Research Report

Sulforaphane protects brains against hypoxic–ischemic injury through induction of Nrf2-dependent phase 2 enzyme

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Neonatal hypoxia–ischemia (HI) brain injury involves reactive oxygen species (ROS) and inflammatory responses. Sulforaphane (SFN), an isothiocyanate found in cruciferous vegetables, has cytoprotective effects against oxidative stress and its effect was mediated by NF-E2-related factor-2 (Nrf2), a transcription factor, and heme oxygenase 1 (HO-1) which is one of Nrf2 downstream target genes. This study was undertaken to investigate the neuroprotective mechanisms of SFN in a neonatal HI rat model. Seven-day-old rat pups were subjected to left common carotid artery ligation and hypoxia (8% oxygen at 37 °C) for 90 min. SFN (5 mg/kg) was systemically administered 30 min before HI insult. Brain injury was assessed by 2,3,5-triphenyltetrazolium chloride (TTC), Nissl, TUNEL staining, malondialdehyde (MDA), 8OH-dG level, and caspase-3 activity in the cortex and hippocampus. SFN pretreatment increased the expression of Nrf2 and HO-1 in the brain and reduced infarct ratio at 24 h after HI. The number of TUNEL-positive neurons as well as activated macroglia and the amount of 8OH-dG, were markedly reduced after SFN treatment, accompanied by suppressed caspase-3 activity and reduced lipid peroxidation (MDA) level. These results demonstrated that SFN could exert neuroprotective effects through increasing Nrf2 and HO-1 expression.

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1. Introduction

Hypoxia–ischemia (HI) insult occurs frequently in the perinatal period and can lead to neuron death (Vannucci et al., 1999). Reactive oxygen species (ROS), such as the hydroxyl radical (‘OH), hydrogen dioxide (H2O2), superoxide anion, nitric oxide (NO), and peroxynitrite (ONOO−) are known to play a critical role in neuron damage. Among all the ROS, ‘OH is much more reactive and easily reacts indiscriminately with cellular macromolecules, including DNA, proteins and lipids, and gives rise to HI injury-originated brain damage. Moreover, free heme derived from degraded hemoglobin protein during

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Abbreviations: ANOVA, analysis of variance; HI, hypoxic–ischemic; HO-1, heme oxygenase 1; MDA, malondialdehyde; Nrf2, NF-E2-related factor-2; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SFN, sulforaphane; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; Keap1, Kelch-like-Ech-associated-protein 1

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HI injury has been implicated as a source of catalytic iron that participates in the Fenton reaction which converts H₂O₂ to *OH and promotes more severe tissue damage by propagating peroxidation. There is no known detoxification system for *OH and therefore, scavenging *OH is a critical antioxidant process (Sheu et al., 2006).

Sulforaphane (SFN) is an isothiocyanate found in cruciferous vegetables such as broccoli that has gained attention as a chemopreventative compound (Zhang et al., 1992). Its cytoprotective effects (against oxidative stress) has been found to be mediated by NF-E2-related factor-2 (Nrf2), a transcription factor, which can induce the expression of Nrf2-dependent phase enzymes such as heme oxygenase-1 (HO-1), NAD(P)H, quinone oxidoreductase 1, glutathione reductase, and glutathione peroxidase (Shinkai et al., 2006). These enzymes were confirmed to participate in adaptive and protective responses to oxidative stress. Neuron membranes are rich in polyunsaturated fatty acids, and neonatal brain is susceptible to oxidative stress (Ikeda et al., 2002). This vulnerability is reduced when these cells are transduced with a functional Nrf2 construct (Kraft et al., 2004; Lee et al., 2003; Lee and Johnson, 2004). However, the potential neuroprotective effect and mechanisms of SFN against neonatal HI brain injury have not been reported. The present study aimed to investigate the potential mechanism underlying the protective effects of SFN in a neonatal HI rat model.

