Overexpression of COX6B1 protects against I/R-induced neuronal injury in rat hippocampal neurons

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COX (19). Additionally, previous studies reported that abnormal alternations of COX6B1 markedly affect the function of COX, potentially leading to the occurrence of cerebromyopathy, hydrocephalus and other diseases (16,17). COX6B1 protein may be associated with the development of the central nervous system (16). Previous studies reported that COX6B1 may protect the myocardium against I/R injury by regulating mitochondrial function (20,21); however, whether COX6B1 protects hippocampal neurons against I/R injury has not been reported, and the molecular mechanisms underlying the role of COX6B1 in the development of the nervous system remains unclear.

In the present study, a model of I/R injury was constructed using rat hippocampal neurons. Additionally, the molecular mechanisms underlying the role of COX6B1 following I/R injury in hippocampal neurons was investigated.

Materials and methods

Animals. A total of 6 pregnant Sprague-Dawley (SD) rats at gestational day 18 (age, 2 months; weight, 180-200 g) were obtained from Guangdong Medical Laboratory Animal Center (Foshan, China) and maintained at 22±1˚C, 40‑70% humidity, with free access to food and water, under a 12-h light/dark cycle. The animal experiments were approved by The Ethics Committee of The Nanchuan People's Hospital Affiliated to Chongqing Medical University (Chongqing, China).

Extraction of hippocampal neurons. Pregnant SD rats were anesthetized by ether, and 12 fetal rats were extracted. The fetal rats were transferred to Petri dishes containing 75% ethanol and the heads were removed using a guillotine (cat. no. 7950; Ugo Basile SRL, Gemonio, Italy). Subsequently, the hippocampi were dissected and digested with 2.5 g/l trypsin (Beyotime Basile Srl, Gemonio, Italy). Subsequently, the hippocampi were subjected to reoxygenation treatment for 60 min, as aforementioned, without reoxygenation; cells were exposed to reoxygenation treatment for 60 min. Based upon the results of this experiment, OGD-60 treatment was selected to be the I/R injury model in subsequent experiments. Cells were transfected with the plasmids prior to OGD/R.

Oxygen-glucose deprivation/reoxygenation (OGD/R) in vitro model establishment. Hippocampal neurons (1x10^6 cells/ml) were cultured in neurobasal medium containing 2% B-27 supplement at 37˚C with 5% CO_2 for 10 days and cells were exposed to OGD by replacing the neurobasal medium with Earle's balanced salt solution (EBSS) without glucose. The cells were maintained in an anaerobic chamber (YQX-II; Shanghai CIMO Medical Instrument Co., Ltd., Shanghai, China) with 95% N_2 and 5% CO_2 for 60 min at 37˚C. Subsequently, cells were subjected to reoxygenation treatment (incubation in neurobasal medium in an incubator with 5% CO_2 at 37˚C) for 10, 30 or 60 min prior to further experimentation.

Cell viability analysis. Following the aforementioned OGD/R treatment, a Cell Counting Kit-8 (CCK-8) was performed to determine the viability of cells following OGD and OGD/R treatment. The hippocampal neurons (2x10^3 cells/well) were seeded into 96-well plates and maintained in an incubator for 24 h at 37˚C. Following incubation, 10 µl CCK-8 reagent (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was added to each well. Cells were transferred into the incubator and maintained for 2 h at 37˚C. A microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to detect the absorbance at 450 nm.

Cell transfection. The pcDNA3.1(+)-empty vector (EV) and pcDNA3.1(+)-COX6B1 vector were purchased from GenomeDitech Co., Ltd. (Shanghai, China). Cells were seeded (2x10^3 cells/well) into 6-well plates, serum-starved overnight, and subsequently transfected with COX6B1 vector (20 nM) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were transfected for 36 h at 37˚C prior to subsequent experimentation. Cells were transfected with the plasmids prior to OGD/R.

