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Original Article

Sulforaphane exerts neuroprotective effects via suppression of the inflammatory response in a rat model of focal cerebral ischemia

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Abstract: Inflammatory damage plays an important role in cerebral ischemic pathogenesis and may represent a promising target for treatment. Sulforaphane exerts protective effects in a rat model of focal cerebral ischemia/reperfusion injury by alleviating brain edema. However, the possible mechanisms of sulforaphane after cerebral ischemia/reperfusion injury have not been fully elucidated. Therefore, in the present study, we investigated the effect of sulforaphane on inflammatory reaction and the potential molecular mechanisms in cerebral ischemia rats. We found that sulforaphane significantly attenuated the blood-brain barrier (BBB) disruption; decreased the levels of pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β; reduced the nitric oxide (NO) levels and inducible nitric oxide synthase (iNOS) activity; inhibited the expression of iNOS and cyclooxygenase-2 (COX-2). In addition, sulforaphane inhibits the expression of p-NF-κB p65 after focal cerebral ischemia-reperfusion injury. Taken together, our results suggest that sulforaphane suppresses the inflammatory response via inhibiting the NF-κB signaling pathway in a rat model of focal cerebral ischemia, and sulforaphane may be a potential therapeutic agent for the treatment of cerebral ischemia injury.

Keywords: Sulforaphane, inflammatory response, focal cerebral ischemia

Introduction

Ischemic stroke, also known as cerebral infarction, is a life-threatening cerebrovascular disease with substantial morbidity and mortality worldwide [1]. Ischemic stroke is more prevalent than that of hemorrhagic, representing approximately 87% of all stroke cases [2]. In spite of advances in understanding brain ischemia and stroke etiology, the precise mechanism of ischemic stroke remains poorly understood. Therefore, understanding of the pathological mechanism of ischemic stroke is very important for developing effective therapies for ischemic stroke.

There is a considerable body of evidence suggesting that excessive inflammatory response plays an important role in the pathogenesis of cerebral ischemia [3-5]. It has been reported that ischemic/reperfusion injury triggered inflammatory cascades in the brain which may further amplify tissue damage [6, 7]. Therefore, targeting inflammation and related factors could be a crucial therapeutic strategy for preventing and treating brain damage and promoting brain repair.

Sulforaphane (4-methylsulfinylbutyl isothiocyanate), a member of the isothiocyanate family, is found in cruciferous vegetables such as broccoli, Brussels sprouts, and cabbages [8]. Sulforaphane has been known to have a variety of properties including anti-carcinogenic, anti-inflammatory, and anti-microbial properties in experimental models [6, 9, 10]. Most recently, one study reported that sulforaphane significantly decreased cerebral infarct volume following focal ischemia in rodents [11], and the reduction of brain edema in response to sulforaphane administration could be due, in part, to water clearance by aquaporin (AQP4) from the injured brain [12]. However, the possible mechanisms of action of sulforaphane after

cerebral ischemia/reperfusion injury have not been fully elucidated. In the present study, we therefore investigated the effect of sulforaphane on inflammatory reaction and the potential molecular mechanisms in cerebral ischemia rats.

Materials and methods

Animals

Adult male Sprague-Dawley rats (220-250 g) were provided by the Model Animal Research Centre of Zhongshan Hospital, Xiamen University. Rats were housed under controlled environmental conditions with ambient temperature of 25°C, relative humidity of 65%, and 12/12-h light/dark cycle. Food and water were provided ad libitum. All efforts were made to minimize the number of animals used and their suffering.

