H$_2$O$_2$ - mediated SRPED1 Inactivation Contributes to Resisting Ischemia-reperfusion Induced Oxidative Injury to Microglia

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Research Article

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Abstract

Oxidative stress induced by ischemia-reperfusion is an important reason for the loss of neurons in the brain. In addition to direct oxidative toxicity that causes apoptosis of neurons and glial cells, oxidative stress can also cause complex physiological effects of related cells, such as activation of microglia to promote their repair effects on damaged areas. Here, we found that SPRED1, sprouty-related protein with an EVH1 domain 1, was significantly inhibited after ischemia-reperfusion by MCAO in rats, while the inhibition of SPRED1 responded to the up-expressed p65 proteins with the stimulation of H₂O₂. What's more, we further revealed that SPRED1 tended to exert a unique role in maintaining intracellular homeostasis, which can re-activate the instinct of microglia upon the induction of H₂O₂ to resist oxidative toxicity. However, SPRED1 did not show such ability when it is only over-expressed but without H₂O₂ induction. In a word, these data revealed a potential role of SPRED1 in preventing cerebral ischemia-induced oxidative stress injury to nerve cells.

1. Introduction

Cerebral ischemia triggers neuro-inflammatory and oxidative stress in central nervous system (CNS), both of which have been wildly demonstrated to be closely related to mitogen-activated protein kinases (MAPKs) and NF-κB pathway[1–4]. Where, microglial cells play an extremely important role in this process[4]. However, MAPKs and NF-κB signals are regulated by a variety of mechanisms, including positive regulators and negative regulators. As previously reported, Dyrk2 (dual specificity tyrosine phosphorylation regulated kinase 2) is one of the positive regulators in activating p38MAPK by autophosphorylating the critical tyrosine residues and phosphorylating serine/threonine residues[5]. There are many similar regulators, such as DLK[6], MLK[7], etc.

Although the negatives are not as rich as the positive regulators, the discovery of SPRED1 as a representative has attracted widespread attention[8]. Sprouty-related protein with an EVH1 domain 1 (SPRED1), one of the negative regulators to RAS-MAPK-ERK, has been reported to link to neurofibromatosis 1 (NF-1)-like human syndrome[9, 10]. Interestingly, evidences show that Sprouty is a group of broadly acting tyrosine kinase (RTK) signaling inhibitory molecules. Sally et al found SPRED1 was highly enriched in CNS germinal zones during neurogenesis and maintained the renewal of neural stem cells by Ras-MAPK-ERK pathway[10]. Hongbin et al revealed that SPRED1 had an impact on the inflammation after contusion spinal cord injury in rats, indicating a unique role for SPRED1 in pro-inflammatory responses in the CNS[11]. Sprouty proteins have been found in mammals and all of them contain a conserved cysteine-rich domain at the C-terminus, which is closely related to their intracellular localization and function[12].

Herein, we investigated the effect of SPRED1 on the cellular injury induced by cerebral ischemia in microglia. We found that SPRED1 was significantly inhibited in the brain after cerebral ischemia, while oxidative stress simulated by H₂O₂ in vitro also greatly repressed the expression of SPRED1 in BV2 cells. Moreover, we demonstrated that the novel role of SPRED1 in influencing the survival of BV2 cells upon
the H$_2$O$_2$ stimulation. H$_2$O$_2$-mediated SRPED1 inactivation contributes to resisting oxidative stress injury, revealing a potential role of SPRED1 in preventing cerebral ischemia-induced oxidative stress injury to nerve cells.

