4.6. **Measurement of infarction**

At 24 h after neurologic test, rats were reanesthetized and the brains were removed quickly. Coronal brain sections (2 mm thick) were stained with TTC at 37 °C for 20 min (Bederson et al., 1986), followed by fixation with 4% paraformaldehyde. The stained cerebral sections were photographed, ipsilateral and contralateral hemispheric volumes and infarct volumes were quantified with the use of Image Pro-Plus 5.1 analysis system. To compensate for the effect of brain edema, %HLV were

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Fig. 7 – Confocal microscope for Nrf2 in neurons in rats with or without CUR (50 mg/kg or 100 mg/kg, ip) at 24 h after MCAO. Tissues were triple-stained with anti-Nrf2, anti-SMI32 and Hoechst to mark neurons and nucleus. Scale bar, 30 μm. The expression of Nrf2 in neurons was induced in CUR-H (c) vs vehicle control (a) and CUR-L (b), (a1), (b1), and (c1) showed Nrf2; (a2), (b2), and (c2) showed Nrf2 merged SMI32; (a3), (b3), and (c3) showed Nrf2 merged SMI32 and Hoechst.
calculated by the following equations, based on that used by Tatlisumak et al. (1998). %HLV=[(total infarct volume−(right hemisphere volume−left hemisphere volume)]/left hemisphere volume/×100%. Infarct volume measurements were carried out by an investigator blind to the treatment groups.

4.7. Measurement of brain water content

Brain water content was measured using the standard wet-dry method (Hatashita et al., 1988). Rats were reanesthetized and the brains were removed quickly 24 h after MCAO. A coronal brain slice (about 3 mm thick) 4 mm from the frontal pole was cut and the slice was divided into the ipsilateral and contralateral hemispheres. Brain samples were immediately weighed on an electronic balance to obtain wet weight. Then brain samples were dried in an oven at 100 °C for 24 h to obtain the dry weight. Brain water content was calculated as (wet weight−dry weight)×100/wet weight.

4.8. Immunohistochemistry

Brains were removed quickly, immersed with 4% paraformaldehyde in PBS for 24 h at 4 °C. Brain sections (5-μm thick) were blocked in 3% H₂O₂, 3% normal goat serum, and incubated with Nrf2 rabbit polyclonal antibodies (1:100, Santa Cruz Biotechnology), and HO-1 rabbit polyclonal antibody (1:200, Stressgen Biotechnologies, Victoria, Canada) in 0.01 mol/L phosphate-buffered saline over night. The secondary antibodies, secondary biotinylated conjugates and diaminobezidine were from the Vect ABC kit (Zhongshan Biology Technology Company, China). Five visual fields of ischemic region of the infarct were selected and the immunoreactive cells were counted under a 400× light microscope.

4.9. Western blot

Protein extraction for Nrf2 and HO-1 was performed as follows. The tissue was homogenized in ice-cold lysis buffer (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.1 mmol/L EGTA) for 15 min. After adding NP-40, centrifuged 10,000 rpm at 4 °C for 3 min, collected the supernatant as cytoplasmic protein for HO-1. The pellets were homogenized in ice-cold lysis buffer (20 mmol/L HEPES, pH 7.9, 400 mmol/L NaCl, 1 mmol/L EDTA, 0.1 mmol/L EGTA) for 15 min. Then centrifuged 12,000 rpm at 4 °C for 10 min, collected the supernatant and added PMSF to the final concentration 1 mmol/L as the nuclear protein for Nrf2.

The protein concentration of the supernatant was determined using a BCA Protein Assay reagent kit (Novagen, Madison, WI, USA). 50 μg of protein was separated by SDS-PAGE, transferred 2 h on to PVDF membranes, and the non-specific binding of antibodies was blocked with 5% non-fat dried milk in PBS. Membranes were then probed with polyclonal rabbit anti-Nrf2 antibody (1:200, Santa Cruz Biotechnology) and polyclonal rabbit anti-HO-1 antibody (1:500, Stressgen Biotechnologies, Victoria, Canada) overnight at 4 °C.

Fig. 8 – Confocal microscope for Nrf2 in astrocytes in rats with or without CUR (50 mg/kg or 100 mg/kg, ip) at 24 h after MCAO. Tissues were triple-stained with anti-Nrf2, anti-GFAP and Hoechst to mark astrocytes and nucleus. Scale bar, 30 μm. The expression of Nrf2 in astrocytes was induced in CUR-H (c) vs vehicle control (a) and CUR-L (b). (a1), (b1), and (c1) showed Nrf2; (a2), (b2), and (c2) showed Nrf2 merged GFAP; (a3), (b3), and (c3) showed Nrf2 merged GFAP and Hoechst.
After four washes with TPBS, IRDye® 800-conjugated goat anti-rabbit second antibody (1:3000, Rockland, Gilbertsville) was incubated with membranes for 1 h at room temperature. The relative density of bands was analysed on an Odyssey infrared scanner (LI-COR Bioscience). The densitometric values were normalized with respect to the values of actin immunoreactivity to correct for any loading and transfer differences between samples.

4.10. Reverse transcription-polymerase chain reaction

RT-PCR was used to analyse the levels of Nrf2 and HO-1 mRNA. At the 5 time points after MCAO, rats were reanesthetized and brains were removed and frozen in liquid nitrogen. Total RNA from cortex supplied by right MCA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Forward and reverse primers were 5′-TTCCCTCTGCTGCCATTAGTACGT-C′ and 5′-GCTTCCATCTCCAGTGACGTACTG-3′ for Nrf2, 5′-CTGGAAGAGGAGATAGAGCGAA-3′ and 5′-TCTTAGGCTTTCTGTCACCCT-3′ for HO-1, and 5′-GCCATGACCCAGGACATCCA-3′ and 5′-GAACCGCTATTGGCGGATAG-3′ for β-actin. Reverse transcription used reagents from Promega following the manufacturer’s instructions. 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.

4.11. Confocal microscope

Rats were transcardially perfused with saline quickly followed by 4% paraformaldehyde in PBS. Fixed frozen cerebral sections (30-µm thick) were blocked with 10% horse serum for half an hour and then incubated in the primary antibodies, rabbit anti-Nrf2 (1:100, Santa Cruz Biotechnology) together with mouse anti-GFAP (1:500, Chemicon, Temecula), or rabbit anti-Nrf2 together with mouse anti-SMI32 (1:5000, Sternberger Monoclonals Incorporation, Lutherville) antibody for 2 h at 4 °C. After 3 washes by PBS, these were correspondingly incubated with FITC- and TRITC-conjugated secondary antibodies for Nrf2 (1:100, Santa Cruz Biotechnology) together with mouse anti-GFAP, or rabbit anti-Nrf2, mouse anti-GFAP, or rabbit anti-Nrf2 together with anti-mouse RI-2 (1:5000, Sternberger Monoclonals Incorporation, Lutherville) antibody for 2 h at 4 °C. Immunofluorescence was visualized using a Laser Scanning Confocal Microscope (Olympus FV10-ASW, Japan).

4.12. Statistical analysis

Quantitative data were expressed as mean±SD. Statistical comparisons were conducted using one way ANOVA followed by SNK and LSD tests for intergroup comparisons. For neurological deficit, Mann–Whitney U test was used for comparisons between two groups. Differences with P<0.05 were considered statistically significant.

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