Int J Clin Exp Med 2016;9(2):4929-4938
www.ijcem.com /ISSN:1940-5901/IJCEM0012915

Original Article

Effects of sulforaphane on TNF-α-induced proinflammatory cytokine production and reactive oxygen species generation in mouse brain endothelial cells

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Received July 15, 2015; Accepted January 16, 2016; Epub February 15, 2016; Published February 29, 2016

Abstract: This study is to investigate the effects of sulforaphane (SFN) on TNF-α-induced proinflammatory cytokine production and reactive oxygen species (ROS) generation in bEnd.3 mouse brain endothelial cells. The bEnd.3 cells were stimulated with TNF-α, with or without SFN treatment, for 24 h. Interleukin-1β (IL-1β) and endothelin contents were detected by ELISA. ROS level was assessed by flow cytometry. The mRNA and protein expression levels of heme oxygenase (HO)-1 were detected by real-time PCR and Western blot, respectively. SFN (0-30 μg/mL) did not induce toxic effects on bEnd.3 cells. ELISA showed that, IL-1β and endothelin levels in bEnd.3 cells were significantly increased by the TNF-α treatment. However, SFN treatments significantly decreased the IL-1β and endothelin contents in these cells, in a dose dependent manner. Moreover, intracellular ROS level was significantly enhanced in TNF-α-treated bEnd.3 cells, which could be decreased by SFN treatments. Real-time PCR and Western blot showed that, the mRNA and protein expression levels of HO-1 were significantly increased by the TNF-α stimulation, which could be further elevated by SFN treatments. The reducing effects of SFN on IL-1β and endothelin production were enhanced by the activator of HO-1, while the effects of SFN were reversed by the inhibitor of HO-1, in TNF-α-treated bEnd.3 cells. SFN inhibits TNF-α-induced proinflammatory cytokine production and ROS generation in bEnd.3 cells, which might be mediated by the up-regulation of HO-1. Our findings may provide evidence for the application of SFN in the treatment of brain injuries caused by cerebral ischemia/reperfusion.

Keywords: Sulforaphane (SFN), brain endothelial cells, interleukin-1β (IL-1β), endothelin, reactive oxygen species (ROS)

Introduction

Microvascular endothelial cells are one of the important components of the blood brain barrier (BBB). Damages of these cells induced by ischemia would result in injuries to BBB, further leading to increased brain vascular permeability and vasogenic brain edema [1, 2]. In recent years, the concept of neurovascular unit has been proposed and gradually accepted, including neural cells, endothelial cells, astrocytes, and surrounding extracellular matrix [3-5]. It has been shown that the activation and regeneration of endogenous nerves alone might not give satisfactory outcomes. Therefore, some researchers claim that neurovascular unit should be considered as a whole in the treatment of cerebral ischemia [6, 7].

It has been well accepted that, the severity and prognosis of brain diseases depend not only on the range and degree of cerebral hypoxia-ischemia, but also on the hypoxia, ischemia/reperfusion injury, which involves reactive oxygen species (ROS), cytokines, adhesion molecules, and platelets [8, 9]. The accumulation of ROS would enhance the expression of cytokines (such as TNF-α and IL-1β) and adhesion molecules, and alter the inflammatory responses, resulting in tissue injuries and neuronal death [10, 11]. Therefore, it is of great importance to find or develop anti-oxidative drugs associated with multiple pathways, with low side effects, for the treatment of brain injuries [12-14].

Sulforaphane (SFN) is a kind of isothiocyanate with strong antioxidant activity, which is derived

from cruciferous vegetables [15, 16]. SNF can reduce the DNA damages induced by various carcinogens, and inhibit the proliferation of tumor cells [17, 18]. Moreover, SFN has also been shown to be able to regulate the body immune system, exhibiting neuroprotective effects in animal models of acute or chronic nervous system diseases [19-22]. However, the effects of SFN on endothelial cell injuries have not yet been fully elucidated. In this study, the bEnd.3 mouse brain endothelial cells were stimulated with TNF-α, and the effects of SFN on TNF-α-induced proinflammatory cytokine production and ROS generation in these cells and the related mechanisms were investigated.

