Expression and antioxidation of Nrf2/ARE pathway in traumatic brain injury

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ABSTRACT

Objective: To explore the expression of Nrf2/ARE pathway in hindbrain tissue after the traumatic brain injury (TBI) and its anti-oxidative stress effect in the secondary nerve injury. Methods: The mice with Nrf2 gene knockout were used for the establishment of brain injury model. The experimental animals were divided into four groups: (Nrf2+/+) sham-operation group, (Nrf2+/+) brain injury group, (Nrf2−/−) sham-operation group and (Nrf2−/−) brain injury group. The specimen 24 h after cerebral trauma was selected. Then RT-PCR method was adopted to detect the expression of Nrf2 mRNA in brain; Western blotting method was adopted to detect the levels of Nrf2, HO-1 and NQO1 proteins in brain; ELISA method was adopted to detect the oxidative stress indicators: protein carbonyls, 4-hydroxy-2-nonenal (4-HNE) and 8-hydroxy-2'-deoxyguanosine (8-OHdG).

Results: The Nrf2 mRNA and protein of Nrf2−/− mice were not expressed, and the difference of the relative amount of Nrf2 mRNA between Nrf2+/+ TBI group and Nrf2+/+ sham-operation group was not statistically significant (P>0.05); the level of Nrf2 protein in Nrf2+/+ TBI group increased significantly compared with the Nrf2+/+ sham-operation group (P<0.01); in the sham-operation groups, the levels of HO-1 and NQO1 proteins of Nrf2−/− mice decreased obviously compared with the Nrf2+/+ mice (P<0.01); after brain injury, the levels of HO-1 and NQO1 proteins of Nrf2+/+ mice increased obviously compared with the corresponding sham-operation group (P<0.01); the levels of HO-1 and NQO1 proteins of Nrf2−/− mice in TBI group had no obvious change compared with the corresponding sham-operation group (P>0.05); there was only a little amount of expression of protein carbonyls, 4-HNE and 8-OHdG proteins in brain tissues in the Nrf2+/+ and Nrf2−/− sham–operation groups, and the difference was not statistically significant (P>0.05); after brain injury, the three oxidative stress indicators were significantly up-regulated in the Nrf2+/+ and Nrf2−/− groups, and the up–regulation of the latter group was more significant (P<0.01). Conclusions: After TBI the Nrf2/ARE pathway is activated and the activity of Nrf2 transcription regulation increases. However, the regulation dose not occur in the gene transcription level and only could increase the Nrf2 protein level, while the mRNA expression level has no obvious change. The nerve cell protective effect of Nrf2/ARE pathway in TBI achieves through inhibiting the oxidative stress injuries.

1. Introduction

Traumatic brain injury (TBI) firstly causes the irreversible primary mechanical injury and then causes the secondary nerve injury. It is a kind of complex pathological process with multi–cells and multi–molecules involvement and can lead to cognitive and behavioral dysfunction. There are some researches showing that[1,2] the excessive generation of oxygen free radicals after TBI is the core pathological link leading to the nerve cell injury and apoptosis, and inhibiting the oxygen free radical chain reaction may be the important strategy to treat TBI. However, the signal transduction of this process is still not clear.

NF–erythroid 2–related factor 2 (Nrf2) is a kind of newly
discovered nuclear factor in recent years. In physiological state, it is mainly distributed in the cytolymph and forms a complex combining with cytoplasmic protein Keapl. When oxidative stress stimulus occurs, it is decoupled with Keapl through phosphorylation, transfers into nucleus, combines with antioxidant response element (ARE) sequence, forms Nrf2/ARE pathway and then starts the gene expression of phase II detoxifying enzymes and antioxidants (HO–1, NQO1 etc) regulated by ARE. Nrf2/ARE pathway plays an important role in endogenous anti-oxidation process in vivo[3] and can be induced by external factors. At present, its roles in resisting myocardial ischemia– reperfusion injury, pulmonary fibrosis, drug–induced liver injury, inflammatory bowel disease and other aspects have been confirmed[4–6], and the role in the nervous system has attracted increasing attention. However, its role in TBI and its mechanism are rarely reported. This work explored the role of Nrf2/ARE pathway in TBI through establishing the brain injury model of mice with Nrf2 knockout, in order to provide a reference for thoroughly revealing the pathophysiological mechanism of TBI and seeking for the appropriate therapeutic targets.

