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Down-regulation of Homer1 attenuates t-BHP-induced oxidative stress through regulating calcium homeostasis and ER stress in brain endothelial cells

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ABSTRACT

Endothelial dysfunction in brain endothelial cells contributes to vasogenic cerebral edema and increased mortality after various neurological diseases. The postsynaptic density protein Homer1 plays an important role in neuronal synaptic activity and is extensively involved in neurological disorders. The present study investigated the role of Homer1 in modulating cell survival using an in vitro endothelial dysfunction model in murine brain endothelial cells (mBECs). Treatment with tert-butyl hydroperoxide (t-BHP) induced a dose-dependent toxicity in mBECs, with no effects on Homer1 expression and distribution. Knockdown of Homer1 using specific siRNA significantly alleviated lactate dehydrogenase (LDH) release, increased cell viability, and ultimately decreased apoptosis after t-BHP treatment. Moreover, Homer1 knockdown attenuated t-BHP-induced ROS generation, lipid peroxidation and mitochondrial dysfunction, as evidenced by loss of mitochondrial membrane potential (MMP), ATP synthesis collapse and mitochondrial swelling. The results of Ca^{2+} imaging showed that Homer1 was involved in inositol trisphosphate receptors (IP_3R)- and ryanodine receptor (RyR)-mediated intracellular Ca^{2+} release, and also mediated t-BHP-induced Ca^{2+} release from the endoplasmic reticulum (ER). In addition, knockdown of Homer1 significantly prevented activation of ER stress markers induced by t-BHP exposure. All these results showed that Homer1 is involved in t-BHP-induced endothelial dysfunction in mBECs, and may be an ideal candidate for searching gene intervention strategy for preventing endothelial oxidative stress in vitro.

1. Introduction

The blood-brain barrier (BBB) is formed by brain endothelial cells (BECs), and serves an important role in cerebral homeostasis. It protects the brain from pathogens, and damaged BBB are associated with many neurological disorders, ranging from acute insults, such as traumatic brain injury (TBI) and stroke to chronic neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) [1,2]. Free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), have been demonstrated to play essential roles in the etiology and progression of endothelial dysfunction by creating vascular oxidative stress. Thus, there is an urgent need to decode the signaling pathways involved in free radicals-induced endothelial dysfunction in BECs.

Homer, also known as ves1, is a recently identified family of scaffolding proteins, which can be classified into Homer1, Homer2 and Homer3 [1]. Homer proteins are encoded by an immediate-early gene whose expression is up-regulated following synaptic activity [1,3]. They function as molecular adaptors that play important roles in calcium signaling. Through a coiled-coil structure in the carboxylic-terminal region, Homer1 interacts with many binding partners, such as the postsynaptic protein Shank, inositol 1,4,5-trisphosphate (IP_3) receptors and glutamate receptors [4]. Previous studies showed that Homer1 dynamically decreased after focal cerebral ischemia, which might contribute to the disturbance of synaptic function and subsequent neurological deficits [5]. Recently, down-regulation of Homer1b/c, one splice variant of Homer1, was shown to protect against traumatic neuronal injury

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and glutamate induced excitotoxicity in primary cultured cortical neurons [6,7]. In addition, a recent report showed that Homer1 mediated MPP^+-induced calcium dysfunction in cultured dopamine (DA) neurons [8]. In the present study, we investigated the role of Homer1 in endothelial dysfunction induced by tert-butyl hydroperoxide (t-BHP) in murine brain endothelial cells (mBECs), and determined the potential underlying molecular mechanism with focus on calcium homeostasis and endoplasmic reticulum (ER) stress.

2. Materials and methods

2.1. Cell cultures

The primary mBECs were obtained from 2 month old C57 BL/6 mice. Briefly, the brains were removed from mice, stripped of meninges and finely minced. Tissues were dissociated in a solution containing 20 U/ml papain and 250 U/ml DNase I type IV in MEM-HEPES. The dissociated brain tissues were triturated, added into a 10 ml tube containing 22% bovine serum albumin and centrifuged at 1000 *g* for 20 min. The isolated cells were then washed and resuspended in endothelial cell growth media with endothelial cell growth supplement. The mBECs were maintained at 37 °C in a humidified 5% CO_2 incubator.

2.2. Immunocytochemistry (ICC)

The mBECs were fixed for 30 min with 4% paraformaldehyde, rinsed twice with PBS and subsequently incubated with 1% hydrogen peroxide for 10 min. Following two PBS rinses, cells were incubated with blocking solution for 20 min and incubated with primary anti-Homer1 antibody (1:50) at 4 °C overnight. Cells were then rinsed twice with PBS and incubated with fluorescein isothiocyanate (FITC) labeled secondary antibody (1:500) for 1 h at room temperature. Coverslips were mounted in mounting medium and visualized by a fluorescence microscope. DAPI (10 μg/ml) was used to stain the nucleus.