Materials and methods

Cell culture and drug administration

Mouse endothelial cell line (bEnd.3) was purchased from the American Type Culture Collection (ATCC, USA). These bEnd.3 cells were cultured with DMEM (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, Zhejiang, China), in a 5% CO_2, 37°C incubator. These cells were divided into the following groups: (1) the control group, in which the cells were treated with 0.1% DMSO (Cusabio, Wuhan, Hubei, China) for 14 h; (2) the TNF-α-induced model group, in which the cells were treated with 5 ng/mL TNF-α for 24 h; and (3) the treatment groups, in which TNF-α-induced cells were treated with 5, 10, and 30 μg/mL SFN (Sigma-Aldrich, St Louis, MO, USA), respectively, for 24 h.

Lactate dehydrogenase (LDH) activity assay

The LDH activity was assessed with the LDH kit (Promega, Madison, WI, USA). Cells in the logarithmic growth phase were collected and planted at a density of 1×10^5/mL. After TNF-α induction and/or SFN treatments, cell culture medium was collected. 50 μL substrate was prepared and added into a well on a microplate, and then incubated in dark for 30 min. The stop solution was added, and the absorbance (OD) at 490 nm was recorded. On the other hand, the cells after induction and treatments were lysed by the freeze-thawing method, and the maximum LDH activity was measured. The LDH leakage rate was calculated according to the following equation: LDH leakage rate (%) = $OD_{490\ nm}$ (the experimental group)/$OD_{490\ nm}$ (the maximum release) × 100%.

Quantitative real-time PCR

The mRNA expression levels of HO-1 in bEnd.3 cells were detected with quantitative real-time PCR. Total RNA was extracted with the Trizol agent (Sangon Biotech, Shanghai, China). The reverse transcription was performed to obtain the cDNA template. The primers used for the real-time PCR were synthesized by Sangon Biotech: HO-1, forward 5'-ATGGCCTCCCTGTACCACATC-3' and reverse 5'-TGTTGCGCTCAATCTCCTCCT-3'; β-actin, forward 5'-CATCCTGCGTCTGGACCTGG-3' and reverse 5'-TAATGTCACGCACGATTTCC-3'. The 50 μL PCR system consisted of 1 μL template, 1 μL primer each, 7 μL 25 mM $MgCl_2$, 5 μL 10 × PCR buffer, 0.5 μL 5 U/μL polymerase, 1 μL SYBR Green I, 1 μL 10 mM dNTP, and 32.5 μL ddH_2O. The reaction conditions were as follows: denaturation at 95°C for 2 min; 95°C for 15 s, 60°C for 60 s, for totally 40 cycles; followed by 72°C for 30 s. The relative expression levels of the target gene were calculated by the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-1β and endothelin were detected with the ELISA kit (Neobioscience, Shenzhen, Guangdong, China), according to the manufacturer's instructions. Briefly, 10 μL sample and 40 μL diluent were added into each well, and the microplate was incubated in dark at 37°C for 30 min. After washing, 50 μL enzyme solution was added, and the microplate was incubated in dark at 37°C for 1 h. After washing, 100 μL TMB was added into each well for color development in dark at 37°C for 15-20 min. 50 μL stop solution was added, and the OD at 450 nm was read by a microplate reader (Thermo Multiskan MK3, Labsystems, Helsinki, Finland) within 15 min. The standard curve was obtained with the standard samples provided within the kit, and the sample concentration was determined accordingly.

Intracellular ROS level assessment

The cells were planted onto a 6-well plate at a density of 5×10^4/mL. After induction and drug

