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Sonic hedgehog promotes neurite outgrowth of cortical neurons under oxidative stress: Involving of mitochondria and energy metabolism

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ABSTRACT

Oxidative stress has been demonstrated to be involved in the etiology of several neurobiological disorders. Sonic hedgehog (Shh), a secreted glycoprotein factor, has been implicated in promoting several aspects of brain remodeling process. Mitochondria may play an important role in controlling fundamental processes in neuroplasticity. However, little evidence is available about the effect and the potential mechanism of Shh on neurite outgrowth in primary cortical neurons under oxidative stress. Here, we revealed that Shh treatment significantly increased the viability of cortical neurons in a dose-dependent manner, which was damaged by hydrogen peroxide (H_2O_2). Shh alleviated the apoptosis rate of H_2O_2-induced neurons. Shh also increased neuritogenesis injuried by H_2O_2 in primary cortical neurons. Moreover, Shh reduced the generation of reactive oxygen species (ROS), increased the activities of SOD and and decreased the productions of MDA. In addition, Shh protected mitochondrial functions, elevated the cellular ATP levels and amelioratesd the impairment of mitochondrial complex II activities of cortical neurons induced by H_2O_2. In conclusion, all these results suggest that Shh acts as a prosurvival factor playing an essential role to neurite outgrowth of cortical neuron under H_2O_2 -induced oxidative stress, possibly through counteracting ROS release and preventing mitochondrial dysfunction and ATP as well as mitochondrial complex II activities against oxidative stress.

1. Introduction

Oxidative stress is well known as the unbalance between cellular oxidative and antioxidant systems that involves cellular injury and disruption of redox signaling. The endogenous stable oxidant hydrogen peroxide (H_2O_2) has been extensively used to induce oxidative stress in many different cell types since it is membrane-permeable in living cells [1]. H_2O_2-induced neuronal damage has been demonstrated to be involved in the etiology of several neurobiological disorders, such as ischemic stroke, traumatic brain injury, Alzheimer's disease and Parkinson's disease [2–6].

Neuroplasticity is a term used to describe a range of adaptive changes that occur in the structure and function of cells in the nervous system in response to physiological or pathological perturbations [7]. Neurogenesis is believed to be functionally important as a mechanism of brain plasticity under physiological conditions and in brain repair after injury [8,9], which needs mitochondria to supply ATP to power fundamental developmental processes including the establishment of axonal polarity and the regulation of neurite outgrowth [10,11]. It has become increasingly clear that the motochondrial functions are involved in oxidative neuronal injury. The motochondrial functions are damaged by various oxidative stimuli. The impaired mitochondria induce a substantial portion of damage to mitochondrial electron transport and further disturb ATP generation leading to neuronal damage [12]. Several previous studies have demonstrated that many pharmacological agents exert protective and neurotropic effects against neuronal injury through preservation of mitochondrial functions, and this might be an ideal neuroprotective strategy [13].

Shh, a secreted glycoprotein factor, activities the Shh pathway, and controls cell growth, survival, and differentiation in a wide variety of cells, including neurons [14]. Recent studies have indicated that the activation of the Shh pathway has been implicated in promoting several aspects of brain remodeling process, including neurogenesis, and axonal remodeling [15]. It has been reported that Shh acts as a midline-derived chemoattractant for commissural axons in the spinal cord [16]. A previous study has demonstrated that overexpression of

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Shh in the non-ischemic dentate gyrus increases neurogenesis in the adult rat [17]. Shh expression is up-regulated in neurons during ischemia/hypoxia [18]. A recent report shows that Shh pre-treatment protects cortical neurons against oxidative stress [19]. A study suggests that the inhibition of the Shh pathway leads to decreased expressions of Gli1 and Ptch at mRNA and protein levels, which exacerbated rat ischemic damage [20]. In addition, Shh protected cortical neurons against 3-NP toxicity, which could cause energy metabolic disorders by inhibition of the mitochondrial complex II [21]. Our previous study showed that Shh promoted neurite outgrowth in primary cortical neurons [22]. However, the effect and the potent mechanism of Shh in oxidative stress-induced neuronal injury have not been previously reported.

Therefore, the aim of the present study is to investigate the role of Shh in H_2O_2-induced neuronal injury in primary cultured cortical neurons, as well as the potential mechanism by which focuses on mitochondrial functions and energy metabolism.

2. Methods

2.1. Primary cortical neuron culture

Cortical neurons were obtained from the embryonic brains at embryonic day 15–18 (E15-18) of C57BL/6 mice (Vital River Laboratory Animal Technology Co. Ltd, Beijing, China). The cerebral cortex were dissected and incubated in Hibernate-E (Sigma, USA) containing papain (2.0 mg/ml, Sigma, USA) for 15 min at 37 °C. The dispersed cerebral cortical tissues were then neutralized with Hibernate-E and dissociated into single cell in neurobasal medium containing 2% B-27 supplement minus AO (Invitrogen, USA) and 0.5 mM glutamine (Life Technologies, USA). Neurons were plated onto culture dishes coated with poly-L-lysine (Biocoat, BD Biosciences, USA) at a density of 2×10^5 cells/cm^2 and grown in neuronal culture medium containing 2% B-27 supplement minus AO and 0.5 mM glutamine and incubated in a humidified 5% CO_2 incubator at 37 °C.

