

available at www.sciencedirect.comwww.elsevier.com/locate/brainresBRAIN
RESEARCH

Research Report

1, 5-Dicaffeoylquinic acid-mediated glutathione synthesis through activation of Nrf2 protects against OGD/reperfusion-induced oxidative stress in astrocytes

Xu Cao^{a,b}, Haibing Xiao^b, Ying Zhang^a, Liangyu Zou^a, Yinghao Chu^c, Xiaofan Chu^{a,*}

^aDepartment of Neurology, Second Clinical College, Jinan University, Shenzhen, 518020, People's Republic of China

^bDepartment of Neurology, Union Hospital, Huazhong University of Science and Technology, Wuhan, 430022, People's Republic of China

^cDepartment of Physics, The Hong Kong University of Science and Technology, People's Republic of China

ARTICLE INFO

Article history:

Accepted 24 May 2010

Keywords:

Ischemia/reperfusion

Nrf2

1, 5-Dicaffeoylquinic acid

Glutathione

Oxidative stress

ABSTRACT

Oxidative stress plays an important role in pathological processes of cerebral ischemia followed by reperfusion. The effect of 1, 5-dicaffeoylquinic acid (1, 5-diCQA) on primary culture rat cortical astrocytes induced by oxygen and glucose deprivation (OGD)/reperfusion was evaluated in this study. Appropriate concentration of 1, 5-diCQA pretreatment significantly suppressed cell death, reduced the production of reactive oxygen species, prevented glutathione (GSH) depletion, increased the activity of glutamate-cysteine ligase (GCL), and triggered Nrf2 nuclear translocation in astrocytes induced by 4 h of OGD and 20 h of reperfusion. Interestingly, these protective effects were greatly attenuated in Nrf2 siRNA-transfected cells. We conclude that 1, 5-diCQA has antioxidant signaling properties that upregulate GSH synthesis by stimulating the Nrf2 pathway in astrocytes and protects them from cell death in an *in vitro* model of ischemia/reperfusion.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Although the precise mechanisms involved after ischemic damage are unclear, a number of complex biochemical events appear to be involved, such as calcium overload, cellular acidosis, energy depletion, ion homeostasis breakdown, and so on (Pfeiffer-Guglielmi et al. 2003; Diemel and Hertz, 2005; Juurlink, 1997). Oxygen and glucose deprivation (OGD) can induce cellular ATP depletion and astrocyte death via the massive generation of free radicals and reactive oxygen species (ROS) such as lipid hydroperoxyl, hydroxyl, peroxyxynitrite, and hydrogen peroxide (Ouyang and Giffard, 2004; Haskew-Layton et al. 2005). Oxidative stress is

considered a linchpin of the pathophysiology of ischemic injury that leads to delayed neuronal and astrocyte death (Lerouet et al. 2002; Fisk et al. 2007), and a large number of researchers are working to learn how to protect astrocytes from injuries induced by OGD.

In the course of evolution, cells have developed a rather sophisticated defense system of detoxification and antioxidants. Nuclear erythroid-related factor 2 (Nrf2), a redox-sensitive transcription factor, is involved in the regulation of many detoxification and antioxidant genes (Nguyen et al., 2003). Activated Nrf2 dissociates from its repressor protein (Keap1), translocates into the nucleus, interacts with antioxidant response elements (ARE), and induces

* Corresponding author. Fax: +86 755 25521180.

E-mail address: chuxiaofan@hotmail.com (X. Chu).

subsequent expression of numerous downstream genes including NAD(P)H/quinone oxidoreductase-1 (NQO1) GCL (Mulcahy and Gipp, 1995), and heme oxygenase-1 (HO-1) (Prester et al., 1995). Agents that stimulate the Nrf2/ARE pathway and related antioxidant gene expression would therefore be expected to be a promising approach for neuroprotection after cerebral ischemia.

Recently, much attention has been focused on natural antioxidants in fruits and vegetables (Yang et al., 2009; Kode et al., 2008). Caffeoylquinic acid derivatives are natural compounds that can be isolated from many kinds of traditional medicinal herbs and that possess a broad spectrum of healing properties, such as antioxidants, anti-HIV, antifibrosis, neuroprotective effects, and other biological activities (Hung et al., 2006; Nakajima et al., 2007; Park, 2009). Several researchers have observed different cell lines treated by caffeoylquinic acid derivatives, and these compounds exhibited remarkable protective properties, especially antioxidative potential. However, unequivocal proof of this effect is still lacking.

To discover mechanisms for the protective action of caffeoylquinic acid derivatives, 1, 5-dicaffeoylquinic acid (1, 5-diCQA), a caffeoylquinic acid derivative, was used for this study. We examined 1, 5-diCQA's protective effect on astrocytes with OGD/reperfusion-induced damage and demonstrated the molecular mechanism of 1, 5-diCQA's antioxidative effect as well as the importance of Nrf2 activation in protecting astrocytes.