2. Result

2.1. TTC staining

Fig. 1 showed the representative photographs of TTC-stained sections from rat pups in each group, at 24 h after initial HI insult. The infarct ratio in the HI group (10.01%) was markedly higher (P<0.01) than that in the SFN pretreatment group (0.92%) which was not significantly different from normal controls (P>0.05).

2.2. Nissl staining

Fig. 2 showed the representative photographs of Nissl staining from the cerebral cortex and hippocampus of pups at 24 h after HI insult. Extensive neuronal morphological changes in the cortex and CA1 regions were noticed with features of considerable dark, pyknotic neurons in the HI group (B1–4). More Nissl stained cells (C1–4) were observed in the SFN pretreatment group than in the HI group (P<0.01).

2.3. TUNEL staining and TUNEL-positive cell counting

TUNEL, which is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of fragmented DNA, has become the most widely used in situ test for the study of apoptosis. Fig. 3 showed that the number of TUNEL-positive cells was markedly increased in the cortex (45±3) and hippocampus (48±3) of HI group (B1 and B3). SFN reduced the number of TUNEL-positive cells (C1 and C3) (P<0.01). At higher magnification, the nuclei of cells were clearly stained in both hippocampus and cortex (B2, B4, C2 and C4). A few TUNEL-positive cells were identified in samples from normal control pups (A1–4). Furthermore, there was no significant difference in the number of TUNEL-positive cells in the cortex and hippocampus between SFN pretreatment group (11±2 and 14±3, respectively) and control group (8±3 and 10±3, respectively) (P>0.05).

2.4. Levels of MDA

The levels of MDA were measured at 24 h after HI insult and shown in Fig. 4A. The level of MDA in the HI group (9.44±0.75) was markedly higher (P<0.01) than that in the SFN pretreatment group (1.79±0.59).

Fig. 1 – TTC staining of damaged brains and infarct ratio. (A) Representative samples of TTC-stained coronal sections were derived from 8-day-old neonatal rats after SFN therapy. Marked cerebral infarction was observed in the HI group. (B) Infarct ratio of each group. The infarct ratio was (10.01±0.65) % in HI group and (0.92±0.11) % in SFN group. The results indicated that SFN therapy decreased the infarct volume.
pretreatment group (4.60±0.67) which was not significantly different from normal controls (3.52±0.53) (P>0.05).

2.5. Activity of caspase-3

The activities of caspase-3 were measured at 24 h after HI insult and shown in Fig. 4B. The activity of caspase-3 was 1.31±0.23 in the cortex and 1.42±0.32 in the hippocampus of HI group. SFN significantly reduced the activity of caspase-3 in the cortex (0.15±0.09) and hippocampus (0.19±0.06) (P<0.01 vs. HI).

2.6. Immunohistochemical analysis

In order to determine whether microglia is activated in the brain, sections were prepared at 24 h after HI for immunohistochemical staining with anti-Iba1 antibody. Iba1-positive cells had small nuclei, scant cytoplasm, and thin, branched processes as shown in Fig. 5. Iba1-positive cells were found throughout the parenchyma of white and grey matter. The distinctive morphology and widespread distribution of these cells were highly consistent with the classical descriptions of ramified microglia (Bona et al., 1999; Sheu et al., 2006).

Fig. 2 – Nissl staining of damaged cortex (A1–C2) and hippocampus (A3–C4) and cell counting. (A) Nissl staining. Cortex and hippocampus in each group after SFN therapy were shown at two different magnifications (A1–C1, A3–C3: ×100, A2–C2, A4–C4: ×400). More neuronal loss and dead cells appeared in the HI group after injury. In the CA1 region of control and HI groups, the cell outline was clear and structure compact. Cells were big and had abundant cytoplasm and Nissl body. In the HI group, cells were arranged sparsely and cell outline was fuzzy. The number of cells with eumorphism was significantly reduced. (B) Cell counting. The number of Nissl stained cells in the cortex and hippocampus of HI group was lower than that of SFN group (**P<0.01).

