Association between CIRP expression and hypoxic-ischemic brain injury in neonatal rats

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Abstract. The role of cold inducible RNA-binding protein (CIRP) in mediating ischemic brain injury in neonatal rats under chronic hypobaric hypoxia was investigated. The neonatal rat model of chronic hypobaric hypoxia and the cell culture model of SH-SY5Y cells exposed to hypoxia (1% O_2) were constructed. The expression of CIRP and hypoxia-inducible factor-1α (HIF-1α) was detected after hypoxic exposure, and the apoptosis-related proteins were analyzed via terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) and western blot analysis to detect neuronal apoptosis. Moreover, the effects of CIRP overexpression on HIF-1α and neuronal apoptosis were identified. Chronic hypobaric hypoxia can lead to HIF-1α expression and neuronal apoptosis in the body. CIRP was induced at early exposure (3 d/7 d). However, the CIRP level in the hypoxic group was obviously lower than that in the control group with the prolongation of exposure time (21 d). In addition, the knockdown of HIF-1α significantly reduced the neuronal apoptosis under hypoxic conditions, indicating that HIF-1α may promote apoptosis during exposure. The overexpression of CIRP significantly inhibited the upregulation of HIF-1α during hypoxia and the HIF-1α-mediated neuronal apoptosis. Results of the current study showed that, CIRP is involved in the ischemic brain injury induced by chronic hypoxia through downregulation of HIF-1α expression.

Introduction

According to previous studies, severe and chronic hypoxia leads to neuronal death in the Cornu Ammonis 3 (CA3) and CA4 regions in hippocampus dentate gyrus, indicating that neuronal apoptosis in this brain region is one of the main causes of chronic hypobaric hypoxia-induced cognitive impairment (1).

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Cold inducible RNA-binding protein (CIRP) was screened as the DNA damage-induced gene transcript initially, which plays a key role in controlling the cell response under various environmental stresses, such as low temperature and ultraviolet light (2,3). Previous studies have revealed that CIRP migrates from the nucleus to the cytoplasm under environmental stress, which regulates its target messenger RNA (mRNA) at the post-transcriptional level and exerts a neuroprotective effect (4,5). For example, CIRP can inhibit the neuronal apoptosis through inhibiting the mitochondrial apoptosis pathway during mild hypothermia (6). Besides, CIRP protein in cortical neuron of rats inhibits H_2O_2-induced neuronal apoptosis under low temperature, thereby protecting the brain. There have been reports that CIRP is up-regulated in acute mild (8% O_2) or severe (1% O_2) hypoxia response (7). However, the expression features of CIRP in brain tissues under chronic hypobaric hypoxia remain unclear, and whether CIRP can serve as a neuroprotective factor under chronic hypobaric hypoxia has not been confirmed (8).

As the most important transcription factor in cell hypoxia response, hypoxia-inducible factor-1α (HIF-1α) is closely related to the hypoxia-induced neuronal apoptosis (9). Under hypoxic stress, HIF-1α can inhibit its anti-apoptosis effect through increasing the anti-apoptotic protein, B-cell lymphoma 2 (Bcl-2) (10-12). Besides, HIF-1α is related to neuronal apoptosis after brain injury through regulating p53 and Bcl-2 nineteen-kilodalton interacting protein 3 (BNIP3) in apoptotic neurons (13). To weaken the hypoxia-induced neuronal apoptosis in the brain region of cognitive function, clarifying the detailed regulatory mechanism of HIF-1α under hypoxic stress and searching for protective factors are of medical significance.

Chang *et al* (14), found that CIRP can bind to mRNA of HIF-1α and several protein translation factors on polysomes, and increase the protein translation under cell stress. Considering that CIRP plays an important role in the stress-induced neuronal apoptosis, it is assumed as a neuroprotective factor. CIRP can be involved in HIF-1α-mediated neuronal apoptosis under chronic hypobaric hypoxia and exert a neuroprotective effect. A microRNA (miRNA) is a small and non-coding RNA, which plays a vital role in the regulation of such biological processes as cell differentiation, proliferation and apoptosis. Under hypoxic conditions, a hypoxia-sensitive miRNA family named hypoxamiRs will

be induced, and these miRNAs are specifically involved in controlling various processes, such as tumorigenesis, angiogenesis and apoptosis.