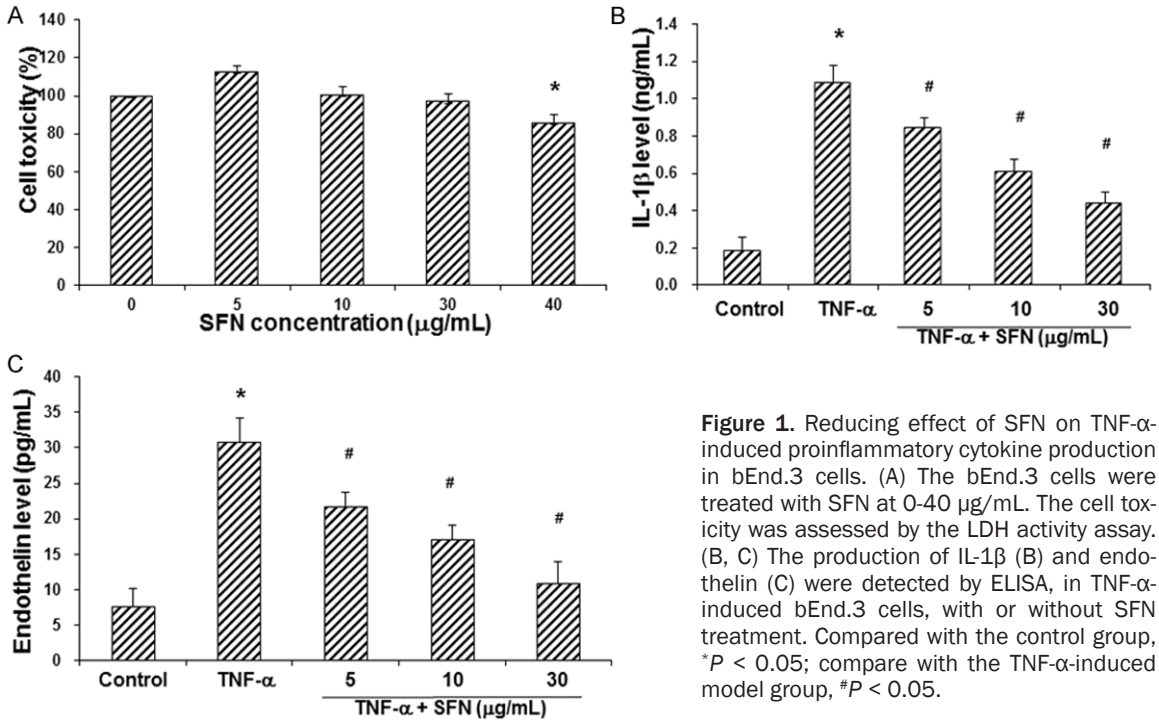


Figure 1. Reducing effect of SFN on TNF-α-induced proinflammatory cytokine production in bEnd.3 cells. (A) The bEnd.3 cells were treated with SFN at 0-40 μg/mL. The cell toxicity was assessed by the LDH activity assay. (B, C) The production of IL-1β (B) and endothelin (C) were detected by ELISA, in TNF-α-induced bEnd.3 cells, with or without SFN treatment. Compared with the control group, $^{*}P < 0.05$; compare with the TNF-α-induced model group, $^{\#}P < 0.05$.

treatments, the cells were collected into an EP tube, and re-suspended with 1 mL DCFH-DA (Molecular Probes, Eugene, OR, USA) solution. The cell suspension was incubated in dark for 40 min. The cells were collected by centrifugation at 1500 rpm for 5 min, and re-suspended with PBS. The fluorescence was detected with flow cytometry, with the excitation wavelength of 488 nm and the emission wavelength of 535 nm. The relative intracellular ROS level was calculated accordingly.

Western blot analysis

The protein expression level of HO-1 in the bEnd.3 cells was detected with the Western blot analysis. Cells were lysed with the lysis buffer, and centrifuged at 4°C at 12000 rpm for 2 min. The protein concentration was determined by the BCA kit (Beyotime, Haiman, Jiangsu, China). 10 μL protein was separated on 10% SDS-PAGE, and then transferred onto a NC membrane. The blot was blocked with 5% fat-free milk at room temperature for 2 h, and then incubated with mouse anti-human anti-HO-1 polyclonal antibody (Santa Cruz, Santa Cruz, CA, USA) at 4°C overnight. Then the membrane was incubated with HRP-conjugated goat anti-mouse polyclonal secondary antibody (Boster, Wuhan, Hubei, China) at 37°C for 1-2 h. The colorization was performed with the electrochemiluminescence (ECL) method.