2. Results

2.1. 1, 5-DiCQA pretreatment inhibits cell death, suppresses ROS production, and prevents GSH depletion in astrocytes induced by OGD/reperfusion

Inducing cells using OGD/reperfusion led to a $50.61\% \pm 6.50\%$ reduction in cell viability, as determined using the MTT method. Compared to the control, the cell viability of groups pretreated with 1, 5-diCQA at different concentrations (10 μM , 20 μM , 50 μM , and 100 μM) before OGD/reperfusion significantly increased to $58.67\% \pm 7.17\%$, $64.04\% \pm 5.49\%$, $74.20\% \pm 5.43\%$, and $77.47\% \pm 6.61\%$, respectively (Fig. 1A). A similar tendency could be seen in the results for LDH release (Fig. 1B). These data indicate that appropriate concentrations of 1, 5-diCQA pretreatment can reduce cell death and elevate cell viability.

To elucidate the antioxidant ability of 1, 5-diCQA, the production of ROS and intracellular GSH levels was measured. OGD/reperfusion induced a significant accumulation of ROS; however, a marked decrease in ROS was observed in 1, 5-diCQA (10 μM , 20 μM , 50 μM , and 100 μM) pretreatment groups, suggesting that 1, 5-diCQA has the ability to quench free radicals (Fig. 1D). The average GSH content in the control was 13.86 ± 1.7 nmol/mg protein. After OGD/reperfusion, the GSH content decreased to 5.20 ± 0.90 nmol/mg protein. 1, 5-DiCQA (10 μM , 20 μM , 50 μM and 100 μM) significantly prevented the depletion of GSH under OGD/reperfusion insult (Fig. 1C).

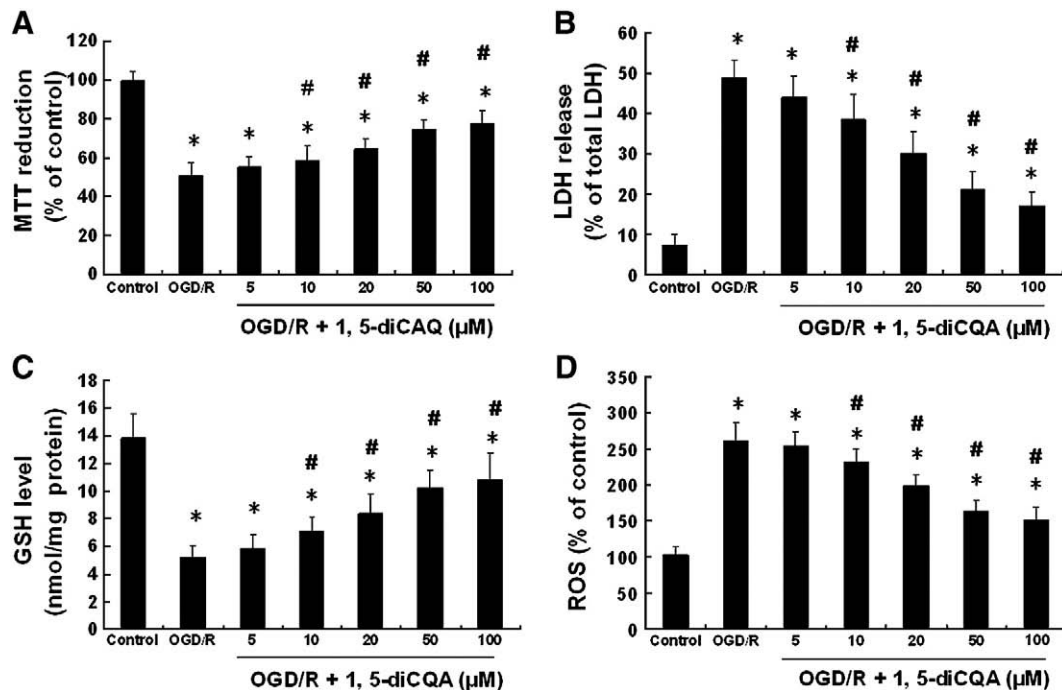


Fig. 1 – Modulation of OGD/reperfusion-induced cell death and oxidative damage by 1, 5-diCQA. Astrocytes induced by OGD/reperfusion in the absence or presence of various concentrations of 1, 5-diCQA. Cell survival was measured using the MTT assay (A) and LDH release (B). The GSH content and ROS levels are shown in C and D, respectively. Data are shown as mean \pm SD from three separate experiments; * $p < 0.05$ compared to control; # $p < 0.05$ compared to OGD/reperfusion.

There were no significant differences in MTT reduction, LDH release, ROS production, or GSH depletion between groups pretreated with 1, 5-diCQA at 50 μ M and 100 μ M; therefore, 50 μ M 1, 5-diCQA was used in subsequent experiments.