To confirm the above hypothesis, dynamic changes in CIRP/HIF-1α expression and neuronal apoptosis were detected in rats exposed to chronic hypobaric hypoxia and SH-SY5Y cells exposed to hypoxia (1% O_2). To investigate the potential association between CIRP change and hypoxia-induced neuronal apoptosis, the effects of CIRP overexpression on HIF-1α expression and neuronal apoptosis were detected.

Materials and methods

Materials

Main reagents. Rabbit anti-human CIRP, HIF-1α, Bax, Bcl-2, caspase-3 and β-actin polyclonal antibodies were purchased from ProteinTech Group, Inc. (Chicago, IL, USA) (1:300; cat. nos. 10209-2-AP, 20960-1-AP, 50599-2-Ig, 12789-1-AP, 19677-1-AP, 20536-1-AP, respectively), rabbit anti-human cleaved caspase-3 (1:200; cat. no. 9661, Cell Signaling Technology, Danvers, USA) and Opti-MEM Medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were also used.

The present study was approved by the Ethics Committee of Liaocheng Third People's Hospital (Liaocheng, China).

Model establishment

In vivo hypobaric hypoxia animal models. Newborn male Sprague-Dawley rats (n=40) were kept in an animal room of the Research Institute in cages with 12/12 h dark-light cycle before exposure to hypobaric hypoxia and provided with sufficient pellet feed and water at 23˚C. The humidity was 60%. All rats were randomly divided into normal control group (n=6) and hypoxia group (n=6).

In vitro chronic 1% hypoxic cell models. Human neuron-like SH-SY5Y neuroblastoma cells (ATCC® CRL-2266™) were placed in the RPMI-1640 medium containing 2 mM L-glutamine supplemented with 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin/streptomycin. The culture was kept in a standard wet incubator with 5% CO_2 at 37˚C, and the original medium was replaced with fresh medium once every 2 days. When 90% cells were fused, the medium was divided as 1:4. Cells were placed in the calibration gas containing 1% O_2 or 3% O_2 (the concentration of CO_2 was adjusted to 5% under these two conditions) and the cells were placed in a humidified microaerophilic culture system (DWS HypOxystation) to prepare the anaerobic environment. The cells were kept in an incubator at 37˚C at different times. Control culture was kept for the same time under normal oxygen content.

Methods

Terminal-deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) analysis. TUNEL was carried out to evaluate cell apoptosis according to the manufacturer protocol. The procedure was as follows: Induced apoptotic cells were fixed in 4% paraformaldehyde phosphate-buffered saline (PBS) at room temperature for 30 min, washed with PBS 3 times and then incubated on ice using 0.1% Triton X-100. After that, the treated cells were mixed with TUNEL reaction mixture, followed by reaction in the dark for 1 h at 37˚C. The nuclei were labelled with Hoechst-33342. Subsequently, the cells were observed under a fluorescence microscope to count the proportion of cell apoptosis.

Western blot analysis. After exposure for the specified time, 3 rats in each group were decollated and western blot analysis was performed to determine HIF-1α, CIRP, cleaved caspase-3/caspase-3 and Bax/Bcl-2 levels in the hippocampus. The hippocampus was removed from brain tissue of rats after cervical dislocation and rapidly placed in prepared pre-cooled 0.9% NaCl solution. Resected tissue was preserved in liquid nitrogen. Samples of tissue and cells were lysed and homogenized, after which the concentration of protein obtained was determined. Western blot analysis was carried out and FluorChem FC2 imaging system (ProteinSimple, San Jose, CA, USA) based on ECLO was used to detect the immune response signal. Gray values of bands in each group were analyzed using ImageJ software. Each protein band was normalized into β-actin value and presented as the intensity ratio. Western blot analyses were performed in triplicate.

Flow cytometry for analysis of cell apoptosis. Flow cytometry was performed for further analysis of cell apoptosis. After hypoxic exposure, SH-SY5Y cells were treated with trypsin, centrifuged at 3,000 x g for 8 min at 4˚C and washed twice. Then, the cells were re-suspended using binding buffer, and added with 5 ml FITC-labeled Annexin-V and 5 ml PI, followed by incubation in the dark at room temperature for 15 min. Samples were analyzed within 1 h after staining.