Focal cerebral ischemia-reperfusion model

Rats were randomly divided into five groups: sham-operated group, I/R group, and sulforaphane groups (10, 20 and 40 $mg{\cdot}kg{\cdot}d^{-1}$ respectively). The sulforaphane groups were injected intraperitoneally for 14 days. After 1 h of the last administration, all rats were anaesthetized with chloral hydrate (400 mg/kg, i.p). Then the focal cerebral ischemia-reperfusion was induced by right MCAO as described previously [13]. In brief, a 4-0 silicone-coated nylon filament was advanced from the external carotid artery into the lumen of internal carotid artery until the rounded tip reached the entrance to the middle cerebral artery. The reduction of the middle cerebral artery blood flow was confirmed by a laser Doppler flow meter (LDF; Perimed PF5000, Stockholm, Sweden) immediately after the occlusion. The filament was withdrawn very carefully after 2 h of occlusion, the wound was sutured and rat was placed in their normal environment with free access of food and water. Sham group underwent sham surgery except MCAO. After 24 h of reperfusion, the rats were anesthetized and then decapitated. The brains were immediately frozen in liquid nitrogen for the following studies.

BBB permeability measurement

BBB permeability was evaluated by Evans blue extravasation. Rats were injected with Evans blue (Sigma-Aldrich, St. Louis, MO, USA; 2% in saline, 3 ml/kg) through the tail vein at 18 h after reperfusion. Six hours later, rats were anesthetized with 10% chloral hydrate (350 mg/kg, ip) and transcardially perfused with saline to remove the intravascular dye. Brains were quickly removed and separated the ipsilateral hemispheres, and then weighed and homogenized in N,N-dimethylformamide (2.0 ml, Sigma-Aldrich, St. Louis, MO, USA), incubated at 37°C for 72 h and then centrifuged at 15,000 rpm for 15 min. The supernatant was measured for absorbance at 620 nm by spectrophotometry. The extravasation was expressed as lg of Evans blue per g of wet tissue weight.

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted from brain sample using Trizol reagents (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed to obtain single-strand cDNA using a Reverse Transcription System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Single-strand cDNA was amplified by PCR in a 100-µl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, 2 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM sense and antisense primers, and 2.5 units Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The primer sequences were as follows: tumor necrosis factor (TNF)-α, (primers, sense 5'-AAATGGGCTCCCTCTCATCAGTTC-3'; antisense 5'-TCTGCTTGGTGGTTTGCTACGAC-3'), interleukin (IL)-1β, (primers, sense 5'-CACCTCTCAAGCAGAGCACAG-3'; antisense 5'-GGGTTCCATGGTGAAGTCAAC-3'), inducible nitric oxide synthase (iNOS), (primers, sense 5'-ATCCCGAAACGC TACACTT-3'; antisense 5'-TCTGGCGAAGAACAATCC-3'), cyclooxygenase-2 (COX-2), (primers, sense 5'-GAGAGATGTATCCTCCCACAGTCA-3'; antisense 5'-GACCAGGCACCAGACCAAAG-3'), β-actin as an internal standard (primers, sense 5'-CACCCTGTGCTGCTCACCGAGGCC-3'; antisense 5'-CCACACAGATGACTTGCGCTCAGG-3'). The reactions were initially heated at 94°C for 4 min, then at 94°C for 40 s, 55°C for 40 s, and 72°C for 50 s, totally 40 cycles, finally stopped at 72°C for 7 min. Gene expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method as previously described [14].

Western blot

Rats were anesthetized and the brains were removed quickly. A coronal brain section 2 mm

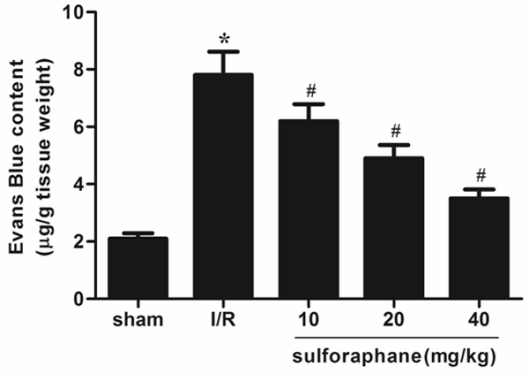

Figure 1. Effects of sulforaphane on the BBB permeability following I/R injury. Sulforaphane doses (10, 20 and 40 mg/kg) were administered i.p. at 1 and 12 h after the onset of ischemia, respectively. Sham-operated and I/R model groups received vehicle (25% DMSO in normal saline) 10 ml/kg according to the same protocol. Animals were sacrificed at 24 h after reperfusion for measurement of BBB permeability which was evaluated by Evans blue extravasation. Data are expressed as mean ± SD; n = 5 for each experimental group. $^{*}P<0.05$, vs. the sham-operated group, $^{\#}P<0.05$, vs. the I/R model group.