2.2. 1, 5-DiCQA induced nuclear translocation of Nrf2 in OGD/reperfusion treated astrocytes

Western blotting analysis was performed to determine the nuclear translocation of Nrf2 in response to OGD/reperfusion with or without 1, 5-diCQA pretreatment. Pretreatment of 50 μ M 1, 5-diCQA effectively decreased the cytoplasmic Nrf2 with augmentation of nuclear Nrf2. However, OGD/reperfusion resulted in the significant reduction of both cytoplasmic and nuclear Nrf2. It was found that the relative expression level of cytoplasmic Nrf2 of the 1, 5-DiCQA+OGD/reperfusion group was lower than that of the OGD/reperfusion group, and besides, the level of nuclear Nrf2 was significantly higher than that of OGD/reperfusion group (Fig. 2). 1, 5-DiCQA may induce Nrf2 translocation into nuclei to activate the ARE–Nrf2 pathway.

2.3. 1, 5-DiCQA induced increased GCL activity, and the effect was lost in Nrf2 siRNA-transfected cells

The de novo synthesis of GSH depends on the activity of GCL. Having established that 1, 5-diCQA maintains the content of intracellular GSH, we next measured GCL activity as an indicator of ARE–Nrf2 activity in astrocytes. Data showed that GCL activity was suppressed by OGD/reperfusion and significantly improved by 1, 5-diCQA pretreatment (Fig. 3C). To determine the role of Nrf2 in the regulation of GCL, we used Nrf2 siRNA to knock down the expression of Nrf2 in astrocytes. The effect of the knockdown was confirmed by Western

blotting analysis. In cells transfected with nontargeting siRNA, no evidence of silencing was seen, and Nrf2 levels were similar to the control (Figs. 3A and B). In Nrf2 siRNA-transfected cells, the marked drop in GCL activity suggested that the Nrf2 knockdown resulted in downregulation of GCL activity. Similarly, 1, 5-diCQA-mediated upregulation of GCL activity was not seen in Nrf2 knockdown astrocytes (Fig. 3C).

2.4. Knockdown of Nrf2 eliminated the protective effects of 1, 5-diCQA

To further confirm the important effect of Nrf2 in antioxidative stress, cell viability and GSH levels were measured in Nrf2-silenced astrocytes induced by OGD/reperfusion with or without 1, 5-diCQA pretreatment. Compared to the OGD/reperfusion group, knockdown of Nrf2 almost completely abrogated the protective effect of 1, 5-diCQA because the cell viability and GSH level were both slightly changed (Fig. 4).

3. Discussion

In the present study, we investigated the mechanism of OGD/reperfusion-mediated oxidative stress and the protective role of 1, 5-diCQA in primary culture rat astrocytes. It has been established that a primary factor in the initiation of the pathological response to ischemia injury is the generation of ROS, and the increase in the levels of ROS produced upon ischemia/reperfusion appears to be essential for the development of astrocyte dysfunction and delayed death (Chan, 1996; Piantadosi and Zhang, 1996; Bains and Shaw, 1997; Liu et al., 1999). Therefore, the use of antioxidants may be effective targets for intervention (Dawson et al., 1994). In this experiment, according to the MTT assay and ROS measurement, pretreatment with proper concentration of 1, 5-diCQA before