Plasmid construction and transfection. CIRP complementary deoxyribonucleic acid (cDNA) was cloned in pEGFP-N2 vector and control transfection was performed via pEGFP-N2 without CIRP. Overexpression of CIRP in cells was confirmed via western blot analysis using anti-CIRP antibody in accordance with the protocol. According to procedures provided by the manufacturer, SH-SY5Y cells were transfected with CIRP cDNA using Lipofectamine 2000 transfection reagents. In brief, the cells were inoculated into a 6-well plate with $3x10^5$ cells in each well and grew overnight until 80% of cells were fused. Transfection complex composed of 2.5 μg pEGFP-N2 vector plasmid DNA or pEGFP-N2-CIRP plasmid DNA and 6 μl Lipofectamine reagent was added into the well with Opti-MEM medium. Transfection efficiency and viability of cells were analyzed at 48 h after lipid transfection.

Statistical analysis. Continuous variables were presented as mean ± standard error of mean (SEM) and Student's t test was applied for analysis. Statistical analyses were completed using GraphPad Prism v.5.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Exposure to chronic hypobaric hypoxia led to the increased apoptotic rate of hippocampal neurons in rats and significant changes in CIRP expression. To investigate the effect of exposure to chronic hypoxia on hypoxia-sensitive hippocampal