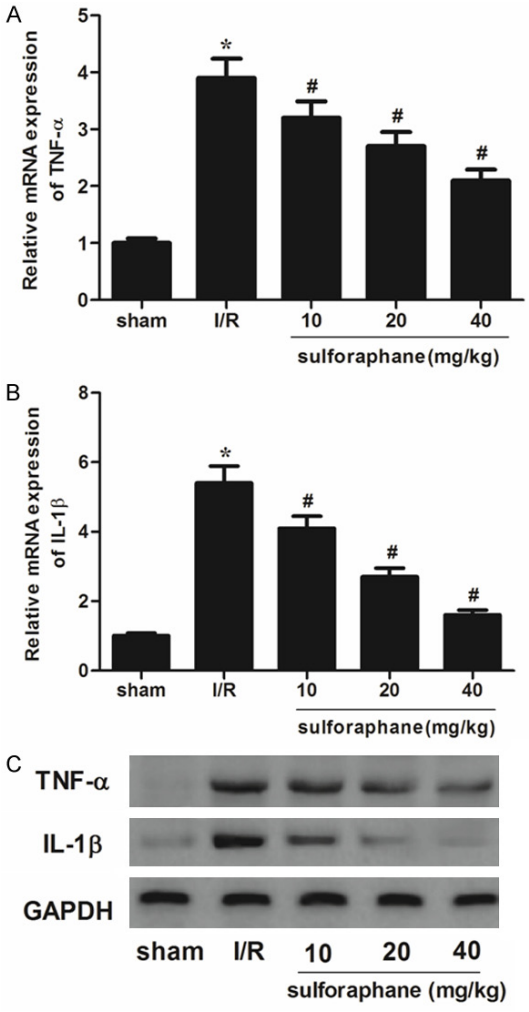

Figure 2. Effects of sulforaphane on the levels of TNF-α and IL-1β following I/R injury. Sulforaphane doses (10, 20 and 40 mg/kg) were administered i.p. at 1 h and again at 12 h after the onset of ischemia, respectively. Sham-operated and I/R model groups received vehicle (25% DMSO in normal saline) 10 ml/kg according to the same protocol. A and B. The mRNA levels of TNF-α and IL-1β were determined by RT-PCR. C. The protein levels of TNF-α and IL-1β were determined by Western blot. Data are expressed as mean ± SD; n = 4 for each experimental group. $^{*}P<0.05$, vs the sham-operated group, #$P<0.05$, vs the I/R model group.

from the frontal pole was cut and the slices were divided into the ipsilateral and contralateral cortex. Total proteins were extracted using tissue protein extraction reagents (Takara Biotechnology, Dalian, China). Protein extracts were resolved by SDS-polyacrylamide gel electrophoresis, and transferred onto a blotting membrane. The membranes were incubated with antibodies against TNF-α, IL-1β, iNOS, COX-2, p-NF-κB p65, NF-κB p65 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Specific protein bands were visualized by enhanced chemiluminescence.

NO levels and iNOS activity assay

NO levels and iNOS activity were evaluated using the assay kits from Jiancheng-Bioeng Ins. (China) according to the manufacturer's instruction. Protein concentration was determined by the Coomassie blue protein-binding assay using bovine serum albumin as a standard.

Statistical analysis

The quantitative data were expressed as mean ± SD. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Bonferroni test for multiple groups or Student t test between two groups. A *P*-value of <0.05 was considered to indicate a statistically significant difference.