Statistical analysis

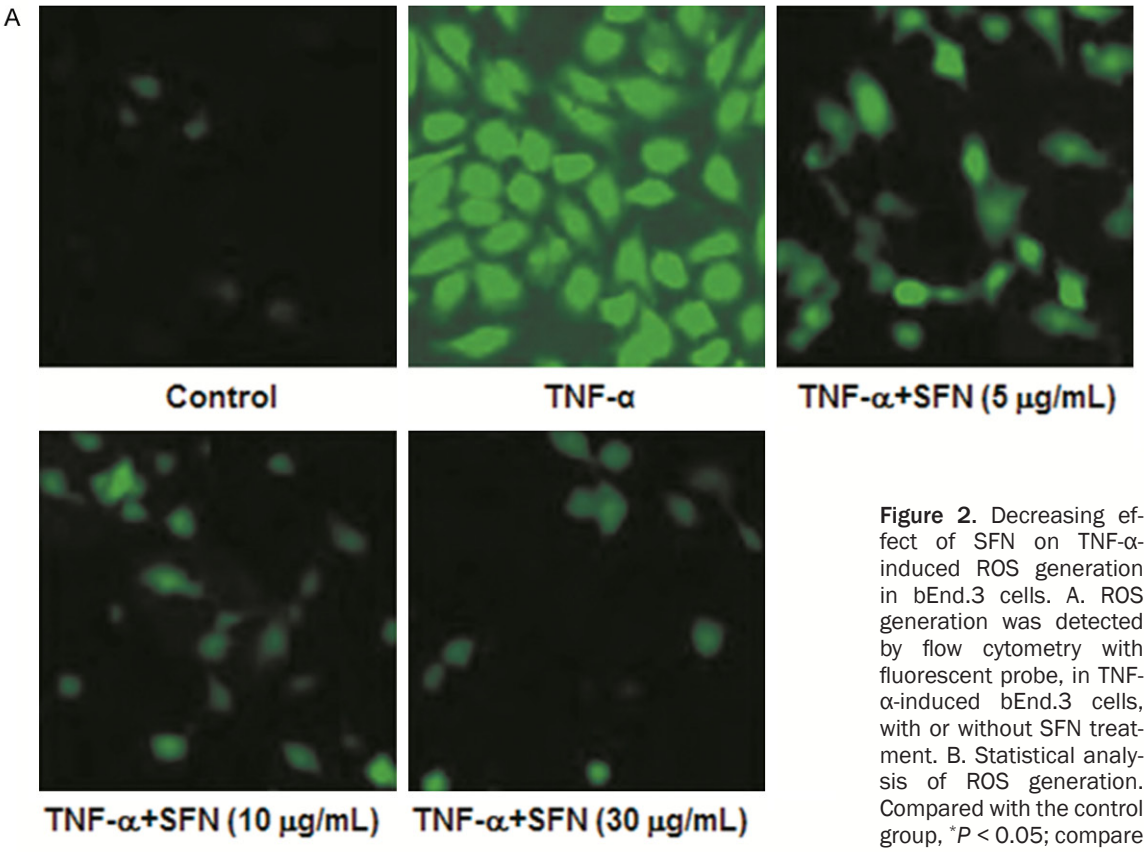
Data are expressed as mean ± SD. SPSS13.0 software was used for the statistical analysis. The *t*-test was used for the comparison between groups. $P < 0.05$ was considered as statistically significant.

Results

SFN reduces TNF-α-induced IL-1β and endothelin production in bEnd.3 cells

We first tried to find out the optimum treatment concentration of SFN on bEnd.3 cells. These cells were treated with SFN at indicated concentrations (0, 5, 10, 30 and 40 μg/mL), and then the toxicity was assessed by the LDH activity assay. Our results showed that, SFN at the concentrations of 0-30 μg/mL did not induce significant toxic effects on the viability of the bEnd.3 cells (**Figure 1A**). Based on these results, the treatment concentrations of SFN were set at 5, 10, and 30 μg/mL for the following experiments.

To investigate the effects of TNF-α and SFN on the bEnd.3 cells, the contents of IL-1β and