2. Materials and methods

2.1. Experimental animals

The male ICR–Nrf2+/− and ICR–Nrf2−/− mice with body weight of 25–30 g were provided by the Animal Center of Nanjing General Hospital of Nanjing Military Command (Introduction by Johns Hopkins University). The license key: SCXK (Su) 2009–0004.

2.2. Main reagents

PCR primers were all synthesized by Shanghai Sangon Biotech Co., Ltd; dNTPs, Taq DNA polymerase and 10×PCR buffer were purchased from Promega Company; protein lysate (Ameresco Company), Trizol Reagent (Invitrogen Company), rabbit anti-rat Nrf2 polyclonal antibody (Santa Cruz Company), rabbit anti-rat HO–1 polyclonal antibody (Epitomics Company), rabbit anti-rat NQO1 polyclonal antibody (Santa Cruz Company), protein carbonyls ELISA kit, 4-hydroxy-2-nonenal (4-HNE) ELISA kit, 8-hydroxy-2-deoxyguanosine (8-OHdG) ELISA kit were purchased from Cell Biolabs Company.

2.3. Main instruments

Electromagnetic cerebral cortex contusion impact instrument (Benchmark Company), Thermo MK3 ELISA (Thermo Labsystems Company), constant temperature table concentrator (Forma Scientific Company), PCR cycler (MJ Research Company), nucleic acid electrophoresis apparatus (BIO–RAD Company), nucleic acid detector (BIO–RAD Company), Gel Doc EQ imaging analysis system (BIO–RAD Company), and low–temperature and high–speed centrifugal machine (Beckman Company).

2.4. Methods

2.4.1. Animal grouping and modeling

The experimental animals were divided into four groups: (Nrf2+/−) sham–operation group, (Nrf2−/−) brain injury group, (Nrf2+/−) sham–operation group and (Nrf2−/−) brain injury group, with 10 mice in each group. The TBI model was built based on the controlled cortical impact injury method established by Brody et al[7]. After anesthesia, the mice were made in prone position and fixed in the stereotactic head frame. The scalp was cut along the midline under sterile condition and the right parietal bone was exposed. One mm cut was made along the midline of sagittal suture, a bone hole with 5 mm diameter was drilled between the coronal suture and lambdoid suture, and cerebral dura mater was exposed. The control arm of brain stereotactic instrument was used to form an eight degree angle between the impact head and the sagittal plane of mouse head. The impact head was parallel to the coronal plane and fixed vertically in the mouse cerebral dura mater, with the end of impact head vertically contacting with the cerebral dura mater. The impact head with 3 mm diameter was adopted to produce the moderate TBI, with 4 m/s impact velocity, 1 mm impact depth and 100 ms impact time. The mice in sham–operation group received the above anesthesia and operation procedure, but did not go through the impact injury process. After 24 h, the animals were executed, and the surrounding tissues of injury foci in injury side were taken, with liquid nitrogen quick freezing, kept in −70 °C icebox.

