Ginsenoside Rd Attenuates DNA Damage by Increasing Expression of DNA Glycosylase Endonuclease VIII-like Proteins after Focal Cerebral Ischemia

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Abstract

Background: Ginsenoside Rd (GSRd), one of the main active ingredients in traditional Chinese herbal Panax ginseng, has been found to have therapeutic effects on ischemic stroke. However, the molecular mechanisms of GSRd’s neuroprotective function remain unclear. Ischemic stroke-induced oxidative stress results in DNA damage, which triggers cell death and contributes to poor prognosis. Oxidative DNA damage is primarily processed by the base excision repair (BER) pathway. Three of the five major DNA glycosylases that initiate the BER pathway in the event of DNA damage from oxidation are the endonuclease VIII-like (NEIL) proteins. This study aimed to investigate the effect of GSRd on the expression of DNA glycosylases NEILs in a rat model of focal cerebral ischemia.

Methods: NEIL expression patterns were evaluated by quantitative real-time polymerase chain reaction in both normal and middle cerebral artery occlusion (MCAO) rat models. Survival rate and Zea-Longa neurological scores were used to assess the effect of GSRd administration on MCAO rats. Mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damages were evaluated by the way of real-time analysis of mutation frequency. NEIL expressions were measured in both messenger RNA (mRNA) and protein levels by quantitative polymerase chain reaction and Western blotting analysis. Apoptosis level was quantitated by the expression of cleaved caspase-3 and terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling assay.

Results: We found that GSRd administration reduced mtDNA and nDNA damages, which contributed to an improvement in survival rate and neurological function; significantly up-regulated NEIL1 and NEIL3 expressions in both mRNA and protein levels of MCAO rats; and reduced cell apoptosis and the expression of cleaved caspase-3 in rats at 7 days after MCAO.

Conclusions: Our results indicated that the neuroprotective function of GSRd for acute ischemic stroke might be partially explained by the up-regulation of NEIL1 and NEIL3 expressions.

Key words: DNA Damage; DNA Glycosylase; Endonuclease VIII-like Proteins; Ginsenoside Rd; Ischemic Stroke

Introduction

Stroke, of which ischemic stroke is the most common type, remains one of the major causes of mortality and disability worldwide. Ischemic stroke-induced oxidative stress plays an important role in neuronal injury by causing damage to cellular proteins, lipids, and DNA. Oxidative DNA damage can trigger dysfunction and death of brain neurons and eventually lead to poor outcomes. Maintaining genome integrity of neurons after ischemia is considered to be an important strategy to improve the prognosis of ischemic strokes. The predominant DNA repair pathway for oxidative DNA damage is base excision repair (BER). BER is initiated by DNA glycosylases, which recognize and excise the damaged bases from the DNA. The endonuclease VIII-like (NEIL) proteins, NEIL1, NEIL2, and NEIL3, are three of the five major DNA glycosylases that remove oxidative base lesions. Previous data have indicated that NEIL gene knockout models exhibit genomic instability.

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Received: 04-04-2016 Edited by: Xin Chen
How to cite this article: Yang LX, Zhang X, Zhao G. Ginsenoside Rd Attenuates DNA Damage by Increasing Expression of DNA Glycosylase Endonuclease VIII-like Proteins after Focal Cerebral Ischemia. Chin Med J 2016;129:1955-62.
gene knockout mice, for example, showed an increased level of DNA damage compared to wild-type mice, and NEIL2 gene knockout mice displayed an accumulation of oxidative genomic damage.\textsuperscript{[7,4]} Evidence also suggested that NEILs may affect stroke prognosis. For instance, the NEIL1 gene knockout mice with middle cerebral artery occlusion (MCAO) exhibited impaired memory retention, and NEIL3 gene knockout mice showed poor outcomes after ischemic stroke, suggesting that NEILs might be essential elements for better outcomes.\textsuperscript{[7,9]}