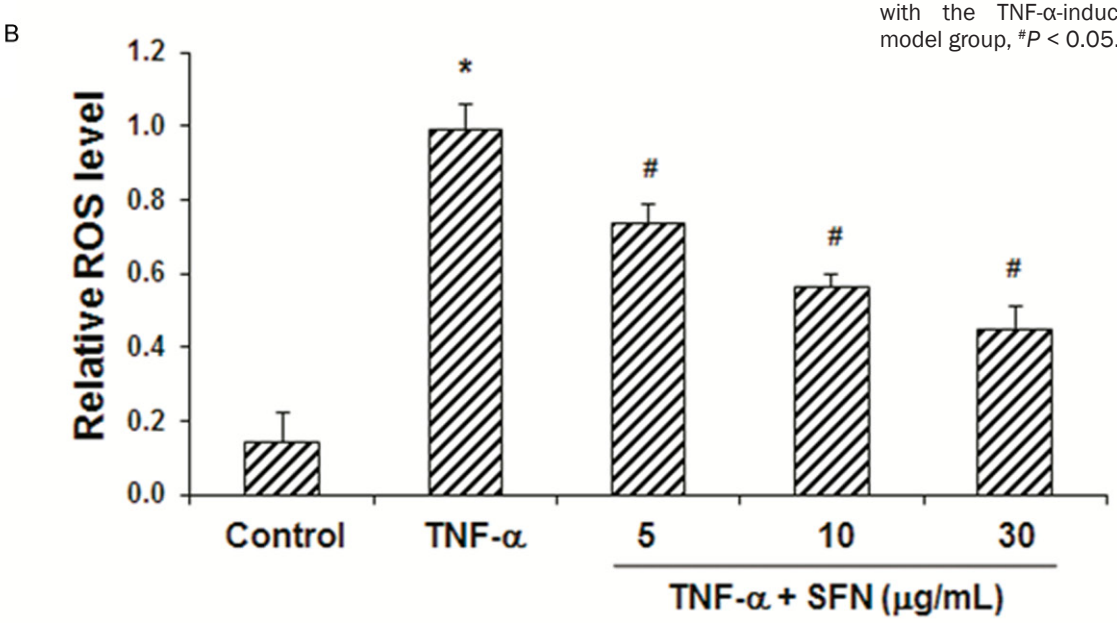


Figure 2. Decreasing effect of SFN on TNF-α-induced ROS generation in bEnd.3 cells. A. ROS generation was detected by flow cytometry with fluorescent probe, in TNF-α-induced bEnd.3 cells, with or without SFN treatment. B. Statistical analysis of ROS generation. Compared with the control group, $^{*}P < 0.05$; compare with the TNF-α-induced model group, $^{\#}P < 0.05$.

endothelin were detected by ELISA. Our results showed that, compared with the control group, 5 ng/mL TNF-α significantly increased the levels of IL-1β and endothelin in bEnd.3 cells (both $P < 0.05$). However, the treatments of SFN significantly decreased the levels of IL-1β and endothelin in TNF-α-induced bEnd.3 cells, in a dose dependent manner (all $P < 0.05$) (**Figure 1B**, **1C**). These results suggest that, SFN treatment could reduce the TNF-α-induced IL-1β and endothelin production in bEnd.3 cells, in a dose dependent manner.

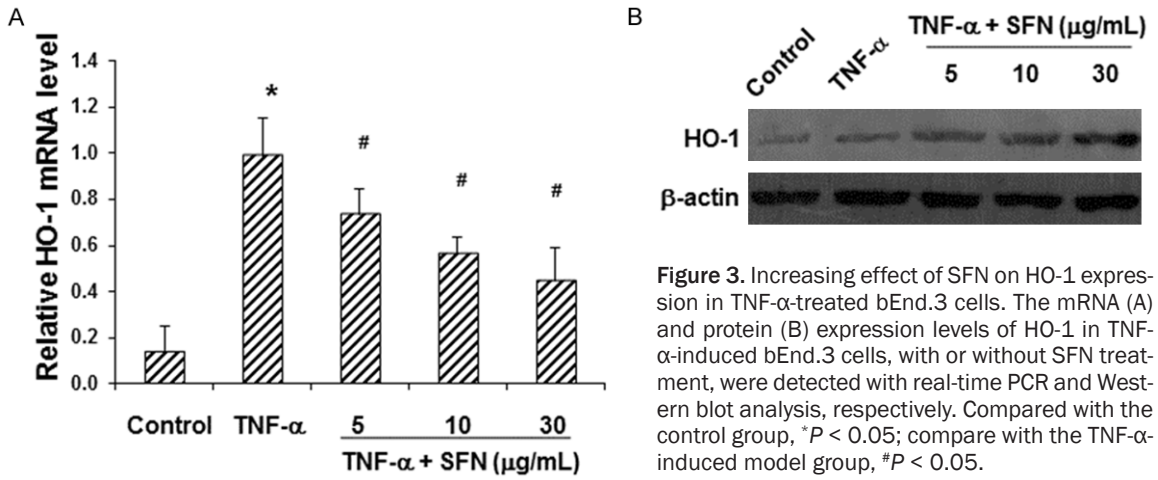


Figure 3. Increasing effect of SFN on HO-1 expression in TNF-α-treated bEnd.3 cells. The mRNA (A) and protein (B) expression levels of HO-1 in TNF-α-induced bEnd.3 cells, with or without SFN treatment, were detected with real-time PCR and Western blot analysis, respectively. Compared with the control group, $^{*}P < 0.05$; compare with the TNF-α-induced model group, $^{\#}P < 0.05$.

