Redox Biology 11 (2017) 135–143

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research paper

Specificity protein 1-zinc finger protein 179 pathway is involved in the attenuation of oxidative stress following brain injury

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ARTICLE INFO

Keywords:
Zinc finger protein 179
Specificity protein 1
Reactive oxygen species
Traumatic brain injury
Nerve growth factor

ABSTRACT

After sudden traumatic brain injuries, secondary injuries may occur during the following days or weeks, which leads to the accumulation of reactive oxygen species (ROS). Since ROS exacerbate brain damage, it is important to protect neurons against their activity. Zinc finger protein 179 (Znf179) was shown to act as a neuroprotective factor, but the regulation of gene expression under oxidative stress remains unknown. In this study, we demonstrated an increase in Znf179 protein levels in both *in vitro* model of hydrogen peroxide (H_2O_2)-induced ROS accumulation and animal models of traumatic brain injury. Additionally, we examined the sub-cellular localization of Znf179, and demonstrated that oxidative stress increases Znf179 nuclear shuttling and its interaction with specificity protein 1 (Sp1). Subsequently, the positive autoregulation of Znf179 expression, which is Sp1-dependent, was further demonstrated using luciferase reporter assay and green fluorescent protein (GFP)-Znf179-expressing cells and transgenic mice. The upregulation of Sp1 transcriptional activity induced by the treatment with nerve growth factor (NGF) led to an increase in Znf179 levels, which further protected cells against H_2O_2-induced damage. However, Sp1 inhibitor, mithramycin A, was shown to inhibit NGF effects, leading to a decrease in Znf179 expression and lower cellular protection. In conclusion, the results obtained in this study show that Znf179 autoregulation through Sp1-dependent mechanism plays an important role in neuroprotection, and NGF-induced Sp1 signaling may help attenuate more extensive (ROS-induced) damage following brain injury.

1. Introduction

Traumatic brain injury (TBI) is a serious public health problem resulting in death or disability [1], due to damages caused by both immediate and secondary injuries. Secondary injuries generate the most damage because they develop and progress over many hours and months after the immediate injury. Environmental stressors include ischemic sugar/oxygen deprivation, inflammatory cytokine release, the release of excitatory neurotransmitters, and metabolic depression. This leads to the accumulation of reactive oxygen species (ROS) [2,3], which plays a major role in the pathophysiology of various kinds of brain damage. The accumulated ROS can damage cellular organelles and induce apoptosis of brain cells if untreated. Therefore, preventing ROS-mediated cellular damage is important in the therapy of neurological disorders.

Zinc finger protein 179 (Znf179, also known as Zfp179/RNF112/Bfp) is predominantly expressed in human nervous system [4], and it is necessary for the embryonic nervous system development [5]. When retinoic acid (RA) is used to induce P19 EC cells to differentiate into neural cells, Znf179 expression was shown to be upregulated, which results in the increase of p35 and p27 protein levels, leading to the cell cycle arrest at the G0/G1 phase and the initiation of cell differentiation. The inhibition of Znf179 expression was shown to significantly suppress neuronal differentiation [5]. Recently, the results of our study

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http://dx.doi.org/10.1016/j.redox.2016.11.012
Received 3 October 2016; Received in revised form 8 November 2016; Accepted 15 November 2016
Available online 29 November 2016

indicated that Znf179 acts as a novel neuroprotector mitigating cell death after hydrogen peroxide (H_2O_2)-induced oxidative stress, and its accumulation correlates with H_2O_2 exposure time [6]. However, the mechanism of H_2O_2-induced elevation of Znf179 expression remains unclear.

Specificity protein 1 (Sp1) is a transcription factor, which is essential for the regulation of the expression of many genes involved in cell growth, angiogenesis, and survival [7]. Previous studies indicated that Sp1 in neurons acts as a pleiotropic oxidative stress response protein [8–10]. It was shown that the exposure of primary cortical neurons to ischemia-like oxygen-glucose deprivation, Sp1 expression increased, which led to the accumulation of this protein, and the protection of neurons against ischemic damage [9]. Additionally, Na +/Ca2+ exchanger 1 (NCX1), was shown to represent a Sp1 target protein [11], and it is important for the reduction of brain damage following cerebral ischemia [12]. Therefore, Sp1 activation may provide neuroprotection and neurorestoration to cells *in vitro* and in the animal models of brain ischemia.

In this study, we investigated the mechanisms of Znf179 upregulation during the exposure to stressful conditions. Our results demonstrated that Znf179 positively autoregulates its own expression through Sp1-dependent activation of transcription, and that the increase in nerve growth factor (NGF)-induced Sp1 activity significantly increases Znf179 levels and improves cell survival after H_2O_2 treatment. These findings may have potential therapeutic value in the treatment of ROS-induced damage in neurotraumatic diseases.