Panax ginseng is a well-known traditional herbal medicine which has been widely used in China for centuries, and ginsenoside Rd (GSRd) is one of its main active ingredients.\textsuperscript{[10]} In our previous studies, GSRd has been shown to protect against ischemic cerebral damage via various mechanisms both in vitro and in vivo.\textsuperscript{[10]} Furthermore, a randomized, double-blind, placebo-controlled, multicenter, phase II clinical trial, wherein 386 patients with acute ischemic stroke received a 14-day intravenous infusion of GSRd or placebo within 72 h after the onset of stroke, found that GSRd-treated patients showed significantly improved primary outcome over the control group.\textsuperscript{[11]} The molecular mechanisms that underpin GSRd’s neuroprotective function, however, have not yet been fully understood. Thus, as an extending study to further elucidate the underlying mechanisms, this study investigated the effect of Rd on the expression of DNA glycosylases NEILs in a rat model of focal cerebral ischemia.

Methods

Experimental animals and middle cerebral artery occlusion model

Male Sprague-Dawley rats, weighing 270–320 g, were provided by the Animal Center of the Fourth Military Medical University (Xi’an, China). Animal experiments were approved by the Ethics Committee for the Animal Care and Use Committee of the Fourth Military Medical University. A total of 96 rats were evenly and randomly allocated to four experimental groups: sham (n = 24), sham + GSRd (n = 24), MCAO (n = 24), and MCAO + GSRd (n = 24). MCAO was performed as described previously.\textsuperscript{[10]} In brief, rats were anesthetized with 10% chloral hydrate by intraperitoneal injection (0.35 ml/100 g). First, the right common carotid artery and into the middle carotid artery. Body temperature was maintained with a thermostatically controlled heating blanket to maintain 37.0 ± 0.5°C. Sham-surgery rats were subjected to the same surgical procedures except that the monofilament was not advanced into the middle carotid artery. Rats in the sham + GSRd and MCAO + GSRd groups, GSRd with a purity of 98% was obtained from Tai-He Biopharmaceutical Co. Ltd. (Guangzhou, China). Stock solutions were prepared in saline containing 10% of 1, 3-propanediol (v/v). For rats in the sham + GSRd and MCAO + GSRd groups, GSRd with a concentration of 30 µg/kg was applied intraperitoneally 1 h before MCAO and 10 mg kg\textsuperscript{-1} d\textsuperscript{-1} until the rats were sacrificed. Rats in the sham and MCAO groups were intraperitoneally injected with the same amount of sterile saline water at the same intervals as the sham + GSRd and MCAO + GSRd groups.

Zea-Longa neurological deficit scores

The neurological scores were blindly assessed by two pretrained technicians independently at 2 h, 8 h, and then every 24 h up to 7 days after MCAO according to the Zea-Longa neurological deficit scores.\textsuperscript{[12]} The Zea-Longa assessment criteria are as follows: score 0, normal, no neurological sign; score 1, cannot completely stretch contralateral forelimbs; score 2, contralateral circling when walking; score 3, contralateral fall over when walking; and score 4, cannot walk and lowering of consciousness. Rats were first evaluated 2 h after MCAO and the model was disqualified when it scored 0 or 1.

Quantitative real-time polymerase chain reaction

The total RNA was isolated using RNeasy kit (Qiagen, Germany) and RNA was reversely transcribed with high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). Subsequently, quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in a total volume of 20 µl, containing 11 µl of SYBR-Green I PCR master mix (Applied Biosystems, California, USA), 6 µl of cDNA (10 ng/µl), and 1 µl of 2.5 µM forward and reverse primers, using a StepOnePlus Real-Time PCR Machine (Applied Biosystems) in standard cycling conditions. All Ct values were first normalized to reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and relative gene expression levels were calculated using the standard 2(–Delta Delta C (t)) method. The primers used in quantitative polymerase chain reaction were as follows: NEIL1 forward 5’-TGGAGTATGAAAGGTTGAGGAGAGA-3’ and reverse 5’-GCCGATTTCAATTGAAGAACTC3’; NEIL2 forward 5’-CAGTGCCCAGGGCTGGTACTC-3’ and reverse 5’-CTCAGTGGAACCTTTTCAGACAAGA-3’; and NEIL3 forward 5’-GTGGAGAAGACCCCAAAGAGT-3’ and reverse 5’-TTTACGGCACCCTGTAGAAGA-3’.