2. Materials And Methods

2.1 Rats and cell cultures

Sprague Dawley (SD) Rats were purchased from Gempharmatech Co., Ltd (Nanjing, China). Animals were group-housed under standard rearing conditions with a 12 h light-dark cycle and a temperature of 25°C. Animals were euthanized 24 h after 1h of brain occlusion. Brains were obtained on ice after rats were anesthetized for the analysis. All animal experiments were reviewed and approved by the Ethics Committee for Animal Investigation of Soochow University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

BV2 cells were purchased from Qingqi Biotechnology Development Co., Ltd. (Shanghai, China) and cultured in DMEM medium with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin. The viability of BV2 cells was assessed using the CCK-8 kit (Abcam, cat# 228554, Cambridge, MA). For transfection, HG-Trans293™ transfection reagent (Genomeditech, Shanghai, China) was used according to the manuals. The transfected plasmids included pcDNA3.1-SPRED1 expression of SPRED1. Cells were harvested 24 h post transfection for further analysis.

2.2 MCAO on Rats

Briefly, a filament with silicone tip was inserted into the middle cerebral artery of rats for 60 minutes, and then the filament was removed for reperfusion to form the model. The sham group rats were operated as the same as the MCAO except for the embolism with filaments.

2.3 TTC staining

Middle cerebral arteria occlusion (MCAO) in rats was performed following a standardized experimental procedure. Brains were frozen at -20°C and cut into 2 mm thick sections for TTC (2,3,5-triphenyltetrazolium chloride) staining. TTC staining allows the precise delineation of lesioned and non-lesioned brain areas.

2.4 Immunohistochemical assays

Tissue sections were deparaffinized thick in xylene and rinsed in ethanol gradient buffers. After antigen retrieval with citrate, tissue sections were incubated with antibody SPRED1 overnight, and the secondary antibody was incubated at room temperature for 3 hours. Finally, tissue sections were stained with 3,3-diaminobenzidine (DAB, ZSGB-BIO, Beijing, China). Cells with tan cytoplasm were counted as positive cells.

2.5 Enzymatic activity assays
The activities of SOD and catalase were measured following the manufacturer’s protocols, respectively. The assay kits were purchased from Beyotime Biotech (Nanjing, China).

2.6 Enzyme-linked immunosorbent assay (ELISA) for TNF-α and IL-1β

After treating BV2 cells with \( \text{H}_2\text{O}_2 \) according to different requirements, the cells were lysed and the supernatant was collected. A microplate reader (Bio-Tek, ELx800, USA) was used to check the optical density (OD) value at a wavelength of 450 nm and to draw a standard curve. Each step was performed according to the manufacturer’s TNF-α ELISA kit and IL-1β ELISA kit (Proteintech, Wuhan, China).

2.7 Apoptosis analysis by flow cytometry

BV2 cells were cultured and treated under different conditions, cells were harvested by centrifugation and resuspended in buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl\(_2\)), and \( 10^5 \) cells were stained with Annexin-V-FITC and PI, and incubated at room temperature for 15 min in the dark by the Apoptosis Detection Kit (BD Pharmingen TM). After addition of 400 µL binding buffer, the cells (~30000 cells per assay) were then analyzed by flow cytometer within one hour period.

2.8 Western blot analysis and antibodies information

Cells were thoroughly lysed with lysis buffer (1% NP-40, 40 mM Tris and 4 M NaCl and protease inhibitor cocktail) and the supernatant was taken. Protein concentrations were quantified by Bradford buffer. Protein samples (20–40 µg/lane) were run in SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking the membrane with nonfat milk and incubating with primary and secondary antibodies for protein detection, the target band was detected. Antibody information: anti-SPRED1 (Bioss, cat# 17685R, Beijing, China), anti-eNOS (Abcam, cat# 00071,), anti-iNOS (Abcam, cat# 178945),anti-p38 (Proteintech, cat# 14064-1-AP), anti-p65 (Abcam, cat#32536), anti-SOD1 (Proteintech Group Inc., cat#10269-1-AP), anti-SOD2 (Proteintech Group Inc., cat#24127-1-AP), anti-Bax (Abcam, cat#32503), anti-Bcl-2 (Abcam, cat#182858), anti-β-Actin (Bioworld; cat#AP0060, Bloomington, MN), anti-GAPDH ((Proteintech Group Inc., cat# 60004-1-Ig). Quantification of bands was performed with ImageJ (http://rsb.info.nih.gov/ij/).