SFN significantly reduced the number of Iba1-positive cells and 8OH-dG-positive cells, when compared with HI group (Fig. 5). Furthermore, the expression of Nrf2 protein was significantly increased accompanied by up-regulation of HO-1 in the SFN pretreatment group, compared to the HI group (data were not shown).

3. Discussion

SFN has been studied mostly for its anti-carcinogenic effects (Zhang et al., 1992). In the present study, we examined the potential neuroprotective effects of SFN in a neonatal HI rat model. We observed that SFN significantly increased Nrf2 and HO-1 expression which was accompanied by reduced infarct volume, and decreased number of apoptotic cells. Further pieces of evidence for SFN induced neuroprotection in this animal model included reduced caspase-3 activity, suppressed activation of microglia, decreased levels of MDA and 8OH-dG in SFN pretreated animals. These observations indicate that SFN may be a potential candidate for the treatment of neonatal brain injury due to its anti-oxidative and anti-inflammatory properties.
SFN, a substance present in extracts of cruciferous vegetables such as broccoli, is a major and probably the principal inducer of Nrf2-dependent phase 2 enzyme (Zhang et al., 1992). SFN induces antioxidant response element (ARE) expression through disruption of the Keap1–Nrf2 complex (Kwak et al., 2003a,b; McMahon et al., 2001, 2003). SFN can interact directly with sulfhydryl residues on the Keap1, causing release of Nrf2. Alternatively, SFN can activate the MAPK pathway, causing phosphorylation of Keap1 and release of Nrf2 (Hu et al., 2004).

Once released, Nrf2 enters the nucleus, where it trans-activates ARE-responsive genes and induces the phase 2 response (Dinkova-Kostova et al., 2002; Marrot et al., 2008). Phase 2 enzymes such as HO-1, NAD(P)H, quinone oxidoreductase 1, glutathione reductase, and glutathione peroxidase participate in adaptive and protective responses to oxidative stress and various inflammatory stimuli (Shinkai et al., 2006). Up-regulation of HO-1 has been known to protect against HI injury (Tanaka et al., 2007) and HO-1 has been shown to exert potent endogenous anti-oxidative, anti-inflammatory and anti-apoptotic properties (Fujita et al., 2001).

ROSA play a critical role in neuron damage. Ferrous ion can convert H₂O₂ to ⋅OH (Fenton reaction) which leads to more severe tissue damage by propagating DNA and lipid peroxidation. However, HO-1 can exert potent indirect anti-oxidative function by catabolizing heme to biliverdin, iron, and CO. Furthermore, these byproducts of heme degradation are believed to be effector molecules underlying the potent cytoprotection observed in the HO system (Amersi et al., 2002; Brouard et al., 2000). The byproducts have their own significance in essential cellular metabolism and contribute to the suppression of oxidative stress (Berberat et al., 2003; Nakao et al., 2005a, b). 8OH-dG is a product of DNA oxidation and had been used as a marker for DNA oxidation. MDA is a product of lipid peroxidation. In the present study, intraperitoneally injected SFN markedly reduced MDA and 8OH-dG levels. These findings strongly supported the potent cytoprotection of HO-1 against oxidative stress and its important role in the HI insult.

HI triggers inflammatory responses due to the generation of ROS and other inflammatory cytokines. These initiators lead to microglia activation which is readily accumulated in the injured issues following HI (Bona et al., 1999; Ivacko et al., 1996) and facilitates the production of more cytokines (Cowell et al., 2002), causing up-regulation of adhesion molecules in the cerebral vasculature. Adhesion molecules promote
adhesion of circulating leukocytes to vascular endothelia and filtration of these cells into brain parenchyma. HO-1 can suppress the up-regulation of adhesion molecules by removing free heme (Beri and Chandra, 1993). Fig. 4 showed that the number of activated microglia was profoundly reduced by SFN suggesting that SFN exerted anti-inflammatory effects on neonatal HI through inducing the expression of Nrf2-dependent phase 2 enzyme such as HO-1. Furthermore, SFN also reduced the activity of caspase-3 in the present study. The results from TUNEL and Nissl staining also demonstrated that HO-1 system ameliorated neuropathological damage after brain HI insult.