Western blotting analysis

After MCAO, the ipsilateral cortex and hippocampus were homogenized on ice in the radioimmunoprecipitation assay buffer (Sigma, USA) with 0.5 mmol/L phenylmethanesulfonyl fluoride (Sigma). Western blotting analysis was performed according to the study of Zhang et al.\textsuperscript{[10]} The primary antibodies were NEIL1 antibody (1:100; Santa Cruz Biotechnology Inc., Texas, USA; sc-47611), NEIL2 antibody (1:100, Santa Cruz Biotechnology Inc., sc-135243), NEIL3 antibody (1:100, Santa Cruz Biotechnology Inc., sc-134835), and cleaved caspase-3 antibody (1:500; Cell Signaling Technology Inc., Beverly, Massachusetts, USA; Asp175). The secondary antibodies were anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody (1:5000, Cell Signaling Technology Inc., 7074), and donkey anti-goat IgG HRP antibody (1:5000; Abcam, Cambridge, USA; ab97110). Target protein signals were detected by...
the chemiluminescence system (GE Healthcare, Amersham, UK), and ImageJ 1.41 (National institutes of health, Maryland, USA) was used for quantitative analysis. The β-actin (1:5000, Abcam, ab8227) or GAPDH (1:10,000, Abcam, ab8245) was used as a loading control.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining**

To assess the number of apoptotic cells, brain sections were stained with the In Situ Cell Death Detection Kit, Fluorescein (Roch Inc., Mannheim, Germany), according to manufacturer’s instructions. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells in rat’s ipsilateral cortices at 7 days after MCAO were observed through Zeiss LSM 510 Confocal Microscope (Carl Zeiss, Chicago, USA) with a 40× objective and counted by two independent investigators. The total number of TUNEL-positive cells was quantified in five randomly selected fields.

**DNA damage quantification**

DNA damage was evaluated with real-time analysis of mutation frequency (RAMF).[10] In brief, the genomic DNA was isolated with DNeasy blood and tissue kit (Qiagen, Germany) and 6 ng of DNA was added to either non-Taq1 or Taq1 containing SYBR Green PCR master mix. The primers to detect mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damage were as follows: 12S forward 5’-ACTCAAAAGGACTTTGGCGTA-3’ and reverse 5’-AGCCCCATTCTCTCCATTCC-3’; and NDUF4A9 forward 5’-TGGTATCTAATCAAGGAGC-3’ and reverse 5’-TTCCGGTCGTAATTGTTT-3’, respectively. The qRT-PCR program was initiated at 65°C for 15 min, followed by 94°C for 10 min, plus 40 cycles at 94°C for 15 s and 60°C for 1 min. DNA damage frequency was calculated as 2exp-(CT\text{WT} - CT\text{NT}).

**Statistical analysis**

Each assay consisted of three independent experiments. Statistical analysis was performed using two-tailed Student’s t-test. For multiple comparisons, Kruskal-Wallis test followed by Dunn’s multiple comparison test was used. All data were analyzed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). A P < 0.05 was considered statistically significant.

**RESULTS**

**Ginsenoside Rd improved the survival rate and neurological scores of rats after middle cerebral artery occlusion**

We found that GSRd administration increased survival rate in the MCAO rat model [Figure 1a]. At the end of the study, 10 rats (out of 22) and 3 rats (out of 20) died in the MCAO and MCAO + GSRd groups, respectively. The survival rate of the MCAO + GSRd group (85%) was significantly improved over the control group (55%; \(\chi^2 = 4.53, P = 0.03\)). The effects of GSRd administration on neurological scores of the MCAO and MCAO + GSRd groups were evaluated according to the Zea-Longa neurological deficit scores. There were no significant differences at 2 h, 8 h, 1 day, and 2 days after MCAO. However, GSRd reduced the neurological deficit scores at 3–7 days after MCAO [Table 1 and Figure 1b].