2.3. Measurement of cell viability

The cell viability assay was performed using the WST-1 assay kit following the manufacture's protocol (Roche, Basel, Switzerland). Briefly, mBECs were cultured at a concentration of 3×10^5 in microplates in a final volume of 100 μl culture medium. After various treatments, 10 μl WST-1 was added into each well and incubated for 4 h at 37 °C. Then, 100 μl/well culture medium and 10 μl WST-1 was added into one well in the absence of cells, and its absorbance was used as a blank value. Cells were shaken thoroughly for 1 min on a shaker and the absorbance of the samples was measured using a microplate (ELISA) reader.

2.4. Lactate dehydrogenase (LDH) assay

Briefly, 50 μl of supernatant from each well was collected, incubated with reduced form of nicotinamide-adenine dinucleotid (NADH) and pyruvate for 15 min at 37 °C and the reaction was stopped by adding 0.4 M NaOH. The activity of LDH was calculated from the absorbance at 440 nm and background absorbance from culture medium that was not used for any cell cultures was subtracted from all absorbance measurements.

2.5. Flow cytometry

The mBECs were harvested 24 h after various treatments, washed with ice-cold Ca^{2+} free PBS, and resuspended in binding buffer. Cell suspension was transferred into a tube and double-stained for 15 min with the Alexa Fluor 488-conjugated annexin V (AV) and propidium iodide (PI) at room temperature in the dark. After addition of 400 μl binding buffer, the stained cells were analyzed by an FC500 flow cytometer with the fluorescence emission at 530 nm and >575 nm. The CXP cell quest software (Beckman-Coulter, USA) was used to count the number of AV^+/PI^- and AV^+/PI^+ cells, and analyzed the results.

2.6. Measurement of ROS generation

Intracellular ROS levels were quantified using the H_2DCF-DA probe (Sigma). Briefly, mBECs were incubated with H_2DCF-DA (10 μM) for 1 h at 37 °C in the dark, and then re-suspended in PBS. Intracellular ROS production was detected using the fluorescence intensity of H_2DCF-DA in an Olympus BX60 microscope and fluorescence was read using an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

2.7. Measurement of lipid peroxidation

Malonyldialdehyde (MDA) and 4-hydroxynonenal (4-HNE), two index of lipid peroxidation, were determined by using assay kits from Cell Bio labs and strictly following the manufacturer's instruction. The absorbance of the samples was measured by a microplate (ELISA) reader. The results were presented as the fold of control.

2.8. Measurement of antioxidant enzyme activity

The enzyme activities of catalase (CAT), superoxide dismutase (SOD) and lutathione (GPx) were measured according to the technical manual of the detection kits (Cayman Chemical, USA). Protein concentration was determined by using BCA protein kit. The enzyme activities were then normalized to the corresponding protein concentration for each group, and expressed as the percentage of control.

2.9. Measurement of mitochondrial membrane potential (MMP)

MMP was measured using the fluorescent dye Rh123 as reported previously [7]. Rh123 was added to mBECs to achieve a final concentration of 10 μM for 30 min at 37 °C after the cells had been treated and washed with PBS. The fluorescence was observed by using an Olympus BX60 microscope with the appropriate fluorescence filters (excitation wavelength of 480 nm and emission wavelength of 530 nm).

2.10. Detection of mitochondrial swelling

Mitochondria swelling was measured following a previously published protocol [9]. Briefly, isolated mitochondria were suspended in fresh swelling buffer (0.2 M sucrose, 10 mM Tris-MOPS, pH 7.4, 5 mM succinate, 1 mM phosphate, 2 μM rotenone, and 1 μM EGTA-Tris, pH 7.4) at 0.5 mg/ml, and the swelling of mitochondria was monitored by a decrease in absorbance at 540 nm in the presence of $CaCl_2$ (200 μM).

2.11. Measurement of ATP synthesis

Isolated mitochondria were utilized to measure ATP synthesis with a luciferase/luciferin-based system as described elsewhere [10]. Thirty μg of mitochondria-enriched pellets were resuspended in 100 μl of buffer A (150 mM KCl, 25 mM Tris-HCl, 2 mM potassium phosphate, 0.1 mM $MgCl_2$, pH 7.4) with 0.1% BSA, 1 mM malate,

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