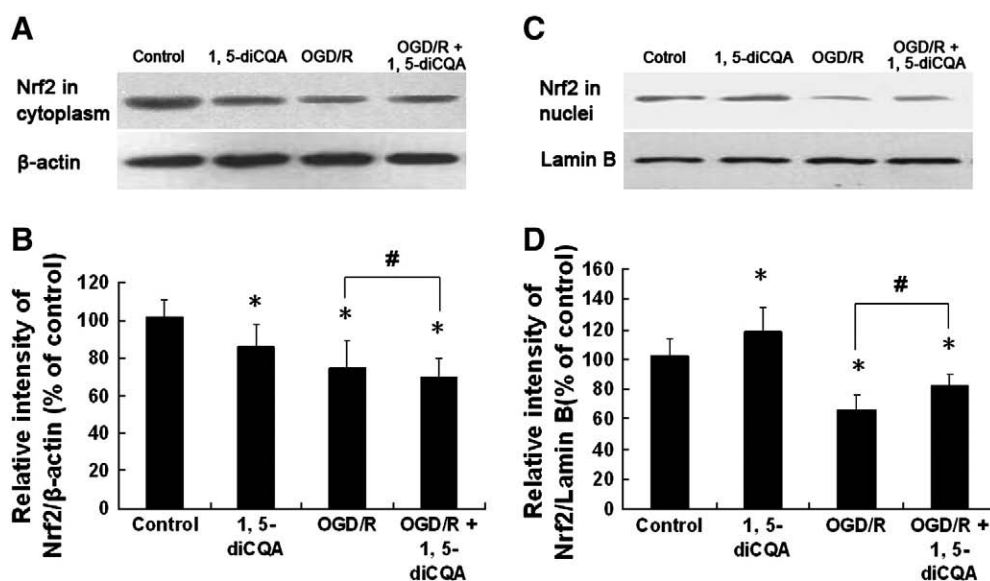


Fig. 2 – Effect of 1, 5-diCQA on the nuclear translocation of Nrf2 in astrocytes induced by OGD/R. (A and C) Representative pictures of Nrf2 expression in the cytoplasm and nuclei of astrocytes, respectively. (B and D) Relative density of Nrf2 protein levels in astrocytes. Data are shown as mean \pm SD from three separate experiments; * p < 0.05 compared to control; # p < 0.05 compared to OGD/reperfusion.

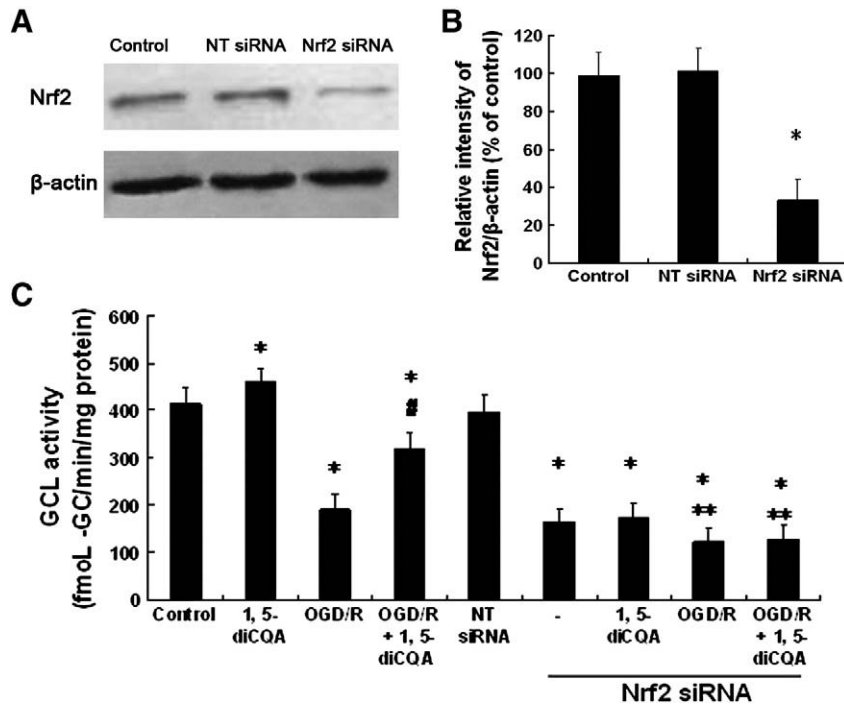


Fig. 3 – Pretreatment of 1, 5-diCQA affected GCL activity, and the effect was lost in Nrf2-knockdown astrocytes. (A and B) Knockdown of Nrf2 led to decreased Nrf2 protein levels in astrocytes. (C) Changes in GCL activity in each group. Data are shown as mean \pm SD from three separate experiments; * p < 0.05 compared to control; # p < 0.05 compared to OGD/reperfusion; ** p < 0.05 compared to Nrf2 siRNA-transfected astrocytes.

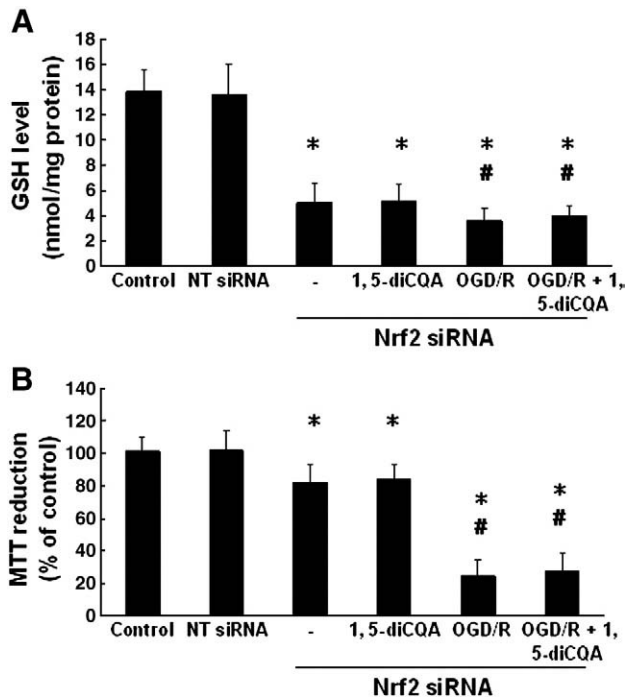


Fig. 4 – The protective effect of 1, 5-diCQA was lost in Nrf2-knockdown astrocytes. GSH levels and cell viability are shown in A and B, respectively. Data are shown as mean \pm SD from three separate experiments; * p < 0.05 compared to control; # p < 0.05 compared to the Nrf2 siRNA-transfected astrocytes.