SFN decreases TNF-α-induced ROS generation in bEnd.3 cells

We nest investigated the effects of TNF-α and SFN treatments on ROS generation in bEnd.3 cells. Our results showed that, compared with the control group, the intracellular ROS level was significantly enhanced in the bEnd.3 cells treated with TNF-α ($P < 0.05$). However, the treatments of SFN significantly decreased the intracellular ROS levels in TNF-α-treated bEnd.3 cells, in a dose dependent manner (all $P < 0.05$) (**Figure 2**). These results suggest that SFN treatment could decrease the TNF-α-induced ROS generation in bEnd.3 cells.

SFN increases HO-1 expression levels in TNF-α-treated bEnd.3 cells

To investigate whether the effects of SFN on TNF-α-treated bEnd.3 cells were related to HO-1, the mRNA and protein expression levels of HO-1 in these cells were detected with real-time PCR and Western blot analysis, respectively. Our results from the real-time PCR showed that, the mRNA expression level of HO-1 was significantly increased by TNF-α treatment ($P < 0.05$). Moreover, the mRNA expression levels of HO-1 were further elevated by SFN treatments in TNF-α-treated bEnd.3 cells, in a dose dependent manner (all $P < 0.05$) (**Figure 3A**). Similar results were obtained for the Western blot analysis. The protein expression levels of HO-1 were significantly increased by the TNF-α treatment ($P < 0.05$), which were further enhanced by the SFN treatments (all $P < 0.05$) (**Figure 3B**). Taken together, these results suggest that SFN treatment could increase the mRNA and protein expression levels of HO-1 in TNF-α-treated bEnd.3 cells.

HO-1 mediates the effects of SFN on TNF-α-treated bEnd.3 cells

To further investigate the role of HO-1 in the effects of SFN on TNF-α-treated bEnd.3 cells, the activator and inhibitor of HO-1 were used to treat the bEnd.3 cells, respectively, and the contents of IL-1β and endothelin were assessed. Our results showed that, the reducing effects of SFN on the IL-1β and endothelin contents in TNF-α-treated bEnd.3 cells could be significantly enhanced by the HO-1 activator. CoPP (both $P < 0.05$) (**Figure 4**). In contrast, the effects of SFN on the IL-1β and endothelin contents could be reversed by the treatment of the HO-1 inhibitor, ZnPP, in TNF-α-treated bEnd.3 cells (both $P < 0.05$) (**Figure 4**). These results suggest that HO-1 mediates the reducing effects of SFN on TNF-α-enhanced IL-1β and endothelin production in bEnd.3 cells.

Discussion

Proinflammatory cytokine TNF-α plays an important role in the activation of inflammatory responses. TNF-α could stimulate the synthesis and release of IL-1β, to further synergistically induce the expression of intermolecular adhesion molecules in vascular endothelial cells and the endothelial leukocyte adhesion molecules. Thereafter, a large number of leucocytes would infiltrate into and accumulate in the brain parenchyma, which could release inflammatory mediators and exacerbate the inflammation. Moreover, the endothelial cells