2.4.2. Detection of Nrf2 mRNA expression in brain by RT–PCR

Brain tissues were taken. The total RNA was extracted by Trizol method and then used to synthesize cDNA through reverse transcription. The cDNA was regarded as the follow board to augment Nrf2 by PCR. The upstream primer was 5′–TTCCCTCTCGTCATGCATGTC–3′ and the downstream primer was 5′–GTGCTTAGCAGTACTGTG–3′. PCR reaction system: the total volume was 25 μL, with 0.5 μL Taq DNA polymerase, 0.5 μL upstream primer, 0.5 μL downstream primer, 0.5 μL dNTP, 1.0 μL cDNA and 2.5 μL 10× PCR buffer (containing 20 mM Mg2+). PCR reaction condition: prededegeneration for 3min at 94 °C; denaturation for 30 s at 94 °C, annealing for 30 s at 57 °C and elongation for 30 s at 72 °C, with 35 cycles in total; elongation again.
for 10 min at 72 °C and end at 4 °C. A pair of β-actin internal reference primers (the upstream primer was 5′-AGTGTGACGTGGACATCCGT-3′ and the downstream primer was 5′-GCCAGAGCAGTAATCTCCTCCTT-3′) was added when target gene augmentation, and the internal reference DNA was augmented as control. Amplified product analysis: 10 μL amplified product was put into 2% sepharose gel. After 20 min electrophoresis, Gel Doc EQ imaging analysis system was adopted for the scanning and analysis of the strips, and the absorbance ratio of target strip and internal reference β-actin was calculated.

2.4.3. Detection of the expression of Nrf2, HO-1 and NQO1 proteins in brain by Western blotting

The total protein in brain tissues was extracted and the protein lysate was added. After schizolysis for 1 h at 4 °C and then centrifugalization for 20 min in the speed of 12 000 r/min at 4 °C, the supernatant was taken, and Coomassie Brilliant Blue protein assay was used to detect the concentration of Nrf2, HO-1 and NQO1 proteins. After the denaturation of 50 μg sample of total protein for 5 min, SDS–PAGE gel electrophoresis was made, then transmembrane and sealing. The diluted primary antibody (Nrf2, HO-1, and NQO1) monoclonal antibodies were added, with incubation for 16 h at 4 °C and washing membrane for three times. The second antibodies labeled by horseradish peroxidase (Nrf2, HO-1 and NQO1) were added, with incubation for 2 h at 37 °C and washing membrane for three times. Chemiluminescence coloration detection was used for analysis of the results.

2.4.4. Detection of oxidative stress injury

After brain tissues were taken, ELISA method was used to detect the concentrations of protein carbonyls, 4-HNE and 8-OHdG.

2.5. Statistical method

The experimental data were analyzed by SPSS 15.0 statistical software and expressed by mean ± sd. t test was used for the comparisons between groups and P<0.05 indicated that the difference had statistical significance.

3. Results

3.1. Expression of Nrf2 mRNA in brain tissues

RT-PCR results showed that the Nrf2 mRNA of Nrf2+/− mice in TBI group and sham-operation group did not express; the relative amount of Nrf2 mRNA of Nrf2+/+ mice in TBI group was 1.12±0.09 and that in sham-operation group was 1.08±0.07, without statistically significant difference between the two groups (t=1.11, P>0.05). The results were shown in Figure 1 and Table 1.

| Groups     | Nrf2+/β-actin mRNA | Nrf2+/β-actin protein |
|------------|---------------------|-----------------------|
| Sham-operation group | 1.08±0.07           | 0.00±0.00             |
| TBI group   | 1.12±0.09           | 1.39±0.08*            |

Note: compared with the sham-operation group having the same indicator, *P<0.01.

Figure 1. Expression of Nrf2 mRNA in brain tissues (RT-PCR picture). 1: Nrf2+/− sham-operation group; 2: Nrf2+/− sham-operation group; 3: Nrf2+/+ TBI group; 4: Nrf2+/- TBI group.

3.2. Expression of Nrf2 protein in brain tissues

The results of Western blotting showed that the Nrf2 protein of Nrf2+/− mice did not express; the relative amount of Nrf2 protein of Nrf2+/+ mice in TBI group was 1.39±0.08 and that in sham-operation group was 0.56±0.07, with statistically significant difference between the two groups (t=24.69, P<0.01). The results were shown in Figure 2 and Table 1.