OGD damage can significantly reduce cell death. We speculate that the protective effect was due to the antioxidant properties of 1, 5-diCQA.

GSH is a central component in the antioxidant defense of cells, acting both to directly detoxify ROS and as a substrate for various peroxidases (Schulz et al., 2000; Dringen, 2000; Fernández-Checa et al., 1991). In comparison with OGD/reperfusion damage alone, we found that 1, 5-diCQA pretreatment significantly decreased OGD/reperfusion-induced ROS production and maintained the level of cellular GSH, which was associated with increased levels of GCL activity. This suggests that 1, 5-diCQA can increase the de novo synthesis of GSH.

Nrf2 is the principal transcription factor that regulates antioxidant response element-mediated expression of phase II detoxifying antioxidant enzymes (Moi et al., 1994). Activation of Nrf2 can result in neuroprotection through translocation into the nucleus from the cytoplasm, interaction with antioxidant response elements, and subsequent expression of numerous antioxidant genes (Li et al., 1995). In the present study of OGD-injured astrocytes, 1, 5-diCQA pretreatment facilitated the activation and nuclear translocation of Nrf2. It is easy to conclude that the upregulation of GCL activity induced by 1, 5-diCQA may occur through an Nrf2-dependent mechanism. For verification of this theory, Nrf2 knockdown astrocytes were used. Our data showed that siRNA-mediated knockdown of Nrf2 resulted in a decrease in GCL activity and that 1, 5-diCQA lost the ability to upregulate GSH levels in astrocytes injured by OGD/reperfusion. These findings consolidated our hypothesis that 1, 5-diCQA plays a protective role in OGD/reperfusion damage by inducing the de novo synthesis of GSH through an

Nrf2-dependent mechanism; therefore, pharmacologic stimulation of antioxidant gene expression may be a promising approach to neuroprotection in cerebral ischemia.

In conclusion, our experiments revealed that 1, 5-diCQA rescues astrocytes from death caused by OGD/reperfusion *in vitro* by quenching ROS and by maintaining the GSH content through activation of Nrf2 nuclear translocation. Although research has confirmed that GSH loss is a critical step in the development of infarction (Moi et al., 1994; Anderson and Sims, 2002), focusing on a simple enzymatic mechanism may provide only a partial answer since there are multiple endogenous antioxidants participating in the pathogenesis of ischemia (Jaiswal, 2000; Liu et al., 2007). Whether other antioxidant agents can be upregulated by 1, 5-diCQA-induced Nrf2/ARE pathway activation in astrocytes is still an enigmatic question. Further studies are needed to elucidate the precise mechanisms by which 1, 5-diCQA blocks the death of astrocytes. The findings of this study may lead to a novel therapeutic strategy to reduce ischemic and/or reperfusion-related neuronal injuries.

4. Experimental procedures

4.1. Primary culture of rat cerebral cortical astrocytes

Astrocytes were obtained from neonatal rats using a modification of a previously described method (Ciccarelli et al., 2004; Suzuki et al., 2009). Briefly, cerebral cortices were harvested from neonatal Sprague-Dawley rats. The dissociated cells were seeded onto poly-L-lysine-coated 50-cm² flasks (Corning, USA) at a density of 2×10^6 cells/ml and cultured in high-glucose DMEM (Gibco, USA) supplemented with 10% fetal calf serum, 10% newborn calf serum (Gibco, USA), and 2 mM glutamine (complete medium). The medium was changed every 3 days. After 10 days or so, the confluent cultures were agitated at $200 \times g$ at 37 °C for 12 h to separate astrocytes from the remaining microglia and oligodendroglia. The adherent cells were replated in the complete medium. More than 95% of the cultured cells were astrocytes, as identified by immunofluorescent staining for glial fibrillary acidic protein (GFAP) (Santa Cruz, USA). The following experiments were performed on *in vitro* cultures aged between 18 and 21 days when they reach maximal sensitivity to cell death caused by OGD.

4.2. *In vitro* model of ischemia and 1, 5-diCQA pretreatment

The processing of an *in vitro* model of ischemia/reperfusion was described previously (Bondarenko and Chesler, 2001; Yu et al. 2001; Lee et al. 2009). The cultures were washed three times with deoxygenated glucose-free DMEM and placed into an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA) with an atmosphere of 10% H₂, 85% N₂, and 5% CO₂ for 4 h. Then the glucose-free DMEM was replaced with complete medium, and the culture plates were maintained at 37 °C in a humidified 5% CO₂-containing atmosphere for an additional 20 h. As a pretreatment, different concentrations of 1, 5-diCQA (Academy of Military Medical Sciences, Beijing, China) were added to the media before OGD for 2 h.