2.9 Statistical analysis

All the data are presented as the mean ± SEM. The statistical analysis was carried out by SPSS software via the Student’s t-test by using Graphpad-Prism Software Version 8.2.1 (La Jolla, CA). P-value < 0.05 was considered to be significant.

3. Results And Discussion

3.1. The expression of SPRED1 protein in cerebral cortex is down-regulated after cerebral ischemia
To investigate the role of SPRED1 in cerebral ischemia, we established MCAO model in rats to simulate the ischemia-reperfusion of brain. A filament with silicone tip was inserted into the middle cerebral artery of rats for 60 minutes, and then the filament was removed for reperfusion to form the model[1]. Representative images of brain slices stained with TTC and ischemic infarct area of white part showed the success of MCAO model (Fig. 1A). Neuro-inflammation and oxidative damage were two important reasons for the loss of nerve cells after ischemia-reperfusion of brain [13, 14]. In the cerebral cortex tissue, the activation of iNOS and the down-regulated SOD1 SOD2 indicated the increased levels of inflammation and stress in brain tissue (Fig. 1B). Associated with this, malondialdehyde (MDA), one of the most important products of intracellular lipid peroxidation after oxidative damage [15], was expanded significantly (Fig. 1C). By contrary, the antioxidant enzyme activity of superoxide dismutase (SOD) and catalase [16] were substantially impaired (Fig. 1D, E). These data suggested that ischemia-reperfusion successfully triggered the oxidative stress to the brain.

Interestingly, the ischemic brain tissues and the sham brain tissues were sectioned respectively and then stained them through IHC experiment by SPRED1 antibody. As shown in Fig. 1F, SPRED1 is high expressed in sham group but low expressed in the cerebral cortex area of MACO rats. Meanwhile, we split the tissues of cerebral cortex and extract the protein to perform the immunoblotting analysis, results demonstrated again that SPRED1 protein level decreased obviously after cerebral ischemia (Fig. 1G, H). This indicates that cerebral ischemia represses the expression of SPRED1 in brain.

3.2. Oxidative stress induced the down-regulation of SPRED1 in BV2 cells

At the SPR domain of C-terminus, SPRED1 proteins contain a conserved cysteine-rich domain (Fig. 2A), indicating that SPRED1 is very likely to be regulated by oxidative stress that is one of the main secondary damages to nerve cell after ischemia-reperfusion. Thus, we explored the effect of H$_2$O$_2$ induction on SPRED1 in BV2 cells. As shown in Fig. 2B, C, the expression of SPRED1 emerged a trend of continuous decrease first and then recovery by the treatment of 100µM H$_2$O$_2$ for 24h. Interestingly, with the treatment of H$_2$O$_2$, corresponding changes in both SOD1 and SOD2 was similar to SPRED1 protein, especially the behavior of SOD2 is almost identical to SPRED1 (Fig. 2D, E). This reveals that the expression of SPRED1 is strictly regulated by the oxidative stress.

Additionally, as the negative regulator of MAPK, the down-regulation of SPRED1 failed to support the expression of p38MAPK, one of the key kinases responding to inflammation signal and stress signal after cerebral ischemia [17]. However, with the treatment to 12h and 24h, the p65 subunit of NF-κB gets a consistent recovery along with the SPRED1 (Fig. 2D, F). NF-κB similarly plays an important role in transducing the signal of neuro-inflammatory, oxidative stress and cell survival in the CNS[18]. This result prompts a potential correlation between oxidative stress, SPRED1 and NF-κB.
3.3. SPRED1 over-expressed can enhance the induction of p65 upon H$_2$O$_2$ treatment