2.2. Drug treatment

Because H_2O_2 is a precursor to hydroxyl radicals and possesses signaling capacities [23], we used 100 μM H_2O_2 (Sigma, USA) to induce oxidative insult. To assess the role of Shh on cortical neurons under oxidative stress, exogenous recombinant Shh (Sino Biological Inc., China) dissolved in phosphate-buffered saline (PBS) was used to treat cells. Cells were pre-treated with 100 μM H_2O_2 for 2hr, subsequently with the treatment of Shh (5/25/50/500 ng/ml) for 24 h. The control experiment was performed with no Shh or H_2O_2 treatment, and the H_2O_2-injury group was treated with 100 μM H_2O_2 for 2 h only.

2.3. Cell viability assay

The viability of cortical neurons was evaluated by Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan). At 24 h after treatment, 10 μl CCK-8 was added to each well and the plates were incubated additional for 2 h at the end of the experiments. Optical Density (OD) at a wave length of 450 nm was measured by a microtiter plate reader (Tecan, Switzerland). These values were represented as OD values in percentage change compared with the control. Each group was done with 6 replicates (n=6), and the experiment was replicated three times. Additional, The nuclear DNA of neurons was stained with Hoechst 33342 at a final concentration of 5 μg/ml. Cells were observed immediately with a fluorescence microscope (Olympus, Tokyo, Japan).

2.4. Assay of neurite outgrowth

After 24 h culture of drug treatment, neurons were fixed with 4% paraformaldehyde for 20 min followed by permeabilization using 0.3% Triton-X-PBS for 15 min at room temperature. Nonspecific binding was blocked with 10% normal donkey serum diluted by 1% bovine serum albumin (BSA) (IgG free) (Sigma, USA) for 1 h at room temperature. Primary cortical neurons were then incubated with mouse monoclonal anti-β-III-tubulin antibody (Tuj-1, 1:500, Sigma, USA) overnight at 4 °C, followed by donkey anti-mouse IgG, FITC, Conjugated (1:200, CWBIO, Beijing China) for 1 h at 37 °C. The images were captured by using an upright fluorescence microscope (Olympus, Tokyo, Japan). The length of the longest neurite of Tuj-1-positive cell was measured by using Image J software. Sixty Tuj-1-positive cells per group were measured.

2.5. Sholl analysis

Neurite morphology was measured using Sholl analysis [24]. The Sholl analysis procedure was adapted according to the previously reported [25,26]. Briefly, for the analysis, β-III tubulin immunofluorescent stained coverslips were viewed at 200× on an fluorescence microscope. For a coverslip to be utilized in Sholl analysis the neurons must display a healthy network phenotype, eight individual neurons from each coverslip were imaged. The Sholl profile represents the total number of neuritic branches for any segment plotted against the distance from the cell soma.

2.6. Western blot analysis

Total proteins of cultured neurons were extracted according to the manufacturer's instructions (Applygen Technologies Inc, Beijing). The concentrations of protein were quantified with the Bicinchoninic Acid Protein Assay (BCA, Thermo Fisher Scientific, USA). Proteins were loaded by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (Bio-Rad Laboratories, USA). Membranes were blocked with 5% non-fat milk and then incubated with primary antibodies overnight at 4 °C. The membranes were then rinsed with Tris-buffered saline plus 0.1% Tween (0.1% TBST) and subsequently incubated in 0.1% TBST containing fluorescent labeling second antibodies (IRDye® 800-conjugated rabbit anti-mouse 1:12000 dilution; Rockland, Gilbertsville, PA, USA) for 1 h at room temperature. After triple washes, bands were visualized by an enhanced chemiluminescent substrate (Thermo Fisher Scientific, USA). For internal loading control, a mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5000, Sigma) was used. Western blot data were quantified by densitometric film scanning and normalized to GAPDH levels.

2.7. Immunofluorescence staining of GAP-43

After 24 h culture of drug treatment, the cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.3% Triton-X-100 for 15 min, and blocked in 10% normal donkey serum diluted by 1% BSA in PBS for 1 h at room temperature. The cells were then incubated with anti-GAP-43 antibody in 1% BSA at 4 °C overnight. followed by secondary antibody–donkey anti-rabbit IgG, TRITC, Conjugated (1:200, CWBIO, Beijing China) for 1 h at room temperature. The immunofluorescence images were acquired by an upright fluorescence microscope (Olympus, Tokyo, Japan).

2.8. Measurement of ROS

Intracellular ROS levels were quantified by reactive oxygen species assay kit (beyoime, China). Briefly, cortical neurons were incubated with 10 μM 2, 7-dichlorofluorescein diacetate (DCF-DA) for 1 h at 37 °C in the dark, and then resuspended in PBS. Intracellular ROS production was detected using the fluorescence intensity of the probe 2,7-dichlorodihydro-fluorescein diacetate (H_2DCF-DA) by a luminescence spectrometer with the excitation source at 488 nm and emission