4.3. Assessment of cell survival and cell viability

The magnitude of LDH efflux has been shown to correlate in a linear dependency with the extent of cell injury (Goldberg et al., 1987). The colorimetric method quantifies cell injury based on the release of LDH from the cytosol of damaged cells using a CytoTox96 nonradioactive cytotoxicity assay kit (Promega, USA) according to the manufacturer's instructions. The released LDH can be normalized as a percentage of the total LDH that was in the cells before injury.

The viability of astrocytes was measured by the MTT reduction method. Briefly, the cells were rinsed with phosphate-buffered saline, pH 7.2, and incubated with 5 mg/ml MTT reagent for 3 h at 37 °C. The medium was removed, and the cells were lysed with 1 ml of dimethyl sulfoxide. The absorbance was measured at 540 nm by a microplate reader (Synergy 2, Bio-tek, USA).

4.4. Measurement of ROS levels and cellular GSH

Intracellular levels of ROS were measured with DCFH-DA (2', 7'-dichlorodihydro-fluorescein diacetate; Molecular Probes, USA). At the end of the treatment, the medium was replaced with fresh serum-free medium containing 10 μg/ml DCFH-DA. The cells were incubated for 30 min at 37 °C and then washed three times with serum-free medium. The DCF fluorescence was measured in a microplate reader with excitation at 485 nm and emission at 520 nm.

The cellular GSH level was assessed using a GSH/GSSG assay kit (Beyotime, China). The cells were collected and resuspended in the buffer for lyticase (Sigma, USA) at 30 °C for 30 min, and then the lysate was clarified by centrifugation ($9000 \times g$, 5 min, 4 °C), and the supernatant was assayed spectrophotometrically following the manufacturer's instructions though use of thiol-reactive fluorescent dye CMFDA by a microplate reader at 405 nm.

4.5. Measurement of glutamate cysteine ligase (GCL) activity

The measurement of GCL enzyme activity was described previously (Kim et al., 2008; Toroser and Sohal, 2005). The whole cellular lysate is prepared using a RIPA Lysis Buffer (Beyotime, China) added to a reaction buffer containing 0.1 M Tris (pH 8.2), 0.15 M KCl, 10 mM ATP, 10 mM L-glutamate, 20 mM MgCl₂, and 2 mM EDTA at 37 °C for 3 min, and then 5 mM cysteine is added at 37 °C for 15 min. The production of glutamylcysteine was immediately quantified for HPLC analysis by O-phthalaldehyde derivatization. GCL activity is presented in units of femtomoles of -GC produced per milligram of protein per minute.

4.6. Western blotting analysis

To prepare the cytoplasmic and nuclear proteins, cells were lysed using a nuclear and cytoplasmic protein extraction kit (Beyotime, China) according to the manufacturer's instructions. The lysates were ultracentrifuged at $12,000 \times g$ for 10 min at 4 °C, and the supernatants were collected as the cytoplasmic fraction. The pelleted nuclei were resuspended in a buffer

containing 1 mM PMSF. After 30 min at 4 °C, lysates were centrifuged, and supernatants containing the nuclear proteins were stored at -80 °C. Whole-cell extracts were obtained as mentioned previously. The concentration of protein was measured using Bradford's reagent (Bio-Rad, USA).

Proteins were separated by 6% SDS-PAGE (Beyotime, China), and the separated proteins were electrically transferred to a nitrocellulose sheet (Pell, USA). After blocking for 1 h using 5% nonfat milk in TBST, each membrane was sequentially incubated in the presence of polyclonal anti-Nrf2 antibody (Abcam, UK) overnight at 4 °C and secondary antibodies for 1 h at RT. The protein bands were visualized using chemiluminescent reagents (Pierce, UK) according to the manufacturer's instructions. Lamin B and β -actin were used as loading controls for nuclear and whole cell extracts, respectively.

4.7. siRNA transfection

For Nrf2 knockdown, a select predesigned siRNA duplex [5'-UUAAGACACUGAACUCGGAAUGG-3' (sense) and 5'-CCAUCCCGAGUUACAGUGUCUUA-3' (antisense)], whose knockdown effect has been demonstrated previously, was used (Chen et al., 2006; Usami et al., 2005). Stealth™ RNAi negative control duplex (Invitrogen, CA) was used as a negative control. The cells were transfected according to the manufacturer's instructions, and the transfection efficiency was determined by Western blotting analysis as described above.

4.8. Statistical analysis

All experiments were repeated at least three times, and the results were analyzed by SPSS 11.0. Data are presented as means \pm SD. One-way analysis of variance (ANOVA) was used for comparing multiple groups, and the Student's t-test was used for comparing two groups. Statistical significance was set at $P < 0.05$.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (grant numbers 30971024 and 30870845) and the Guangdong Provincial Natural Science Foundation (grant number 91510515000009).