Figure 2. Expression of Nrf2 protein in brain tissues (protein picture). 1: Nrf2+/− sham-operation group; 2: Nrf2+/− sham-operation group; 3: Nrf2+/+ TBI group; 4: Nrf2+/- TBI group.

3.3. Expression of HO-1 and NQO1 proteins in brain tissues

The results of Western blotting showed that in sham-operation groups, the levels of HO-1 and NQO1 proteins
of Nrf2/− mice significantly decreased compared with the Nrf2/+ mice (P<0.01); the levels of HO-1 and NQO1 proteins of Nrf2/− mice in TBI group significantly increased compared with the sham-operation group (P<0.01); the levels of HO-1 and NQO1 proteins of Nrf2/+ mice in TBI group changed little compared with the sham-operation group (P>0.05). The results were shown in Figure 3 and 4.

![Figure 3. Expression of HO-1 protein in brain tissues.](image)

![Figure 4. Expression of NQO1 protein in brain tissues.](image)

3.4. Anti–oxidative stress injury

The contents of Protein Carbonyls, 4–HNE and 8–OHdG in brain tissues represented the oxidative injury degree of protein, fatty substance and DNA, respectively. Results showed that the levels of Protein Carbonyls, 4–HNE and 8–OHdG in Nrf2/− and Nrf2/+ sham–operation groups expressed a little, without statistically significant difference between the two groups (P>0.05); the three oxidative stress indicators showed obvious increase in Nrf2/+ and Nrf2/+ TBI groups, and the Nrf2/+ group increased more significantly than Nrf2/+ group (z=7.14, 4.04, 4.57, P<0.01). The results were shown in Table 2.

### Table 2

Influence of Nrf2 gene knockout on oxidative stress indicators of mice after TBI (mean±SD).

| Groups                  | Nrf2/+   | Nrf2/−   |
|------------------------|----------|----------|
| Protein carbonyls      | Sham–operation | 0.39±0.06 | 2.53±0.19* |
|                        | TBI group | 0.42±0.07 | 3.17±0.21* |
| 4–HNE (μg/mL)         | Sham–operation | 0.16±0.07 | 0.86±0.15* |
|                        | TBI group | 0.18±0.10 | 1.14±0.16* |
| 8–OHdG (ng/mL)        | Sham–operation | 0.38±0.15 | 1.65±0.20* |
|                        | TBI group | 0.40±0.14 | 2.08±0.22* |

Note: compared with the sham–operation group having the same indicator, *P<0.01; compared with Nrf2/+ group having the same indicator, **P<0.01.

4. Discussion

TBI is a kind of common disease in neurosurgery and its death rate is the highest in trauma. Studies have found that most patients died of post–traumatic secondary brain injury, and effectively preventing the occurrence and development of secondary brain injury is one of the key factors for improving the prognosis of TBI patients. Thus, domestic and foreign scholars have done numerous studies on the pathogenesis of TBI, and found that a variety of pathophysiological processes jointly participate in the secondary nerve injury after TBI and they interact with each other, such as oxidative stress, inflammation damage, intracellular calcium ion overload and metabolic poison accumulation[9–11]. In the past, the effect of clinical treatment only aiming at one pathogenic mechanism was not ideal. Therefore, researchers are trying to find the common targets that can explain various pathological change mechanisms after brain injury.