Results

Effect of sulforaphane on BBB permeability

We first examined the effect of sulforaphane on BBB permeability. As shown in **Figure 1**, at 24 h

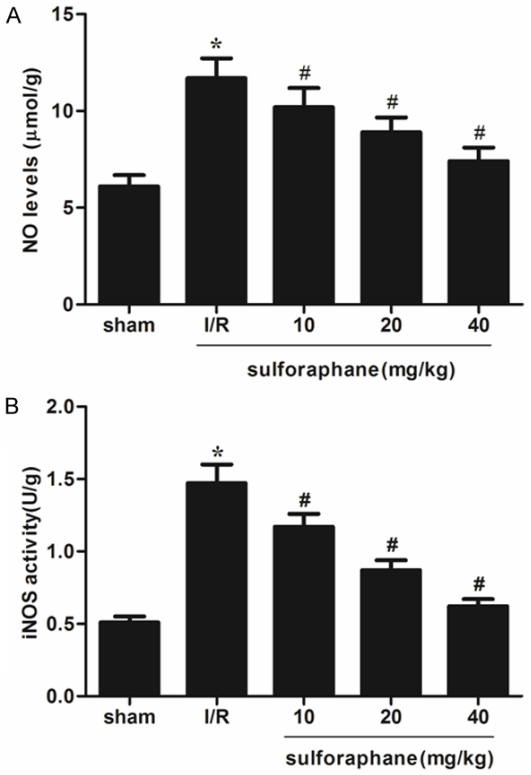

Figure 3. Effects of sulforaphane on the NO content and iNOS activity in the cortex following I/R injury. Sulforaphane doses (10, 20 and 40 mg/kg) were administered i.p. at 1 h and again at 12 h after the onset of ischemia, respectively. Sham-operated and I/R model groups received vehicle (25% DMSO in normal saline) 10 mL/kg according to the same protocol. Animals were sacrificed at 24 h after reperfusion for measurement of NO levels and iNOS activity in the lesioned hemisphere cortex. Data are expressed as mean ± SD; n = 4 for each experimental group. $^{*}P<0.05$, vs the sham-operated group, #$P<0.05$, vs the I/R model group.

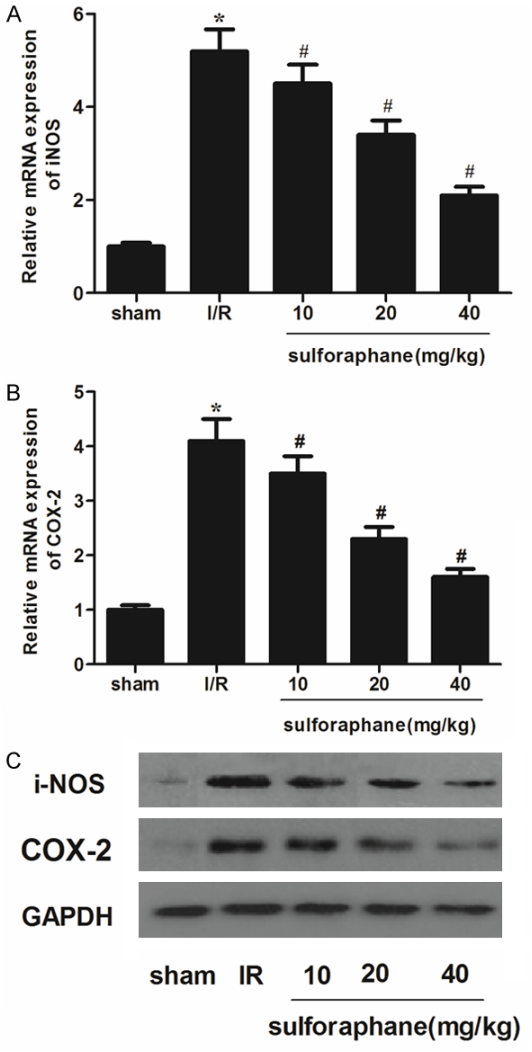

Figure 4. Effect of sulforaphane on the levels of iNOS and COX-2 in cerebral ischemia/reperfusion rats. A and B. The mRNA levels of iNOS and COX-2 were determined by RT-PCR. C. The protein levels of iNOS and COX-2 were determined by Western blot. Data are expressed as mean ± SD; n = 4 for each experimental group. $^{*}P<0.05$, vs. the sham-operated group, $^{\#}P<0.05$, vs. the I/R model group.

after reperfusion, I/R model group showed a significant increase in BBB permeability, as compared with sham-operated group. However, treatment of sulforaphane significantly inhibited Evans Blue extravasation compared with the I/R model group.