REFERENCES

- Anderson, M.F., Sims, N.R., 2002. The effects of focal ischemia and reperfusion on the glutathione content of mitochondria from rat brain subregions. *J. Neurochem.* 81, 541-549.
- Bains, J.S., Shaw, C.A., 1997. Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. *Brain Res. Rev.* 25, 335-358.
- Bondarenko, A., Chesler, M., 2001. Rapid astrocyte death induced by transient hypoxia, acidosis, and extracellular ion shifts. *Glia* 34, 134-142.
- Chan, P.H., 1996. Role of oxidants in ischemic brain damage. *Stroke* 27, 1124-1129.
- Chen, Z.H., Yoshida, Y., Saito, Y., Sekine, A., Noguchi, N., Niki, E., 2006. Induction of adaptive response and enhancement of PC12 cell tolerance by 7-hydroxycholesterol and 15-deoxy-delta (12, 14)-prostaglandin J2 through up-regulation of cellular glutathione via different mechanisms. *J. Biol. Chem.* 281, 14440-14445.
- Ciccarelli, R., D'Alimonte, I., Santavenere, C., D'Auro, M., Ballerini, P., Nargi, E., Buccella, S., NicosiaFolco, S., Caciagli, F., Iorio, P.Di., 2004. Cysteinyl-leukotrienes are released from astrocytes and increase astrocyte proliferation and glial fibrillary acidic protein via cys-LT1 receptors and mitogen-activated protein kinase pathway. *Eur. J. Neurosci.* 20, 1514-1524.
- Dawson, D.A., Graham, D.I., McCulloch, J., Macrae, I.M., 1994. Anti-ischaemic efficacy of a nitric oxide synthase inhibitor and a N-methyl-D-aspartate receptor antagonist in models of transient and permanent focal cerebral ischaemia. *Br. J. Pharmacol.* 113, 247-253.
- Dienel, G.A., Hertz, L., 2005. Astrocytic contributions to bioenergetics of cerebral ischemia. *Glia* 50, 362-388.
- Dringen, R., 2000. Metabolism and functions of glutathione in brain. *Prog. Neurobiol.* 62, 649-671.
- Fernández-Checa, J.C., García-Ruiz, C., Ookhtens, M., Kaplowitz, N., 1991. Impaired uptake of glutathione by hepatic mitochondria from chronic ethanol-fed rats. Tracer kinetic studies *in vitro* and *in vivo* and susceptibility to oxidant stress. *J. Clin. Invest.* 87, 397-405.
- Fisk, L., Nalivaeva, N.N., Boyle, J.P., Peers, C.S., Turner, A.J., 2007. Effects of hypoxia and oxidative stress on expression of neprilysin in human neuroblastoma cells and rat cortical neurones and astrocytes. *Neurochem. Res.* 32, 1741-1748.
- Goldberg, M.P., Weiss, J.H., Pham, P.C., Choi, D.W., 1987. N-methyl-D-aspartate receptors mediate hypoxic neuronal injury in cortical culture. *J. Pharmacol. Exp. Ther.* 243, 784-791.
- Haskew-Layton, R.E., Mongin, A.A., Kimelberg, H.K., 2005. Hydrogen peroxide potentiates volume-sensitive excitatory amino acid release via a mechanism involving Ca^{2+} /calmodulin-dependent protein kinase II. *J. Biol. Chem.* 280, 3548-3554.
- Hung, T.M., Na, M., Thuong, P.T., Su, N.D., Sok, D., Song, K.S., Seong, Y.H., Bae, K., 2006. Antioxidant activity of caffeoyl quinic acid derivatives from the roots of *Dipsacus asper* Wall. *J. Ethnopharmacol.* 108, 188-192.
- Jaiswal, A.K., 2000. Regulation of genes encoding NAD(P)H:quinone oxidoreductases. *Free Radic. Biol. Med.* 29, 254-262.
- Juurink, B.H., 1997. Response of glial cells to ischemia: roles of reactive oxygen species and glutathione. *Neurosci. Biobehav. Rev.* 21, 151-166.
- Kim, S.K., Yang, J.W., Kim, M.R., Roh, S.H., Kim, H.G., Lee, K.Y., Jeong, H.G., Kang, K.W., 2008. Increased expression of Nrf2/ARE-dependent anti-oxidant proteins in tamoxifen-resistant breast cancer cells. *Free Radic Biol Med.* 45, 537-546.
- Kode, A., Rajendrasozhan, S., Caito, S., Yang, S.R., Megson, I.L., Rahman, I., 2008. Resveratrol induces glutathione synthesis by activation of Nrf2 and protects against cigarette smoke-mediated oxidative stress in human lung epithelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* 294, 478-488.
- Lee, W.T., Hong, S., Yoon, S.H., Kim, J.H., Park, K.A., Seong, G.J., Lee, J.E., 2009. Neuroprotective effects of agmatine on oxygen-glucose deprived primary-cultured astrocytes and nuclear translocation of nuclear factor-kappa B. *Brain Res.* 1281, 64-70.
- Lerouet, D., Beray-Berthaut, V., Palmier, B., Plotkine, M., Margail, I., 2002. Changes in oxidative stress, iNOS activity and neutrophil infiltration in severe transient focal cerebral ischemia in rats. *Brain Res.* 958, 166-175.
- Li, R., Bianchet, M.A., Talalay, P., Amzel, L.M., 1995. The three-dimensional structure of NAD(P)H:quinone reductase, a flavoprotein involved in cancer chemoprotection and