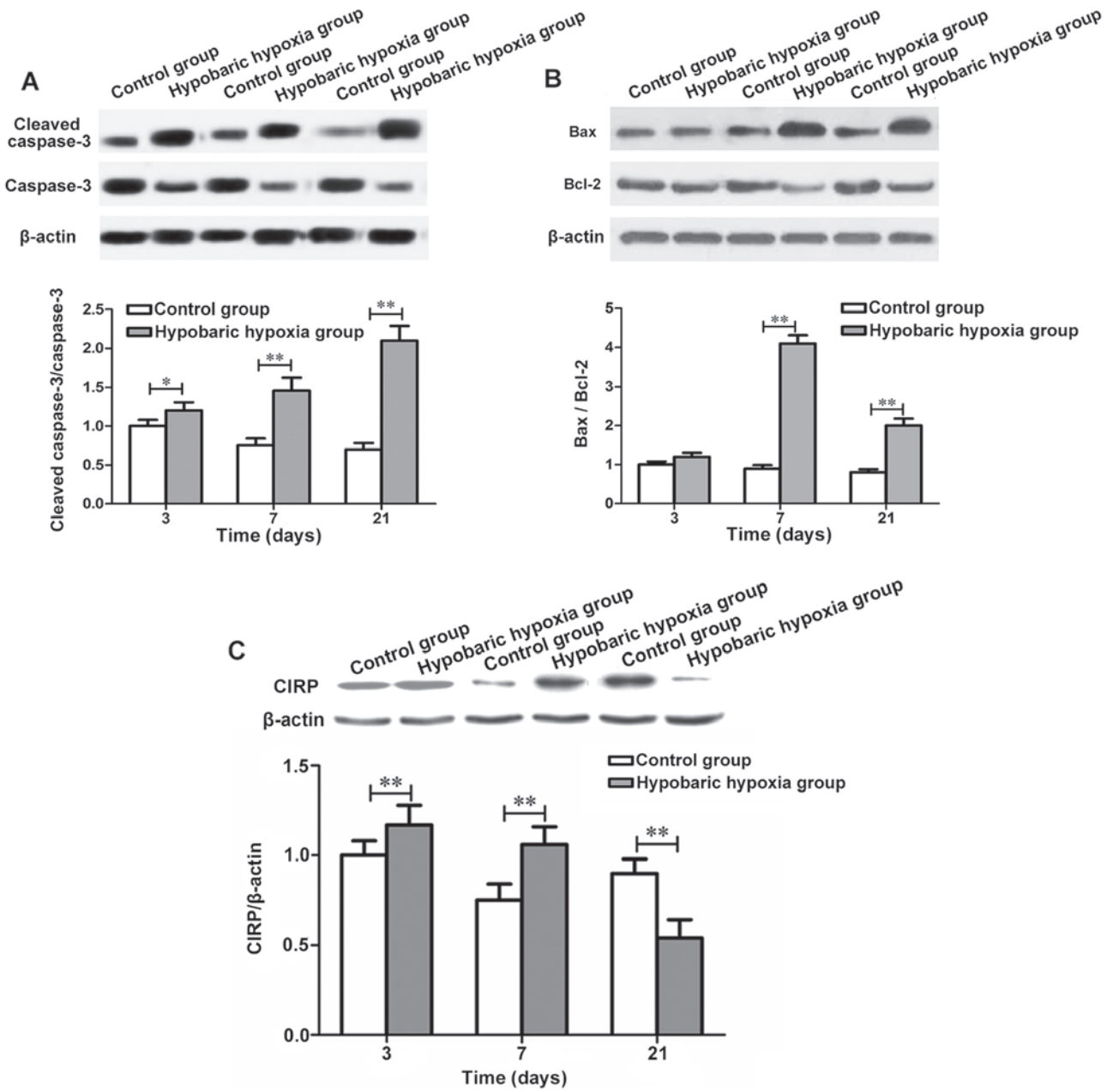

Figure 1. Exposure to chronic hypobaric hypoxia leads to increased apoptosis of hippocampal neurons in rats and significant changes in CIRP expression. (A and B) The relative ratios of cleaved caspase-3/caspase-3 and Bax/Bcl-2 increased significantly in the exposure group at day 7 and 21, compared with the control group. (C) Western blot analysis of CIRP in the hippocampus of rats in control group and hypobaric hypoxia group at day 3, 7 and 21. The content of CIRP in hypoxia group at day 3 and 7 is significantly increased, and it is significantly lower in hypobaric hypoxia group at day 21 than that in the control group. *P<0.05, **P<0.01. CIRP, cold inducible RNA-binding protein.

neurons, adult rats were placed in an animal decompression chamber under 349 mmHg. The expression of apoptosis-related proteins, caspase-3, Bcl-2 associated X protein (Bax) and Bcl-2, in hippocampal neurons were detected, and the CIRP expression during hypobaric hypoxia was also detected at day (d) 3, 7 and 21. Compared with those in control group, the cleaved caspase-3/caspase-3 and Bax/Bcl-2 ratios in hypoxia group at 7 d and 21 d were significantly increased (Fig. 1A and B). Results of western blot analysis revealed that the CIRP levels at 3 and 7 d in hypoxia group were obviously higher than those in control group (P<0.01), while the CIRP level at 21 d in hypoxia group was obviously lower than that in control group (P<0.01). CIRP was induced at the early stage of hypoxia exposure, and inhibited continuously with the prolongation of exposure time (Fig. 1C).

Exposure to chronic hypobaric hypoxia induces apoptosis of SH-SY5Y cells and significantly reduces the CIRP expression. To detect the role of CIRP in hypoxic-related neuronal apoptosis, the *in vitro* chronic hypoxia model was constructed. SH-SY5Y cells were cultured in an anoxic chamber with 1% O_2 for 48 h to simulate the chronic hypoxic condition in tissues. Results of western blot analysis showed that compared with those in control group, the CIRP expression was significantly decreased, and cleaved caspase-3/caspase-3 and Bax/Bcl-2 ratios were significantly increased in hypoxia group (P<0.01;