To investigate the relevance between SPRED1 and p65, BV2 cells were transfected by SPRED1 over-expressed plasmid vector to raise its expression. However, the up-regulated SPRED1 seemingly showed a limited impact on the expression of p38MAPK and p65, but dramatically affected them upon treating by H$_2$O$_2$ simultaneously (Fig. 3A, B). Atsushi Hirao et al. found that the presence of SPRED1 in hematopoietic stem cells is more inclined to maintain intracellular homeostasis, while the deletion of SPRED1 can enhance the self-renewal of cells [19]. Thus, when H$_2$O$_2$ re-suppressed the overexpressed SPRED1, these cells in homeostasis were probably re-activated and thus become more active, triggering a significant increase in p38 and p65 proteins (Fig. 3A, B).

As a major immune cell in the CNS, BV2 cell can secrete pro-inflammatory cytokines such as TNF-α and IL-1β, etc [5]. We found H$_2$O$_2$-treatment weakened their abilities to secrete TNF-α and IL-1β, to some extent, which may be due to the existence of oxidative damage (Fig. 3C, D). Curiously, overexpression of SPRED1 did not alter the secretion of these two cytokines in BV2 cells, but in parallel H$_2$O$_2$ treatment after SPRED1 over-expressed, the secretion of TNF-α and IL-1β fall into a decline instead (Fig. 3C, D). That suggests up-regulated NF-κB after H$_2$O$_2$ induced the degradation of the overexpressed SPRED1 is more inclined to reduce the secretion of pro-inflammatory cytokines.

3.4 H$_2$O$_2$-mediated SPRED1 inactivation contributes to resisting oxidative stress injury

In order to further determine the role of SPRED1 in response to oxidative stress injury in BV2 cells, we examined cell viability and apoptosis after overexpressed SPRED1 as well as in parallel treatment of H$_2$O$_2$. Compared to H$_2$O$_2$ treatment alone, the viability of BV2 cells was enhanced in parallel H$_2$O$_2$ treatment after SPRED1 overexpression (Fig. 4A). Detection of cell necrosis, apoptosis and survival also support a similar conclusion, although overexpression of SPRED1 alone did not improve cell condition, including cellular activity and generative status (Fig. 4B, C). NF-κB mediated Bax/Bcl2 signal is critical for neuronal survival after cerebral ischemia[20]. However, H$_2$O$_2$ induction promoted the level of Bax but repressed Bcl2. SPRED1 overexpression with parallel H$_2$O$_2$ treatment played an opposite role, promoting Bcl2 but repressing Bax (Fig. 4D, E). This finding seems to indicate that SPRED1 is not so important in normal BV2 cells, but when conditionality causes its degradation, it can play a unique regulatory role, re-activating the cell instinct to resist the damage.

Discussion

Cerebral ischemia is a common disease in the elderly, and its pathological manifestations are the impairment of motor, sensory and autonomic functions caused by the loss of central ischemic areas and
peripheral neurons [21]. However, cerebral ischemia and the restoration of cerebral blood flow will lead to the damage of neuronal structure and function in the ischemic area again, where includes many pathological mechanisms like mitochondrial dysfunction, oxidative stress, excitatory amino acid toxicity, inflammatory response, etc [22]. Oxidative stress and its related molecular events play an important role in the pathological process of ischemic stroke, which not only triggers many direct damages such as lipid peroxidation, protein and DNA oxidation, but also leads to the initiation of inflammatory and cell death pathways through multiple cell signaling effects[23–25]. Here, we found that H$_2$O$_2$-mediated SRPED1 inactivation contributes to resisting oxidative stress injury, indicating the potential role of SPRED1 in preventing cerebral ischemia-induced oxidative stress injury to nerve cells.