Even though ROS are implicated in the pathophysiology of the HI induced brain injury, clinically it remains difficult to achieve neuroprotection by application of exogenous antioxidant. However, as mentioned above, SFN activates endogenous biological antioxidant defense system. In the present study, pretreatment with SFN effectively reduced cerebral infarct volume through inducing the expressions of Nrf2 and HO-1, which may indirectly act as therapeutic antioxidants by selectively reducing cytotoxic oxygen radicals. Therefore, activation of endogenous antioxidant system by SFN may offer an alternative route in clinical management of neonatal hypoxia-ischemia brain injury.

4. Experimental procedures

4.1. Surgery and treatment

Seven-day-old Sprague–Dawley rat pups were randomly assigned to the following 3 groups: 1) control group (no carotid
ligration and hypoxia) (n=20); 2) HI group (carotid ligation and hypoxia) (n=60); and 3) HI+SFN pretreatment group (5 mg/kg SFN) (n=60). Each group was composed of pups from each litter to obtain parity within the groups. The Animal and Ethics Review Committee of the Second Military Medical University evaluated and approved the whole protocol used in this study.

4.2 Hypoxic–ischemic model

The model used in this study was based on the Rice–Vannucci model (Vannucci et al., 1999). Pups weighing 15–18 g were housed with the dam under a 12-h-light/12-h-dark cycle, with food and water available ad libitum throughout the studies. These neonatal rats were anesthetized by inhalation with diethyl ether. The pups were kept at a temperature of 37 °C as the left common carotid artery was exposed and ligated with 5.0 surgical sutures. After operation, the pups were returned to the holding container. Anesthesia and surgery time averaged 5 min per pup. Surgery was completed for an entire little, and the pups were allowed to recover with their dams for 1 h (for rehydration via nursing). Then, they were placed in a jar perfused with a humidified gas mixture (8% oxygen balanced nitrogen) for 90 min. Both the jar and mixture were kept at 37 °C to maintain a constant thermal environment. All surviving pups were returned to their dams after hypoxia exposure.

4.3 SFN administration

SFN (LKT Laboratories, Inc. USA) was dissolved in corn oil and injected intraperitoneally (5 mg/kg) (Zhao et al., 2006) 30 min before the onset of ischemia. Animals in vehicle group received corn oil of equal volume.

4.4 Measurement of infarct ratio

Twenty four hours after HI, the pups were decapitated and the infarct ratio was measured. Briefly, the brains were quickly removed after decapitation and placed in cold saline for 5 min, cut at 2 mm intervals from the frontal pole into 5 coronal sections. After incubation in 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 8 min at 37 °C, the brain slices were fixed in 4% formalin for 24 h. The images of brain slices were captured and the infarct volume was analyzed with an image analysis system (ImageXpress, Molecular Devices). The percentage of infarction was calculated as the number of cells per high-power field.

4.5 Nissl staining

For Nissl staining, the 4 μm sections were hydrated in 1% toluidine blue at 50 °C for 20 min. After rinsing with double distilled water, they were dehydrated and mounted with permount. The images of the cortex and CA1 area of hippocampus from each animal were captured and Imaging-Pro-Plus (LEIKA DMLB) was used to perform quantitative analysis of cell numbers.