Cell grouping. To assess the effects of OGD/R treatment on neurons, cells were divided into five experimental groups: i) Control group, in which untreated neurons were exposed to PBS; ii) OGD group, in which cells were exposed to OGD for 60 min, as aforementioned, without reoxygenation; iii) OGD-10 group, OGD cells exposed to reoxygenation treatment for 10 min; iv) OGD-30 group, OGD cells exposed to reoxygenation treatment for 30 min; v) OGD-60 group, OGD cells exposed to reoxygenation treatment for 60 min. Based upon the results of this experiment, OGD-60 treatment was selected to be the I/R injury model in subsequent experiments. To investigate the role of COX6B1 in OGD/R-induced damage, cells were divided into six groups: i) Control
Cytosolic Ca²⁺ levels analysis. The cytosolic Ca²⁺ levels of cells in the control, EV, COX6B1, I/R, EV + I/R and COX6B1 + I/R groups were determined by flow cytometry. Following transfection, hippocampal neurons were seeded (2x10⁵ cells/well) into 6-well plates and cultured in an incubator for 24 h at 37°C prior to exposure to the aforementioned OGD/R protocol. The staining reagent Fluo-3, AM (Sigma-Aldrich; Merck KGaA) was added into the wells and the cells were incubated at 37°C for 45 min. Subsequently, the cells were incubated with Hank’s Balanced Salt Solution (HBSS; Thermo Fisher Scientific, Inc.) for 40 min at 37°C. Following incubation, the cells were washed with HBSS for 3 times. The cells were resuspended in HBSS and incubated at 37°C for 10 min. The concentration of intracellular Ca²⁺ was assessed by flow cytometry (BD Biosciences, San Jose, CA, USA) and FSC Express version 3 software (De Novo Software, Glendale, CA, USA).

Cell apoptosis analysis. The cell apoptosis of cells in the control, EV, COX6B1, I/R, EV + I/R and COX6B1 + I/R groups were determined by flow cytometry. Following transfection, hippocampal neurons were seeded (2x10⁵ cells/well) into 6-well plates and cultured in an incubator for 24 h prior to exposure to the aforementioned OGD/R protocol. The cells were then incubated with Annexin V-FITC and propidium iodide (Beijing Solarbio Science & Technology Co., Ltd.) in the dark for 20 min at room temperature. A flow cytometer and FSC Express version 3 software were used to measure the level of apoptosis. Advanced apoptotic cells were presented in upper right quadrants, and early apoptotic cells in lower right quadrants. The relative apoptosis rate of total apoptotic cells (early and advanced) was calculated.

Western blotting. A Mitochondria/Cytosol Fractionation kit (Amyle Scientific Inc., Wuhan, China) was used to separate the mitochondrial and cytosolic fractions. Total protein was extracted from neurons (2x10⁶ cells/well in 6-well plates) using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). The protein concentration was determined using Bradford method (Beyotime Institute of Biotechnology). The protein expression level was determined using a Molecular Imager® Gel Doc™ XR+ System (cat. no. 1708195; Bio-Rad Laboratories, Inc.) and ImageJ version 1.46 software (National Institutes of Health, Bethesda, MD, USA). β-actin was used as the loading control for mitochondrial fraction; protein expression was normalized to β-actin and COX4I1.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). A Mitochondria/Cytosol Fractionation kit was used to separate the mitochondrial and cytosolic fractions. Total RNA was extracted from hippocampal neurons (2x10⁴ cells/well in 6-well plates) using TRIzol® reagent (Thermo Fisher Scientific, Inc.). RNA was reverse transcribed to cDNA using TIANscript cDNA Synthesis kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. cDNA was amplified using TeloPrime Full-Length cDNA amplification kit (Lexogen GmbH, Vienna, Austria), according to the manufacturer's protocol. qPCR experiments were performed using a SYBR Premix Ex Taq™ (Takara Bio, Inc., Otsu, Japan). qPCR thermocycling conditions were as follows: Initial denaturation at 95°C for 1 min, followed by 45 cycles at 96°C for 15 sec and at 63°C for 45 sec, with a final extension step at 75°C for 10 min. β-actin was used as the reference gene, except for mitochondrial fraction, for which COX4I1 was used as the reference gene. The primers were purchased from Sigma-Aldrich (Merck KGaA) and are listed in Table I. The relative expression of genes was