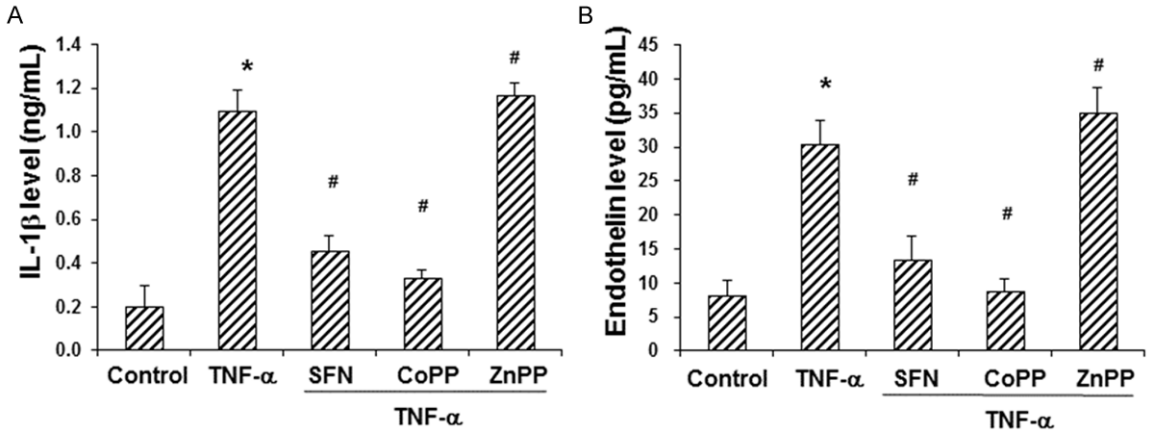


Figure 4. Involvement of HO-1 in the effects of SFN on TNF-α-treated bEnd.3 cells. The TNF-α-treated bEnd.3 cells were pre-treated with the HO-1 activator CoPP (0.3 μmol/L) and inhibitor ZnPP (0.1 μmol/L), respectively, together with SFN (30 μg/mL). Then the production of IL-1β (A) and endothelin (B) were detected by ELISA in these cells. Compared with the control group, $^{*}P < 0.05$; compare with the TNF-α-induced model group, $^{\#}P < 0.05$.

would be stimulated to produce various cytokines, ending up with the ischemia-inflammation-thrombosis cycle. Meanwhile, the excitatory amino acids, NO, and free radicals would also be produced and released [23-25].

Endothelin is one of the most potent and persistent vasoconstrictors, which can specifically bind to receptors on the membrane of vascular smooth muscle cells, activating phospholipase C, and producing inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 facilitates the extracellular Ca^{2+} influx and promotes the intracellular Ca^{2+} mobilization, resulting in the elevated intracellular Ca^{2+} concentration and the subsequent vasoconstriction. On the other hand, endothelin stimulates the proliferation of vascular endothelial cells, smooth muscle cells, and fibroblasts, which might lead to wall thickening and luminal stenosis [26]. Vasoconstriction, metabolic disturbance, and cell proliferation promotion induced by endothelin share common pathogenic factors with the diseases associated with vascular injuries [27]. In this study, our results showed that the treatment of TNF-α could significantly elevate the production of IL-1β and endothelin in bEnd.3 cells, confirming the synergistic effect of endothelin with proinflammatory cytokines in the disease pathogenesis.

ROS is a key factor in the damage of vascular endothelial cells and in the dysfunction of host cells induced by TNF-α. Intracellular ROS level in TNF-α-induced bEnd.3 cells was determined in this study. Flow cytometry showed that, after treated with 5 ng/mL TNF-α for 24 h, the fluorescence intensity (i.e., the intracellular ROS level) in the bEnd.3 cells was significantly higher than in the control group, indicating that TNF-α could induce the ROS generation in these cells. The excessive ROS production might reduce the membrane fluidity, and might induce apoptosis via inhibiting the mitochondrial function. In addition, ROS could inhibit the proliferation of endothelial cells, which is disadvantageous to the repairing of endothelial cell injuries. Accumulated ROS can not only induce the peroxidation of lipids, DNA, and proteins, but also up-regulate the expression of various cytokines, adhesion molecules, which would aggravate the brain damages [28, 29]. These effects might work together to form a complex inflammatory pathway, eventually leading to neuronal necrosis.

Nuclear transcription factor Nrf2 is an important endogenous antioxidant pathway *in vivo*, which could induce the expression of various endogenous antioxidant enzymes and proteins, protecting the host cells from oxidative and inflammatory injuries. HO is one of the most important targets regulated by Nrf2. There are totally three HO isoenzymes: HO-1, HO-2 and HO-3. HO-1 is an inducible isoenzyme, which could be induced by oxidative stress, heavy metal ions, and other factors. HO-1 has been shown to be able to exert protective effects on myocardial cells, hepatic parenchymal cells, fiber cells, and vascular endothelial cells [30,