The transcription factor Nrf2 plays a critical role in some pathological reactions, for examples, inducing the expression of phase II detoxifying enzymes, hemoglobin metabolism and the expression of antioxidant factors[12,13]. At the same time, it can regulate multiple mechanisms of secondary brain injury and may be the ideal treatment target in theory. There are researches showing that[14–16] Nrf2/ARE pathway plays an important neuroprotective role through inhibiting inflammatory cytokines, inducing the expression of detoxifying enzyme and maintaining the homeostasis of calcium ion in the secondary brain injury after TBI. However, there are few studies on the antioxidant protection mechanisms of this pathway. This work selected wild type (Nrf2+/+) and gene knockout (Nrf2/−) mice and adopted the quantitative cerebral cortex contusion method to build the TBI model. The specimen 24 h after TBI was taken, and RT–PCR was used to detect the expression of Nrf2 mRNA in brain. The results showed that the Nrf2 mRNA of
Nrf2−/− mice did not express, and the difference of the Nrf2 mRNA relative amount of Nrf2+/− mice between TBI group and sham-operation group was not statistically significant (P>0.05). Western blotting was used to detect the expression of Nrf2 protein in brain, and the results showed that the level of Nrf2 protein of Nrf2+/− mice in TBI group increased significantly compared with that in sham-operation group (P<0.01). All these indicated that Nrf2 regulation did not occur in the level of gene transcription, which was in accordance with the previous research result that the Nrf2 transcriptional regulatory activity increased and the level of mRNA expression had no obvious change after the oxidizing agent was used to stimulate cells[17].

HO−1 and NQO1 are two important antioxidases in the downstream of Nrf2/ARE pathway. HO−1 is a kind of rate-limiting enzyme and can catalyze hemachrome to produce dehydrobilirubin, thus decreasing the generation of free radicals[18]; NQO1 is a kind of flavin protease, can catalyze the reduction of two electrons, and has degradation effect on quinones and its ramifications, preventing them from participating in the oxidation-reduction reaction[19]. This work found that after knockout of Nrf2, the activity of mouse endogenous antioxidant enzymes HO−1 and NQO1 significantly decreased (P<0.01). Moreover, the protein levels of HO−1 and NQO1 could not be up-regulated after TBI. This indicated that the knockout of Nrf2 gene could limit the gene expression of antioxidase which was regulated by Nrf2 in the downstream, decrease the endogenous antioxidant capacity and thus increase the sensitivity of oxidative stress injury.

Oxidative stress injury is the most crucial pathological link for neuron injury. After brain injury, a large number of oxygen free radicals generate, which in one hand cause the overoxidation of fatty substance, protein and DNA, the destruction of membrane phospholipid structure, protein degradation, breakage of the main chain of nucleic acid, cell disruption and the irreversible changes of cells; on the other hand cause and aggravate the brain injury through stimulating the expression of cytokine and adhesion molecule, mediating immunity and inflammatory reaction[20]. Besides, oxygen free radicals can activate apoptotic signal pathway through inhibiting mitochondrial function and other indirect ways, causing cell apoptosis[21,22]. With collaborative development and infernal circle, these links eventually lead to ischemic neuronal necrosis. Our work confirmed this pathogenesis: the obvious oxidative stress appeared in the surrounding brain tissues of injured foci of mice, expressed as the increase of protein oxidative injury indicator (Protein carbonyls), lipid peroxidation indicator (4-HNE) and DNA oxidative injury indicator (8-OHdG), and obvious degeneration and necrosis of neurons of brain mantle in injured side. However, the oxidative stress injury indicators of Nrf2−/− injured mice rose more significantly than those of Nrf2+/− injured mice (P<0.01), which indicated that Nrf2/ARE pathway can inhibit the oxidative stress injury after TBI.

In summary, this work selected wild type (Nrf2+/−) and gene knockout (Nrf2−/−) mice and adopted the quantitative cerebral cortex contusion method to build the TBI model. It confirmed that Nrf2/ARE pathway was activated after TBI and the Nrf2 transcriptional regulatory activity increased, but this regulation did not occur in the level of gene transcription, with the increase of Nrf2 protein level but no obvious change of mRNA expression level. The nerve cell protective effect of Nrf2/ARE pathway in TBI may be realized through inhibiting the oxidative stress injury.

Conflict of interest statement

We declare that we have no conflict of interest.

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