2. Materials and methods

2.1. Experimental animals

We used 10–12 weeks old male *wild-type* mice (C57BL/6: n =24 and FVB/NJ: n =12, National Laboratory Animal Center, Taipei, Taiwan) and 12 weeks old male Znf179-expressing transgenic mice (n =8) on the C57BL/6 genetic background (Table 1), housed five per cage in an air-conditioned vivarium with free access to food and water. Throughout the study, a 12-h light/dark cycle was maintained with lights on at 8 AM. Each mouse was used for one experiment only. All procedures adhered to the Guidelines for Care and Use of Experimental Animals of the Taipei Medical University (Taipei, Taiwan). Ten C57BL/6 mice were excluded from weight-drop TBI because they: (1) had missed target areas (*wild-type*: n =4) or (2) died during the experimental procedures (*wild-type*: n =5; *znf179* transgenic: n =1) and within 24 h after the impact (*wild-type*: n =1).

2.2. Bacterial artificial chromosome (BAC) transgene construction and transgenic mice generation

By the assistance of National Laboratory Animal Center, the transgenic mouse overexpressing green fluorescent protein (GFP)-Znf179 under control of *znf179* gene promoter presented in a BAC expression vector were generated. Mouse *znf179* gene fused to GFP was inserted into the BAC DNA (RP23-354C18) using homologous recombination in *Escherichia coli*. Subsequently, the recombined BAC clone was injected into fertilized mouse oocytes from C57BL/6 mice, and the oocytes were implanted into the uterus of pseudo-pregnant foster mothers. After birth, potential founders were screened for the presence of the transgene using PCR with primers: 5′-CGCACCATCTTCTTCAAGGACG-3′ and 5′-TTCTCGTTGGGGTC TTTGCTC-3′. Animal positive for the transgene mated to *wild-type* (C57BL/6) mice to stabilize the line and for further characterization.

Table 1
The number of animals used in each group of weight-drop TBI and CCI.

	Weight-drop TBI model (C57BL/6)						CCI model (FVB/NJ)		
	Wild-type			*znf179* transgenic			*Wild-type*		
	sham	TBI	Exclusion of TBI	Sham	TBI	Exclusion of TBI	Sham	TBI	Exclusion of TBI
n	8	7	9	3	4	1	3	9	0

2.3. Weight-drop TBI model

Mice (C57BL/6) weighing 25–30 g were anesthetized lightly by inhalation of isoflurane (3%) in a closed glass chamber for 2 min. The left side of the head, between the eye and ear, was positioned under the guide tube of a weight-drop device and held in place by a sponge. In the device, a cylindrical iron weight (50 g) with a spherical tip was dropped from the full height of the vertical, graduated guide tube (100 cm long). The effect of the injury on the brain was studied at 4 days following the trauma.

2.4. Controlled cortical impact (CCI) model

Mice (FVB/NJ) weighing 25–30 g were anaesthetized and placed in a Kopf stereotaxic head frame (David Kopf Instruments). By using a dental drill, a 5-mm craniotomy was performed over the left parietal cortex between the bregma and lambda. The bone flap was removed and injury was made using a Precision Systems and Instrumentation TBI-0310 (Fairfax Station, VA) that administered a 1 mm cortical compression (3 mm impactor diameter, 2.5 m/s velocity, 150 ms duration dwell time) [13]. Sham animals were anesthetized but no CCI. Body temperature was monitored throughout the surgery by a rectal probe; temperature was maintained at 37.0 ± 0.5 °C using a heated pad.

2.5. Cell culture and transfection

Mouse neuroblastoma Neuro-2a (N2a) cells (ATCC) were cultured in minimum essential medium Eagle (MEM, Invitrogen) containing 10% (vol/vol) fetal bovine serum (FBS), and 1% penicillin/streptomycin in an incubator set at 37 °C with 5% CO2. Cellular differentiation was induced by serum deprivation in MEM/BSA medium (MEM supplemented with 0.1% bovine serum albumin) for 24 h [14], and differentiating N2a cells were used for all experiments. The 80% confluent cells were treated with H_2O_2 (Sigma-Aldrich), NGF (Invitrogen), and/or mithramycin A (Sigma-Aldrich). Transfection of a reporter plasmid (pGL2-Basic-*znf179*), protein-expressing vectors (pEGFP, pEGFP-Sp1, and pEGFP-Znf179) or shRNA plasmids (pLKO-shLuc and pLKO-shSp1) was performed by using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Each transfection experiment was performed more than three times as indicated, and each sample in each experiment was prepared in duplicate.

2.6. Western blot analysis

Protein samples from cells (1×10^6) were separated by electrophoresis on a polyacrylamide gel in the presence of sodium dodecyl sulfate, and then transferred onto a PVDF membrane (Bio-Rad Laboratories). The membrane after transfer was blocked with 5% skim milk in TBST for 1 h and incubated with primary antibodies: Anti-Sp1 (0.5 μg/ml), anti-GAPDH (0.1 μg/ml), anti-actin (1 μg/ml) antibodies from Millipore, anti-p53 (1 μg/ml), anti-phospho-p53 (Ser15) (1 μg/ml), anti-p38 (1 μg/ml), anti-phospho-p38 (Thr180/Tyr182) (1 μg/ml) antibodies from Cell Signaling Technology, and anti-Znf179 (0.5 μg/ml) antibody [5], for 2 h at room temperature. After primary antibody incubation and washing, the membrane was then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (0.15 μg/ml, Santa Cruz Biotechnology) for 1 h at room temperature. Finally, the membranes were washed three times with TBST