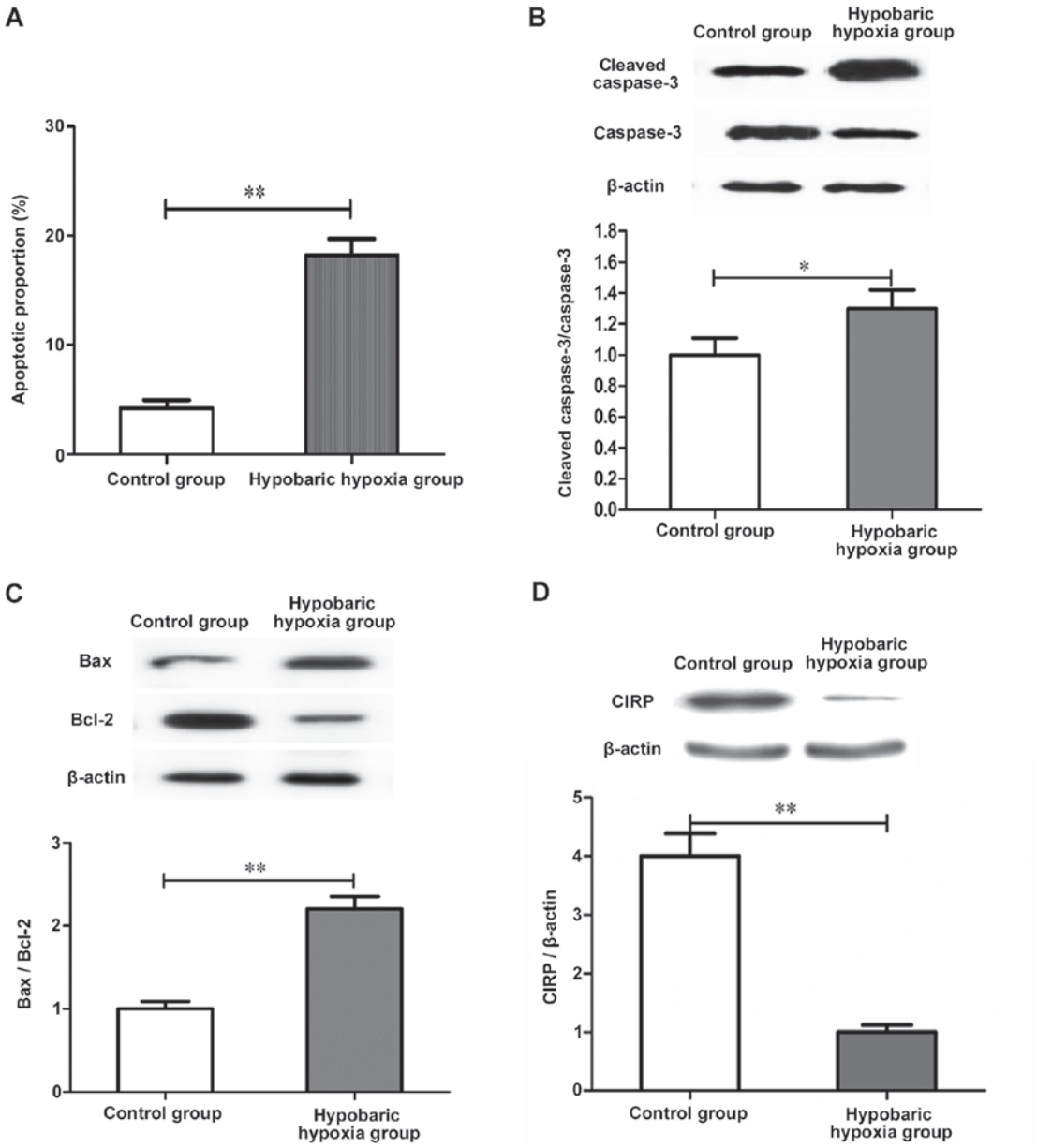

Figure 2. Exposure to chronic hypobaric hypoxia leads to the increased apoptosis of hippocampal neurons in rats and significant changes in CIRP expression. (A) Flow cytometry showed 48 h SH-SY5Y cell apoptosis in control and hypobaric hypoxia group. (B and C) The relative ratios of cleaved caspase-3/caspase-3 and Bax/Bcl-2 in the hippocampus of rats in control group and hypobaric hypoxia group. (D) Western blot analysis of CIRP in the SH-SY5Y cells cultured under normoxia or hypoxia for 48 h. Compared with the normoxic group, the expression of CIRP in the hypoxic group decreased significantly. *P<0.05, **P<0.01.

Fig. 2B and C). Therefore, it is speculated that exposure to 1% hypoxia for 48 h leads to the increased apoptotic rate of SH-SY5Y cells.