| Gene          | Primer sequence (5′→3′)     | Product size (bp) |
|---------------|-----------------------------|-------------------|
| COX6B1        | F: AAGAACTACAAAAACCGCCCCC  | 195               |
|               | R: ATCCAGGCGTGAGACCCTAT     |                   |
| BAX           | F: GAGACACCTGAGCTGACCTTT   | 187               |
|               | R: CGTCTGGAAACATGTGACCT    |                   |
| BCL-2         | F: GCTCTTTTGGATGGCTGTTG    | 221               |
|               | R: CTTGAGCCGCTTTCAGAG      |                   |
| Cyt c         | F: GGAGGCAAGCATAGACTGG     | 210               |
|               | R: TGCTCTTCTCCCTCTCTCT     |                   |
| β-actin       | F: TGGTTGCTCCCTGTATGCG     | 232               |
|               | R: AATGTACGCCAGATTCCCC     |                   |

Table I. Primer sequences used in reverse transcription-quantitative polymerase chain reactions.

BAX, BCL-2 associated X, apoptosis regulator; BCL-2, apoptosis regulator BCL-2; bp, base pairs; COX6B1, cytochrome c oxidase subunit 6B1; cyt c, cytochrome c; F, forward; R, reverse.
calculated using the 2^\Delta\Delta Cq quantification method (22) and normalized to β-actin and COX4i1 expression.

**Statistical analysis.** All experiments were performed at least three times. GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform statistical analysis. Data are presented as the mean ± standard deviation. Statistical comparisons were performed using one-way analysis of variance followed by Tukey’s test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

Cell viability and COX6B1 expression levels are decreased in hippocampal neurons exposed to OGD/R. The cellular identity of the extracted hippocampal neurons was tested by immunofluorescence assay and >95% of cells were identified to be positive for MAP2 (Fig. 1). The effects of OGD and OGD/R on hippocampal neurons were investigated, and cell viability and COX6B1 mRNA and protein expression levels were examined by CCK-8 assay, RT-qPCR analysis and western blotting, respectively. Following exposure to OGD, cell viability decreased significantly in hippocampal neurons compared with the Control group (Fig. 2A). Furthermore, the viability of cells in the OGD-10 and OGD-30 groups was markedly decreased compared with in OGD group, and significantly decreased in the OGD-60 group. The mRNA and protein expression levels of COX6B1 were significantly reduced in the OGD group, compared with the Control group (Fig. 2B); additionally, the mRNA and protein expression levels of COX6B1 were significantly reduced in OGD-30 and -60 groups compared with in the OGD group (Fig. 2C and D). OGD-60 treatment exhibited the most pronounced effects on cell viability and COX6B1 expression, it was selected as the I/R injury model in subsequent experiments.

**Overexpression of COX6B1 decreases the cytosolic levels of Ca^{2+} in hippocampal neurons increased following I/R.** The transfection efficiency of COX6B1 overexpression vectors was detected using RT-qPCR and western blotting. Hippocampal neurons transfected with pcDNA3.1(+)‑COX6B1 exhibited an increase in the mRNA and protein expression levels of COX6B1 compared with the control EV-transfected cells (Fig. 3). To investigate the role of COX6B1 in the intracellular levels of Ca^{2+} in hippocampal neurons, flow cytometry was conducted. The cytosolic levels of Ca^{2+} were 4.19% in the Control group, 4.23 in EV, 3.67 in COX6B1, 8.44 in I/R, 8.15 in EV + I/R and 6.02% in COX6B1 + I/R treated cells (Fig. 4A). The relative concentration of Ca^{2+} was significantly increased in the I/R group compared with the EV group (Fig. 4B); however, COX6B1 overexpression decreased the concentration of Ca^{2+} compared with the EV + I/R group.