Sulforaphane down-regulated the expression of TNF-α and IL-1β in cerebral ischemia/reperfusion rats

To further analyze sulforaphane-induced anti-inflammatory effects, we also examined the mRNA and protein levels of TNF-α and IL-1β using RT-PCR and western blot. As shown in **Figure 2**, the mRNA and protein expression levels of TNF-α and IL-1β were significantly increased in the I/R group. However, sulforaphane obviously prevented I/R-induced the expression levels of TNF-α and IL-1β in a dose-dependent manner.

Effect of sulforaphane on expression of iNOS and COX-2

It has been demonstrated that the inducible effector enzymes such as iNOS and COX-2 play critical roles in ischemic brain regions. Thus, we investigated the effect of sulforaphane on NO

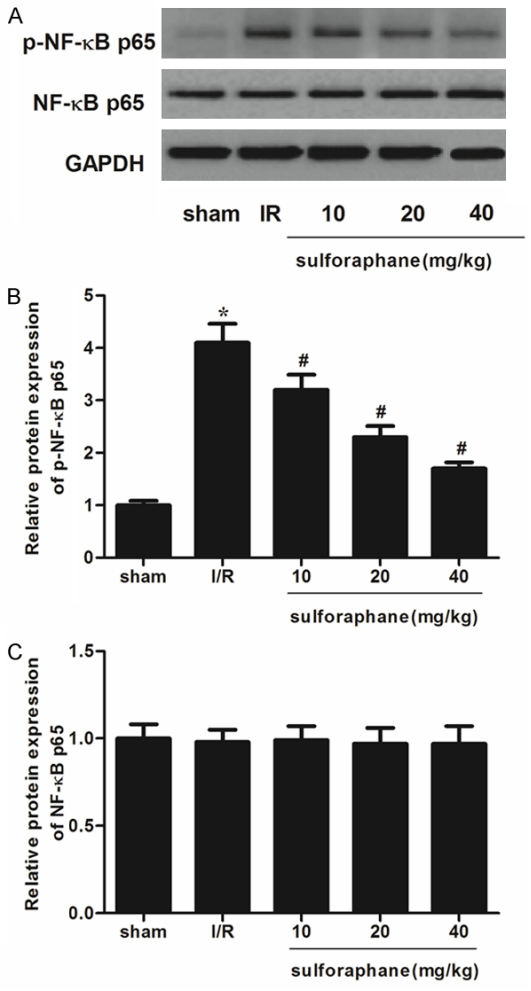

Figure 5. Effect of sulforaphane on protein expression of NF-κB p65 in cerebral ischemia/reperfusion rats. Proteins were extracted from the ischemic cortex. A. The protein levels of p-NF-κB p65 and NF-κB p65 were determined by Western blot; B and C. The relative protein expression levels of p-NF-κB p65 and NF-κB p65 were quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. Data are expressed as mean ± SD; n = 3 for each experimental group. **P*<0.05, vs. the sham-operated group, #*P*<0.05, vs. the I/R model group.

levels and iNOS activity. As shown in **Figure 3**, NO levels and iNOS activity in the lesioned hemisphere were obviously increased after I/R injury, compared with sham-operated group. However, sulforaphane significantly inhibited this increase in NO and iNOS induced by I/R injury.

Furthermore, we investigated the effects of sulforaphane on the expression of iNOS and COX-2 in the cortex region 24 h after reperfusion. As shown in **Figure 4**, the mRNA and protein expression levels of iNOS and COX-2 in the cortex region were markedly increased in the I/R model group, compared with the sham operated group. However, treatment of sulforaphane significantly decreased the expression of iNOS and COX-2, compared with the I/R model group.