4.6 TUNEL staining

TUNEL staining was performed on the paraaffin-embedded sections using the in situ cell death detection kit (Roche). According to the standard protocols, the sections were deparaffinized and rehydrated by heating the slides at 60 °C. Then these sections were incubated in a 20 μg/ml proteinase K working solution for 15 min at room temperature. The slides were rinsed with PBS thrice followed by incubation with TUNEL reaction mixture for 1 h at 37 °C. Area around sample was dried and Converter-AP was added on the samples followed by incubation for 1 h at 37 °C. After rinsing with PBS (5 min, thrice), color development was performed in dark with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

4.7 Cell counting

Six visual fields (0.6 mm²) of the cerebral cortex and CA1 were photographed in each section. The number of staining cells in each field was counted at high magnification (×400). Data were represented as the number of cells per high-power field.

4.8 Lipid hydroperoxide assay

Levels of malondialdehyde (MDA), a product of membrane lipid peroxidation, were determined (Ito et al., 2005). The tissues were homogenized in 10 volumes of 1.15% KCl solution containing 0.85% NaCl and then centrifuged at 1500 × g for 15 min. In brief, the reaction mixture consisted of 40 μl of 8.1% sodium dodecyl sulfate, 300 μl of 20% acetic acid solution adjusted to pH 3.5 with NaOH, 300 μl of 0.8% aqueous solution of thiobarbituric acid, and 40 μl of the tissue supernatant. The mixture was heated at 95 °C for 60 min in a tube (1.5 ml). After cooling on ice, 200 μl of distilled water was added and the sample was centrifuged at 15,000 × g for 20 min. The supernatant was removed and the absorbance was determined at 532 nm.

4.9 Caspase activity assay

Brain samples from the cortex and hippocampus were taken from the impaired hemispheres of neonatal rats at 24 h after SFN therapy. Brain samples were homogenized in ice-cold lysis buffer and kept at 4 °C for 1 h. Brain homogenate was centrifuged (Eppendorf, 5810R) at 12,000 × g for 15 min at 4 °C. The supernatant was removed and stored at −80 °C for use. Protein concentration was measured using the Enhanced BCA Protein Assay Kit. A total of 50 μg of lysate was incubated in a 96-well plate with 2× Reaction Buffer (50 μl). The reaction was started by adding 1 mM DEVD-APC substrate (5 μl). After incubation in dark at 37 °C, the plate was read in a fluorometer equipped with a 400 nm excitation filter and a 505 nm emission filter.

4.10 Immunohistochemical procedures

For immunohistochemistry, sections were blocked in 5% BSA for 30 min at room temperature and stained with anti-8OH-dG (MOG-020P; Japan Institute for the Control of Aging; 1:800)
antibodies overnight at 4 °C. Secondary antibodies conjugated to Alexa Fluor 546 (Molecular Probes, Eugene, OR, USA; 1:200) were applied for 1 h at 4 °C. Nuclei were stained with TO-PRO-3 (Molecular Probes) in a mounting medium.

For the Iba1, Nrf2 and HO-1 staining, sections were washed with PBS, incubated in 0.3% H2O2 in methanol for 30 min to inactivate endogenous peroxidase activity, washed with PBS, and blocked in PBS containing 1.5% normal goat serum and 1% BSA for 2 h at room temperature. The sections were then independently incubated with anti-Iba1 (1:100), Nrf2 (1:50) and HO-1 (1:50) polyclonal antibody (Abcam, UK) overnight at 48 °C, washed with PBS, and incubated with HRP-conjugated secondary antibody (Bio-Rad) for 2 h at room temperature. They were then incubated with 50 mM Tris–HCl (pH 7.2) containing 0.05% diaminobenzidine tetrahydrochloride (DAB) and 0.01% H2O2. For control staining, normal rabbit IgG was used as the primary antibody.

4.11. Statistical analysis

All quantitative data are expressed as mean ± SD. The significance of differences between means was verified by ANOVA followed by Tukey’s test. For analyzing the results of cell counting, a non-parametric Kruskal–Wallis ANOVA was used followed by Dunn’s test. A value of \( P<0.05 \) was considered statistically significant.

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