31]. Previous studies have shown that, the up-regulated HO-1 expression can effectively reduce the oxidative stress-induced injury in pulmonary epithelial cells [32, 33]. Moreover, HO-1 gene-deficient mice are associated with elevated sensitivity to inflammatory lung injuries [34]. Furthermore, the transfection of HO-1 in mouse models could effectively reduce the area of myocardial infarction induced by myocardial ischemia/reperfusion injury [35]. These results suggest that HO-1 is part of the endogenous protective system against cardiovascular diseases. In this study, our results showed that TNF-α treatment could induce the proinflammatory cytokine production and ROS generation in bEnd.3 cells, and promote the expression of HO-1, which might provide a feedback protection mechanism. However, it is usually difficult to balance the *in vivo* redox state only with this kind of compensation mechanism. Therefore, exogenous drugs inhibiting oxidative stress may be an important strategy for the treatment of various brain injuries caused by ischemia/reperfusion.

Sulforaphane (SFN) exists in a large number of cruciferous vegetables like Broccoli. It has been shown that SFN has a variety of biological activities, such as antioxidant, anti-cell proliferation, anti-inflammation, and immune regulating effects [36, 37]. Particularly, increasing attention has been focused upon the antitumor and antioxidant effects of SFN, which might be achieved by indirectly regulating the antioxidant genes [38, 39]. The pharmacological studies have found that, SFN could regulate the activity of transcription factor Nrf2 within cells. The activated Nrf2 would enter the nucleus and bind to ARE, and further induce the expression of antioxidant and detoxification enzymes, such as HO-1 and NQO1, reducing the toxicity induced by ROS and other toxic substance [40]. Other *in vivo* experiments have shown that SFN could up-regulate the phase II detoxification enzymes via activating Nrf2, to exert protective effects against myocardial ischemia/reperfusion injury. It has also been shown that, pre-treatment of SFN could enhance the activities of glutathione, SOD, NQO1, and GST in murine thoracic aortic smooth muscle cells [41, 42]. In addition, SFN could protect pancreatic β cell damages induced by H_2O_2 [43, 44]. In the central nervous system, SFN have also been shown to alleviate the neurological deficits caused by cerebral ischemia, cerebral hemorrhage, and hypoxic encephalopathy, and to some extent protect against MPTP-induced toxicity [45, 46]. Moreover, SFN could up-regulate the expression of HO-1 and NQO1 in immature hippocampal neurons, enhancing the cell tolerance to hypoxic conditions [47]. However, the mechanisms underlying the protective effects of SFN on neurons against cerebral ischemia/reperfusion injury have not yet been fully elucidated. In this study, we found that SFN treatment could significantly elevate the mRNA and protein expression levels of HO-1 in bEnd.3 cells. To further investigate whether HO-1 was involved in the regulation of inflammatory responses, the HO-1 activator CoPP and inhibitor ZnPP were used to incubate these cells, respectively. Our results showed that CoPP could synergistically decrease the production of IL-1β and endothelin in TNF-α-induced bEnd.3 cells with SFN treatment, while ZnPP could significantly elevate the production of IL-1β and endothelin in these cells. These results suggest that HO-1 mediates the inhibiting effects of SFN on IL-1β and endothelin production in TNF-α-treated bEnd.3 cells. It has been widely accepted that oxidative stress is associated with the complex pathogenesis of cerebral vascular diseases. Moreover, HO-1 plays an important role in the host defense against oxidative stress. Phase II enzyme inducer, SFN, could activate the HO-1 pathway to regulate the expression of downstream target antioxidant/detoxifying enzymes, and inhibit the oxidative stress induced by various pathogenic factors, further exerting neuroprotective effects.

In conclusion, our results suggest that SFN treatment could reduce TNF-α-induced IL-1β and endothelin production, and decrease TNF-α-enhanced ROS generation, in bEnd.3 cells. Moreover, the mRNA and protein expression levels of HO-1 could be further increased by SFN treatments in TNF-α-treated bEnd.3 cells. Furthermore, our results indicate that HO-1 mediates the reducing effects of SFN on TNF-α-induced IL-1β and endothelin production in these cells. Our findings would provide evidence for the application of SFN in the treatment of brain injuries caused by cerebral ischemia/reperfusion.

Acknowledgements

This work was supported by Scientific Research Key Project for Universities and Colleges from

the Education Department of Hunan Province (No. 11A106).

Disclosure of conflict of interest

None.

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