Sulforaphane inhibited cerebral ischemia-induced NF-κB pathway activation

To further understand the anti-inflammatory mechanisms offered by sulforaphane, we examined the level of NF-κB p65 expression in the nucleus of the ipsilateral hemisphere cortex after ischemia reperfusion injury. As shown in **Figure 5**, the protein level of p-NF-κB p65 in the nucleus of the ipsilateral hemisphere cortex was obviously increased after I/R injury, as compared with the sham-operated group. However, administration of sulforaphane significantly suppressed I/R injury-induced the expression of p-NF-κB p65.

Discussion

The results of the present study show that sulforaphane treatment is able to significantly attenuate the BBB disruption; decrease the levels of pro-inflammatory cytokines TNF-α and IL-1β; reduce the NO levels and iNOS activity; inhibit the protein expression of iNOS and COX-2. In addition, sulforaphane and suppress the expression of p-NF-κB p65 after focal cerebral ischemia-reperfusion injury.

Pro-inflammatory cytokines, including TNF-α, IL-1β which are secreted in the ischemic region by activated immune cells, are the key cytokines that augment inflammatory reactions in the brain [15, 16]. It has been reported that brain concentrations of TNF-α and IL-1β were increased following transient cerebral ischemia in gerbils [17] and inhibition of TNF-α and IL-1β has been shown to reduce ischemic brain damage [18, 19]. Most recently, Mao et al showed that sulforaphane reduced levels of cytokine gene expression and protein levels of TNF-α, IL-6, and IL-1β in the spinal cord lesions after spinal cord injury [20]. In line with previous studies, in this study, we found that ischemia induced the expression levels of TNF-α and IL-1β, after sulforaphane treatment, the expression levels of TNF-α and IL-1β were significantly down-regulated. Based on these studies, our findings suggest that sulforaphane exerted its

neuroprotective effects against ischemic brain injury likely through the attenuation of intracerebral inflammatory response.

Attenuation of iNOS expression and NO production has been demonstrated to protect brain against ischemic damage [21, 22]. In this study, our data demonstrated that sulforaphane significantly attenuates ischemia-induced NO levels, increase in the lesioned hemisphere. Furthermore, compared with sham-operated group controls, both iNOS activity and protein expression were increased after cerebral ischemia insult. However, treatment with sulforaphane resulted in a significant inhibition of both the activity and protein expression of iNOS.

COX-2 is the key enzyme involved in arachidonic acid metabolism and plays an important role in inflammatory reaction [23]. COX-2 was not expressed in normal brains, but it was highly expressed in cerebral cortex of ischemia [24]. In this study, we observed that COX-2 was activated after cerebral ischemia. Sulforaphane significantly decreased expression of COX-2. The result suggested that sulforaphane could inhibit the activation of COX-2 and the effect might be associated with the inhibition of inflammatory reaction.

NF-κB is an inflammation responsive transcription factor known to be activated in response to ischemic stroke [25]. Inhibition of NF-κB activation results in a significant reduction of brain damage in both transient and permanent cerebral ischemia models [26]. In addition, studies had confirmed that blocking the activation of NF-κB led to inhibit the expression of iNOS and other inflammatory mediators that contributes to the evolution of ischemic brain damage. In the present study, we found that the protein level of p-NF-κB p65 in the nucleus at 24 h after reperfusion in the ipsilateral hemisphere cortex was increased significantly after I/R injury. However, treatment with sulforaphane significantly prevented this increase in p-NF-κB p65 level induced by I/R injury. On the basis of these data, we suggest that sulforaphane can suppress the expression of p-NF-κB p65 in the nucleus of the ipsilateral hemisphere cortex, thereby leading to a reduction in the production of pro-inflammatory mediators and decrease in the inflammation after cerebral ischemic insult.

In conclusion, we have shown that sulforaphane suppresses the inflammatory response via inhibition of NF-κB signaling pathway in a rat model of focal cerebral ischemia. Sulforaphane administration may therefore be a potential therapeutic agent for the treatment of cerebral ischemia injury.

Disclosure of conflict of interest

None.

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