- chemotherapy: mechanism of the two-electron reduction. *Proc. Natl. Acad. Sci. USA* 92, 8846–8850.
- Liu, D., Smith, C.L., Barone, F.C., Ellison, J.A., Lysko, P.G., Li, K., Simpson, I.A., 1999. Astrocytic demise precedes delayed neuronal death in focal ischemic rat brain. *Mol. Brain Res.* 68, 29–41.
- Liu, Y., Kern, J.T., Walker, J.R., Johnson, J.A., Schultz, P.G., Luesch, H., 2007. A genomic screen for activators of the antioxidant response element. *Proc. Natl. Acad. Sci. USA* 104, 5205–5210.
- Moi, P., Chan, K., Asunis, I., Cao, A., Kan, Y.W., 1994. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc. Natl. Acad. Sci. USA* 91, 9926–9930.
- Mulcahy, R.T., Gipp, J.J., 1995. Identification of a putative antioxidant response element in the 5'-flanking region of the human gamma-glutamylcysteine synthetase heavy subunit gene. *Biochem. Biophys. Res. Commun.* 209, 227–233.
- Nakajima, Y., Shimazawa, M., Mishima, S., Hara, H., 2007. Water extract of propolis and its main constituents, caffeoylquinic acid derivatives, exert neuroprotective effects via antioxidant actions. *Life Sci.* 80, 370–377.
- Nguyen, T., Sherratt, P.J., Pickett, C.B., 2003. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annu. Rev. Pharmacol. Toxicol.* 43, 233–260.
- Ouyang, Y.B., Giffard, R.G., 2004. Changes in astrocyte mitochondrial function with stress: effects of Bcl-2 family proteins. *Neurochem. Int.* 45, 371–379.
- Park, J.B., 2009. 5-Caffeoylquinic acid and caffeic acid orally administered suppress P-selectin expression on mouse platelets. *J. Nutr. Biochem.* 20, 800–805.
- Pfeiffer-Guglielmi, B., Fleckenstein, B., Jung, G., Hamprecht, B., 2003. Immunocytochemical localization of glycogen phosphorylase isozymes in rat nervous tissues by using isozyme-specific antibodies. *J. Neurochem.* 85, 73–81.
- Piantadosi, C.A., Zhang, J., 1996. Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. *Stroke* 27, 327–331.
- Prester, T., Talalay, P., Alam, J., Ahn, Y.I., Lee, P.J., Choi, A.M., 1995. Parallel induction of heme oxygenase-1 and chemoprotective phase 2 enzymes by electrophiles and antioxidants: regulation by upstream antioxidant-responsive elements (ARE). *Mol. Med.* 1, 827–837.
- Schulz, J.B., Lindenau, J., Seyfried, J., Dichgans, J., 2000. Glutathione, oxidative stress and neurodegeneration. *Eur. J. Biochem.* 267, 4904–4911.
- Suzuki, Y., Takahashi, H., Fukuda, M., Hino, H., Kobayashi, K., Tanaka, J., Ishii, E., 2009. beta-Hydroxybutyrate alters GABA-transaminase activity in cultured astrocytes. *Brain Res.* 1268, 17–23.
- Toroser, D., Sohal, R.S., 2005. Kinetic characteristics of native gamma-glutamylcysteine ligase in the aging housefly, *Musca domestica* L. *Biochem. Biophys. Res. Commun.* 326, 586–593.
- Usami, H., Kusano, Y., Kumagai, T., Osada, S., Itoh, K., Kobayashi, A., Yamamoto, M., Uchida, K., 2005. Selective induction of the tumor marker glutathione S-transferase P1 by proteasome inhibitors. *J. Biol. Chem.* 280, 25267–25276.
- Yang, C., Zhang, X., Fan, H., Liu, Y., 2009. Curcumin upregulates transcription factor Nrf2, HO-1 expression and protects rat brains against focal ischemia. *Brain Res.* 1282, 133–141.
- Yu, A.C., Wong, H.K., Yung, H.W., Lau, L.T., 2001. Ischemia-induced apoptosis in primary cultures of astrocytes. *Glia* 35, 121–130.