**Ginsenoside Rd decreased apoptosis in rat ipsilateral cortices at 7 days after middle cerebral artery occlusion**

To investigate the effect of GSRd on apoptosis after MCAO, the cleaved caspase-3 was measured by Western blotting analysis [Figure 2a and 2b]. It was found that cleaved caspase-3 levels were significantly higher in the MCAO group and lowered by GSRd treatment. TUNEL staining was performed in rat’s ipsilateral cortices at 7 days after MCAO [Figure 2c and 2d]. The TUNEL-positive cell count in the MCAO + GSRd group was significantly lower than that in the MCAO group, implying that GSRd decreased cell apoptosis in rat’s ipsilateral cortices at 7 days after MCAO.

**Ginsenoside Rd reduced mitochondrial DNA and nuclear DNA damage after middle cerebral artery occlusion**

Using RAMF, the mtDNA and nDNA damages were evaluated and quantitated by RT-PCR in the MCAO and MCAO + GSRd groups [Figure 3]. At 3 and 7 days after MCAO, both mtDNA and nDNA damage levels were significantly increased in the MCAO and MCAO + GSRd groups. The mtDNA damage levels were lower in the MCAO + GSRd group than those in the MCAO group at both 3 and 7 days after MCAO. The nDNA damage levels were also lower in the MCAO + GSRd group versus the MCAO group at both 3 and 7 days after MCAO, but only with a significant difference at 7 days after MCAO.

**Distribution patterns of NEILs in brains of normal and middle cerebral artery occlusion rat**

To evaluate the distribution patterns of NEILs in normal rat brains, the expression levels of NEILs were measured by qRT-PCR in different regions of untreated rat brains, including the cortex, hippocampus, brainstem, cerebellum, spinal cord, corpus striatum, and thalamus [Figure 4a].

**Table 1: Zea-Longa neurological deficit scores of MCAO and MCAO + GSRd groups after GSRd administration**

| Time points | MCAO group | MCAO + GSRd group | t | P |
| --- | --- | --- | --- | --- |
| Numbers | Scores, mean ± SD | Numbers | Scores, mean ± SD |
| 2 h | 22 | 2.8 ± 0.1 | 20 | 2.9 ± 0.3 | 0.23 | 0.82 |
| 8 h | 22 | 2.2 ± 0.3 | 20 | 2.8 ± 0.3 | 1.83 | 0.08 |
| 1 days | 18 | 2.2 ± 0.4 | 19 | 2.2 ± 0.1 | 0.25 | 0.80 |
| 2 days | 17 | 2.6 ± 0.1 | 19 | 2.0 ± 0.9 | 2.00 | 0.07 |
| 3 days | 15 | 2.9 ± 0.1 | 18 | 1.9 ± 0.1 | 4.59 | <0.05 |
| 4 days | 15 | 2.6 ± 0.1 | 18 | 1.8 ± 0.2 | 2.75 | 0.01 |
| 5 days | 14 | 2.5 ± 0.2 | 18 | 1.7 ± 0.3 | 2.28 | 0.03 |
| 6 days | 13 | 2.5 ± 0.1 | 17 | 1.7 ± 0.1 | 3.99 | <0.01 |
| 7 days | 12 | 2.4 ± 0.2 | 17 | 1.7 ± 0.1 | 3.37 | <0.01 |

MCAO: Middle cerebral artery occlusion; GSRd: Ginsenoside Rd; SD: Standard deviation.
NEIL1 and NEIL2 showed ubiquitous expression in all the regions measured. Compared with cortex, NEIL1 was highly expressed in the hippocampus \( t = 2.19, P = 0.04 \) and cerebellum \( t = 2.59, P = 0.02 \); NEIL2 showed a moderate expression increase in the corpus striatum \( t = 2.54, P = 0.02 \); and NEIL3 was dominantly expressed in the hippocampus \( t = 2.29, P = 0.04 \).