Sprouty-related protein with EVH1 domain (SPRED1) is an identified negative Ras-MAPK-ERK regulator associated with neurofibromatosis 1 (NF-1)-like human syndrome and has been found highly enriched in CNS germinal zones during neurogenesis, while SPRED1 knockdown will increases the self-renewal of neural stem cells and promote the proliferation of progenitor cell autonomously [11, 12]. Additionally, SPRED1 has inhibitory effects on the Ras-ERK pathway induced by multiple growth factors, Ras-MAPK pathway is one of the receptor tyrosine protein kinase pathways, which plays an important role in the signal transmission of cell growth, survival, proliferation and differentiation [26]. However, sprouty-related structural domain of SPRED1, the SPR domain, is rich in cysteine residues (Cys) that are easily regulated by H$_2$O$_2$ as well as other ROS[12].

In fact, our investigation revealed that H$_2$O$_2$-simulated oxidative stress readily reduced SPRED1 protein levels in BV2 cells, and SPRED1 in cortical tissues was also shown to be largely inhibited in an MCAO-simulated model of cerebral ischemia-reperfusion injury. However, overexpressed SPRED1 in BV2 cells exhibited a limited effect on p38 and p65 proteins level as well as on the cell viability and apoptosis, seeming to be effective in maintaining the cellular homeostasis. Interestingly, once H$_2$O$_2$ induces the degradation of overexpressed SPRED1 in BV2 cells, the original cytotoxic effect of H$_2$O$_2$ is greatly inhibited, and the cell's instinct to resist oxidative toxicity was instead enhanced. Furthermore, the regulation of intracellular homeostasis by SPRED1 may involve multiple mechanisms, besides the widely known Ras-MAPK pathway, NF-$\kappa$B and the dependent pathways are also the important objects to be regulated.

**Declarations**

**Declaration of interest**

The author reports no conflicts of interest in this work.

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Figure 1

(A) TTC-stained mice brain slice after experimental stroke (MCAO, 20 min occlusion, 24 h reperfusion). (B) The expression levels of eNOS, iNOS, SOD1, SOD2 after MCAO treatment in cerebral cortex. GAPDH was used as a loading control. (C) Quantification of the malondialdehyde (MDA) in cerebral cortex after MCAO treatment. (D, E) The activities of superoxide dismutase (SOD) and catalase were detected in cerebral cortex after MCAO treatment. (*, p < 0.05, n=5) (F) The representative images of immune-histochemical
assays for SPRED1 expression in cerebral cortex. (G) The expression levels of SPRED1 after MCAO treatment in cerebral cortex. GAPDH was used as a loading control. (H) Quantification of the SPRED1 protein level after MCAO treatment. (*, p < 0.05)
(A) Schematic representation of the domains of SPRED1 of human origin. (B, C) Detection of the level of SPRED1 protein in BV2 cells after 100 μM H₂O₂ treatment for 1, 3, 6, 12 and 24 h, and quantified in the right (C). GAPDH was used as a loading control. (D-F) Detection of the levels of p38, p65, SOD1 and SOD2 proteins in BV2 cells after 100 μM H₂O₂ treatment for 1, 3, 6, 12 and 24 h, quantified in the right (E) and below (F). β-actin was used as a loading control.
(A, B) Detection of the levels of SPRED1, p38 and p65 proteins in BV2 cells after transfection, quantified in (B). GAPDH was used as a loading control. (C, D) Detect the levels of TNF-α and IL-1β via ELISA kits in BV2 cells after transfection with H₂O₂ or/and pcDNA3.1-SPRED1 for 24 h. (*, p< 0.05)
(A) The relative value of CCK-8 in BV2 cells after transfection with H₂O₂ or/and pcDNA3.1-SPRED1 for 24 h. (B) Flow cytometer was used to detect the fluorescence intensity of PI-labeled and FITC-labeled after transfection. (C) According to streaming results, the average fluorescence intensity of each group was counted. (D, E) Detection of the levels of Bax and Bcl2 proteins in BV2 cells after transfection, quantified in the right (E). GAPDH was used as a loading control. (*, p< 0.05)