**Overexpression of COX6B1 represses I/R-induced apoptosis of hippocampal neurons and increases cell viability.** To investigate the effects of COX6B1 on apoptosis and viability of hippocampal neurons, flow cytometry and CCK-8 assays were conducted, respectively. Cells exposed to I/R exhibited an increased total apoptotic rate (early + advanced) compared with the EV group (Fig. 5 A and B). Nevertheless, COX6B1 overexpression in hippocampal neurons decreased the apoptosis induced by I/R injury (Fig. 5 A and B). In addition, cell viability was decreased following I/R injury, whereas overexpression of COX6B1 increased the viability of cells following I/R injury (Fig. 5C).

**Overexpression of COX6B1 increases the expression levels of BCL-2 and decreases the expression levels of BAX in hippocampal neurons following I/R injury.** To investigate apoptotic pathway components downstream of COX6B1 in hippocampal neurons, the expression levels...
of apoptosis-associated factors BCL-2 and BAX were investigated by RT-qPCR and western blotting. The mRNA expression levels of BAX in the I/R, EV + I/R and COX6B1 + I/R groups were significantly increased compared with those in the EV group, whereas the expression levels of BCL-2 were significantly decreased compared with in the EV group.
Overexpression of COX6B1 following I/R injury decreased the mRNA expression levels of BAX and increased the expression levels of BCL-2 compared with the EV + I/R group. Similar alterations in the expression of BAX and BCL-2 were observed at the protein level, as assessed by western blotting (Fig. 6C-E).

**Overexpression of COX6B1 increases the expression levels of mitochondrial cyt c and decreases the expression levels of cytosolic cyt c in hippocampal neurons following I/R.** To investigate the role of COX6B1 on the expression levels of cyt c in hippocampal neurons, RT-qPCR and western blotting were performed. The expression levels of mitochondrial cyt c decreased, whereas the expression levels of cytosolic cyt c increased following I/R injury; COX6B1 overexpression reversed the effects of I/R injury (Fig. 7).

**Discussion**

Primary cultured hippocampal neurons are frequently harvested from hippocampi of newborn (<24-h-old) rodents, owing to their localization and the amount of available material (23,24), and these cells have been used to establish experimental models to study the function of neurons in vitro. Therefore, in the present study, the hippocampal neurons from fetal rats were selected as a model to study I/R injury. MAP2 is a specific marker of hippocampal neurons (25). MAP2 protein expression was used to confirm the identity of the extracted cells used in the present study; therefore, the extracted cells were identified as hippocampal neurons. OGD exposure has been used by various previous studies to investigate neuronal ischemia in vitro (26,27). Therefore, in the present study, this method was selected to establish an I/R model in hippocampal neurons. Cell viability decreased by ~50% in cells exposed to OGD for 60 min followed by reoxygenation treatment for 60 min, as assessed by CCK-8 assay.

Massa et al (16) demonstrated that genetic mutations affecting the 20th amino acid residue of COX6B1 protein may cause severe infantile encephalomyopathies. In addition, previous studies reported that knockout of COX5A and COX6A may lead to a decline in the number of neurons in animal models (28,29). Nevertheless, the molecular mechanism underlying COX6B1 function in hippocampal neurons remains unclear. In the present study, the expression levels of COX6B1 decreased following ischemic or I/R injury. Therefore, it was hypothesized that COX6B1 may serve a role in hippocampal neurons following I/R injury. Subsequently, in the present study, COX6B1 was overexpressed in hippocampal neurons exposed to OGD/R, which reversed the I/R-induced decrease in viability of hippocampal neurons.