The expression patterns of NEILs were further tested in samples from rat ipsilateral cortex at 12 h, 1 day, 3 days, and 7 days after MCAO [Figure 4b]. The expression of NEIL1 increased gradually, peaking at 3 days after MCAO with an 87-fold increase over the sham group \( t = 18.18, P < 0.01 \) and falling to a 41-fold increase over sham group \( t = 2.67, P = 0.02 \). NEIL2 showed an increasing trend after MCAO without significant difference among groups \( P > 0.05 \). Similar to NEIL2, the expression of NEIL3 also gradually increased without significant difference among groups for the first 3 days after MCAO, but increased significantly to 82-fold at 7 days after MCAO \( t = 22.65, P < 0.01 \). In summary, NEIL1 showed an early response, NEIL3 showed a later response, and NEIL2 had a less pronounced increase after MCAO. These results suggested that NEILs responded and acted differently after acute ischemic stroke.
Ginsenoside Rd increased NEIL1 expression at 3 days and NEIL3 expression at 7 days after middle cerebral artery occlusion

To evaluate the effect of GSRd administration on the expression of NEILs, both messenger RNA (mRNA) and protein levels of NEILs were measured in rat ipsilateral cortices and hippocampi at 3 days and 7 days after MCAO [Figure 5]. At 3 days after MCAO, the mRNA expressions of NEILs were significantly increased in both cortex and hippocampus compared to the sham and sham + GSRd groups, with the exception of NEIL2 [Figure 5a and 5b]. Only NEIL1 expression was significantly increased in the MCAO + GSRd group over the MCAO group in the cortex [Figure 5a]. At 7 days after MCAO, NEIL1 and NEIL3 mRNA levels were higher in the MCAO and MCAO + GSRd groups in both cortex and hippocampus [Figure 5c and 5d]. However, only NEIL3 expression increased in the MCAO + GSRd group over the MCAO group in both cortex and hippocampus [Figure 5c and 5d]. The changes in NEIL expressions were further confirmed at protein level. The results of Western blotting analysis showed that NEIL1 expression

Figure 3: The effect of GSRd on mtDNA and nDNA damages at 3 days (a) and 7 days (b) after MCAO. Data are presented as mean ± SD from 3 independent experiments ($n = 6$ biological replicates). *$P < 0.01$. mtDNA: Mitochondrial DNA; nDNA: Nuclear DNA; MCAO: Middle cerebral artery occlusion; SD: Standard deviation; GSRd: Ginsenoside Rd.

Figure 4: Regional expression analyses of NEILs in brains of normal rat and MCAO rat by qRT-PCR. (a) The expression of NEILs in normal rat brains. *$P < 0.05$, compared with cortex. (b) The distribution of NEILs in MCAO rat brains. *$P < 0.05$ and †$P < 0.01$, versus sham group. Data are presented as mean ± SD from 3 independent experiments ($n = 3$ biological replicates). Data were normalized to GAPDH expression. NEIL: Endonuclease VIII-like; qRT-PCR: Quantitative real-time polymerase chain reaction; MCAO: Middle cerebral artery occlusion; SD: Standard deviation; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Ginsenoside Rd increased NEIL1 expression at 3 days and NEIL3 expression at 7 days after middle cerebral artery occlusion.
was significantly increased by GSRd administration at 3 days after MCAO in the cortex [Figure 5e], and NEIL3 expression was significantly increased by GSRd administration at 7 days after MCAO in the hippocampus [Figure 5f]. No significant difference in NEIL1 and NEIL3 protein levels was found between the MCAO and MCAO + GSRd groups in the rest of the comparisons and NEIL2 was not detected by Western blotting analysis.

Figure 5: The effect of GSRd on the expression levels of NEILs after MCAO. (a and b) The mRNA expression levels of NEILs at 3 days after MCAO in rat ipsilateral cortices and hippocampi. (c and d) The mRNA expression levels of NEILs at 7 days after MCAO in rat ipsilateral cortices and hippocampi. (e) Western blotting analysis of NEIL1 in rat ipsilateral cortices at 3 days after MCAO. (f) Western blotting analysis of NEIL3 in rat ipsilateral hippocampi at 7 days after MCAO. *$P < 0.05$ and †$P < 0.01$. Data are mean ± SD from three independent experiments ($n = 6$ biological replicates). mRNA: Messenger RNA; NEIL: Endonuclease VIII-like; MCAO: Middle cerebral artery occlusion; GSRd: Ginsenoside Rd; SD: Standard deviation.
**Discussion**