The CIRP expression was detected after exposure to 1% hypoxia, and results of western blot analysis manifested that the CIRP expression in hypoxia group was remarkably decreased compared with that in control group, which was consistent with *in vivo* results (Fig. 2D).

Overexpression of CIRP inhibits the upregulation of HIF-1α in hypoxia and inhibits hypoxia-induced neuronal apoptosis. To study the potential association between CIRP decrease and hypoxia-induced brain injury, the effects of CIRP overexpression on HIF-1α expression and hypoxia-induced apoptosis were detected. SH-SY5Y cells were transfected with p-EGFP-N2-CIRP plasmid, and apoptosis was detected after exposure to hypoxia (1% O_2) for 48 h. The overexpression of CIRP in transfected cells was confirmed via western blot analysis (Fig. 3C), which obviously decreased the HIF-1α expression under 1% hypoxic conditions (P<0.01; Fig. 3B) and significantly reduced neuronal apoptosis induced by hypoxia (Fig. 3A). The above results suggest that the overexpression of CIRP can alleviate hypoxia and induce apoptosis of SH-SY5Y cells by regulating HIF-1α expression.

Discussion

The aim of the present study was to investigate the molecular mechanism of CIRP participating in apoptosis during chronic hypobaric hypoxia stress. It was found that the CIRP

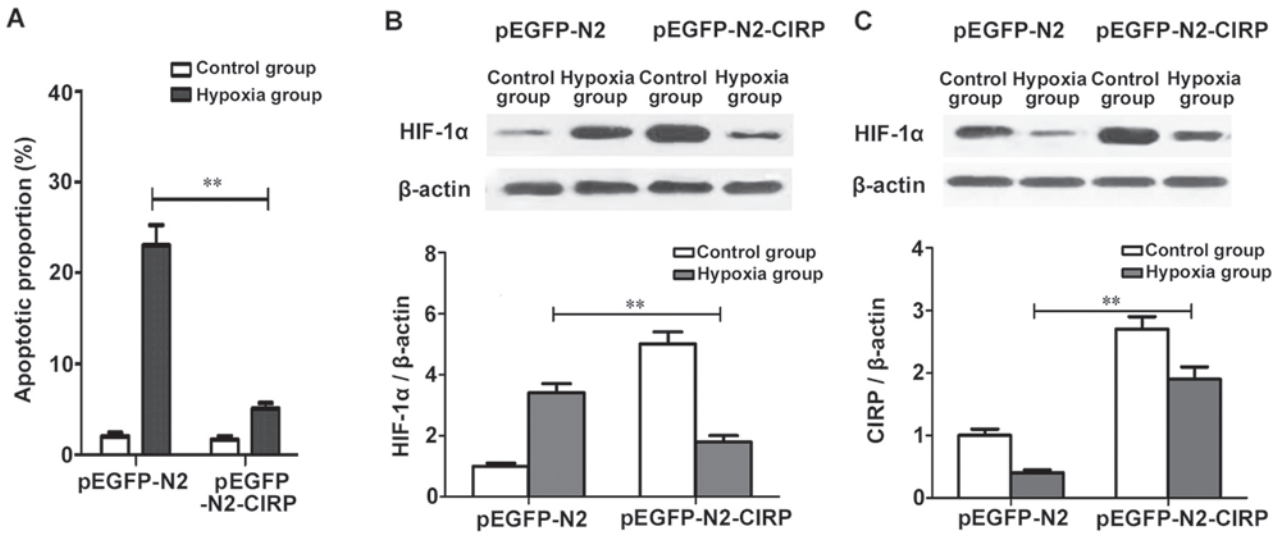

Figure 3. The effect of CIRP overexpression on HIF-1α expression and neuronal apoptosis. (A) The apoptosis of neurons in hypoxic group decreased significantly after pEGFP-N2-CIRP infection (P<0.01). (B) The expression of HIF-1α in hypoxic group decreased significantly after pEGFP-N2-CIRP infection (P<0.01). (C) The expression of CIRP in hypoxic group increased significantly after pEGFP-N2-CIRP infection (P<0.01). **P<0.01.

expression was downregulated in hippocampal neurons and SH-SY5Y cells of rats exposed to hypoxia. Moreover, the overexpression of CIRP could effectively inhibit the upregulation of HIF-1α, thus inhibiting the hypoxia-induced neuronal apoptosis.