Dysregulation of intracellular Ca^{2+} homeostasis is a mechanism of cell death caused by various factors (30,31). Cerebral ischemia leads to membrane depolarization and causes the release of presynaptic excitatory transmitters, including glutamic acid and aspartic acid, and extracellular Ca^{2+} ions enter the cells via voltage-gated channels and N-methyl-D-aspartate receptor-gated channels (32). An increase in intracellular Ca^{2+} may lead to the activation of Ca^{2+}-dependent enzymes, including cytoplasmic phospholipases and proteases, which may cause the degradation of cell membranes and the disruption of the neuronal cytoskeleton, damaging the structure and function of neurons (33,34). In the present study, the levels of cytosolic Ca^{2+} were increased following I/R neuronal injury, whereas overexpression of COX6B1 significantly decreased I/R-induced cytosolic Ca^{2+} levels in hippocampal neurons. The present results suggested that COX6B1 overexpression protected against I/R-induced neuronal damage by inhibiting the cytosolic levels of Ca^{2+}.
A number of previous studies demonstrated that neuronal apoptosis served a principal role in neuronal death following cerebral I/R (26,35,36). In accordance with these previous studies, the present study demonstrated that apoptosis was promoted following I/R injury. Additionally, overexpression of COX6B1 was able to suppress I/R-induced neuronal apoptosis. Subsequently, the molecular mechanism underlying neuronal apoptosis was investigated in the present study. The proteins belonging to the BCL-2 family are involved in the apoptotic pathway, and regulate the permeability of the outer membranes of mitochondria (37). BCL-2 and BAX are antagonistic proteins. BCL-2 serves anti-apoptotic roles, whereas BAX serves a role in promoting apoptosis (38). Aboutaleb et al (39) demonstrated that the expression level of BCL-2 decreased, whereas BAX expression increased in hippocampal neurons following I/R injury (39). In accordance with that previous study, the present results revealed that the expression level of BCL-2 decreased following I/R injury, whereas the expression level of BAX was increased. However, overexpression of COX6B1 significantly reversed the expression levels of BCL-2 and BAX in hippocampal neurons following I/R injury. The present results suggested that COX6B1 overexpression was able to reduce I/R-induced neuronal apoptosis by downregulating the expression level of BAX and by upregulating the expression level of BCL-2.
In addition, Wang et al (40) reported that cyt c was detectable in the cytoplasm of neurons following I/R injury, and appropriate ischemic post-conditioning significantly decreased the cytoplasmic levels of cyt c in neurons. The release of cyt c from mitochondria to the cytoplasm is an important step of apoptosis (41-43). Therefore, in the present study, the levels of mitochondrial and cytoplasmic cyt c in hippocampal neurons were investigated. The results suggested that the expression level of mitochondrial cyt c decreased; however, the expression level of cytosolic cyt c increased in hippocampal neurons following I/R injury. This may be a result of the destruction of the mitochondrial outer membrane induced by I/R injury, leading to the release of cyt c from the mitochondrial membrane space. Conversely, overexpression of COX6B1 reversed the effects of I/R injury; the expression levels of mitochondrial cyt c in the COX6B1
+ I/R group were significantly increased compared with in the EV + I/R group, whereas the expression levels of cytoplasmic cyt c were significantly decreased in the COX6B1 + I/R group compared with in the EV + I/R group. The overexpression of COX6B1 may reduce the IR-induced damage to the mitochondrial outer membrane. Of note, there was no clear difference in the mRNA expression of cytosolic cyt c between the EV and the COX6B1 group, whereas the protein expression of cytosolic cyt c in the COX6B1 group was markedly (but not significantly) increased compared with in the EV group. It may be that the overexpression of COX6B1 promoted a small release of cyt c from the mitochondria to the cytoplasm; however, this would require further investigation. The present results suggested that COX6B1 overexpression reversed I/R-induced neuronal apoptosis by inhibiting the release of cyt c from mitochondria to the cytoplasm. However, the present study presents some limitations; the specific molecular mechanism underlying the function...
of COX6B1 in I/R injury was not investigated. Furthermore, the present experiments were performed in vitro and further in vivo analyses are required to confirm the present observations.

Collectively, the present study results indicated that COX6B1 overexpression was able to partially protect neurons following I/R-induced damage by increasing cell viability and by decreasing the levels of cytosolic Ca2+ and apoptosis. The results may facilitate the development of novel strategies for CVD prevention and treatment.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
SY made substantial contributions to the conception and design of the study, PW, JX and LJ were involved in the acquisition, analysis and interpretation of data. All authors approved of the final version of the manuscript.

Ethics approval and consent to participate
The animal experiments were approved by The ethics committee of chongqing Medical university (chongqing, china). The final version of the manuscript.

Competing interests
The authors declare that they have no competing interests.

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