The previous studies showed that GSRd had a neuroprotective function in ischemic stroke. In the present study, we investigated the effects of GSRd on the expression of DNA glycosylase NEILs in a rat MCAO model. Consistent with the previous findings, our results demonstrated that GSRd improved survival rate and neurological function. Moreover, we found that GSRd upregulated NEIL1 and NEIL3 expression levels. This suggested a correlation between the upregulation of NEIL1 and NEIL3 expression levels and a reduction in mtDNA and nDNA damage and, therefore, cell apoptosis.

Our previous work has revealed that GSRd is a neuroprotective agent through ameliorating oxidative stress after ischemic stroke. Similar results were observed in another study wherein oxidative stress was suppressed by GSRd pretreatment in the dopamine-induced apoptosis PC12 cell model. In seeking to further explore the neuroprotective mechanism of GSRd after ischemic stroke, we found that GSRd upregulated the expression of NEIL1 in the cortex at 3 days after MCAO and NEIL3 in the hippocampus at 7 days after MCAO in a rat model. However, we did not detect NEIL2 by Western blotting analysis, which may be induced by the relative low expression level of NEIL2 in rat brains. The differences in region and time course between NEIL1 and NEIL3 may be due to their different distributions in rat brains and NEILs responses after acute ischemic stroke. We also found that GSRd administration reduced the mtDNA and nDNA damages and expression of cleaved caspase-3. We proposed that by increasing NEIL1 and NEIL3 expressions, GSRd can enhance the BER pathway to reduce oxidative DNA damage, maintaining the genome integrity of neurons and subsequently decreasing apoptosis to improve the outcome of ischemic stroke in the rat model. This also indicated a strong association between the BER capacity and outcome of ischemic stroke. In line with our findings, other studies also found that enhancing the BER pathway can improve the recovery of ischemia and potentially be used as a neuroprotective strategy for ischemic stroke. Nicotinamide adenine dinucleotide (oxidized form; NAD⁺) repletion was reported to confer neuroprotection in a number of models of neurodegenerative disorders. The role of NAD⁺ in neuroprotection is supported by the finding that increasing NAD⁺ levels enhances the BER capacity and reduces DNA damage through increased DNA glycosylase OGG1 expression, which initiated the BER pathway.

Furthermore, we found that NEIL3 was dominantly expressed in rat hippocampi, one of the restricted neurogenesis regions in the adult brain, in both normal and MCAO rats. In line with our results, using in situ hybridization and Northern blotting, Rolseth et al. found that NEIL3 showed expression preference in the stem cell harboring region, including the subventricular zone and rostral migratory stream in the hippocampus. The specific distribution of NEIL3 in the central nervous system suggested that NEIL3 plays an important role in neurogenesis. Neural stem/progenitor cells from aged NEIL3 gene knockout mice showed impaired proliferative capacity and reduced DNA repair activity, indicating that NEIL3 was critical in maintaining hippocampal neurogenesis. Our previous studies found that GSRd promoted neurogenesis in vitro and in vivo and also showed the ability to maintain neural stem cell proliferation in a lead exposure rat model. In this study, we found that GSRd-induced NEIL3 expression in the hippocampus could be one of the mechanisms contributing to neurogenesis promotion, leading to the improvement of survival rates and neurological scores in GSRd-treated MCAO rats. In line with these results, Sejersted et al. found that NEIL3 could promote neurogenesis after hypoxia-ischemia. In contrast to NEIL3, NEIL1 is ubiquitously expressed in the brain and may have a role in protecting all types of neuronal cells against oxidative stress.

In conclusion, this study found that the neuroprotective function of GSRd could be partially explained by the upregulation of NEIL1 and NEIL3 expressions. Future studies are needed to determine the potential role of NEILs as a treatment biomarker for ischemic stroke.

**Financial support and sponsorship**

This study was supported by a grant from the National Natural Science Foundation of China (No. 81371365).

**Conflicts of interest**

There are no conflicts of interest.

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