CIRP is involved in the neuronal apoptosis induced by a variety of environmental stresses, such as low temperature, oxidative stress, inflammation and DNA damage (15,16). In cortical neurons of rats, CIRP inhibits the etoposide-induced apoptosis through regulating levels of p53 and its downstream targets (8). To the best of our knowledge, no studies are available on the role of CIRP under chronic hypobaric hypoxia. It was found in the present study that in the hippocampus of rats exposed to chronic hypobaric hypoxia, CIRP expression was increased at early exposure, decreased after 7 d and continuously inhibited. Accordingly, the neuronal apoptosis and proportion of apoptosis-related proteins in the hippocampal CA3 region were increased from 7 d after exposure. In SH-SY5Y cells exposed to 1% O_2, CIRP expression was increased in the first 12 h of exposure, then decreased at 24 h after hypoxia exposure and continuously inhibited. Therefore, it can be speculated that the downregulation of CIRP may be involved in the chronic hypobaric hypoxia-induced neuronal apoptosis.

The role of HIF-1α, the most important transcription factor in cell hypoxia response, in hypoxia-induced apoptosis has been discussed widely (17). HIF-1α can initiate the hypoxia-mediated apoptosis by increasing the expression of Bcl-2 binding protein, thus inhibiting the anti-apoptotic effect of Bcl-2 (11). Chang *et al* found that CIRP can bind to mRNA of HIF-1α and several protein translation factors on polysomes, and increase the protein translation under cell stress (14). In the present study, the overexpression of CIRP obviously decreased the HIF-1α level and the apoptotic rate of SH-SY5Y cells exposed to 1% O_2, suggesting that the overexpression of CIRP can inhibit the HIF-1α expression and alleviate the hypoxia-induced apoptosis. Recently, Luo *et al* (18), reported several HIF inhibitors under chronic hypoxia, and found that several kinds of genes, such as peroxiredoxin 2 (PRDX2) and PRDX4, inhibit the HIF-1α mRNA level and transcriptional activity. Therefore, it is speculated that CIRP may repress HIF-1α during chronic hypobaric hypoxia-induced neuronal apoptosis.

CIRP significantly increased HIF-1α expression under normoxia compared with that under hypoxia. Several previous studies have proved that HIF-1α accumulates under hypoxia (19-21). Wang *et al* (21), confirmed that the accumulation of HIF-1α under normoxia is possibly related to the increased glycolysis or glutamine dissolution. It has been proved that CIRP is widely involved in cellular metabolism, so it can be inferred that the transfection of CIRP under normoxia can change the cellular metabolism, resulting in the accumulation of HIF-1α. Moreover, HIF-1α is mainly regulated by the protein stability in an oxygen-dependent way. Under normoxia, HIF-1α can be rapidly degraded by the proteasome, failing to exert its functions. In the present study, the apoptotic rate and levels of apoptosis-related proteins in cells transfected with CIRP under normoxia had no difference from those in control group. It can be observed that although CIRP transfection significantly increases the HIF-1α accumulation under normoxia, it seemingly has no effect on apoptosis of SH-SY5Y cells. The detailed mechanism of CIRP in promoting HIF-1α under normoxia remains to be clarified.

In conclusion, the present study indicates that exposure to hypobaric hypoxia leads to hypoxia injury in the hippocampus of rats and neuronal apoptosis. At the same time, hypoxia exposure to 1% O_2 increases levels of HIF-1α and apoptosis-related proteins, and apoptotic rate of SH-SY5Y cells. CIRP is considered to exert a neuroprotective effect under chronic hypobaric hypoxia stress. The overexpression of CIRP can effectively inhibit the HIF-1α expression in cells, thus alleviating the hypoxia-induced apoptosis. However, the CIRP expression decreases gradually with the prolongation of exposure time.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LC assisted with TUNEL analysis and wrote the manuscript. LC and QT were responsible for model establishment. WW performed western blot analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Liaocheng Third People's Hospital (Liaocheng, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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