Leptin Alleviates Endoplasmic Reticulum Stress Induced by Cerebral Ischemia/Reperfusion Injury via PI3K/Akt Signaling Pathway

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Research Article

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Abstract

Cerebral ischemic/reperfusion injury (CIRI) is a key factor affecting the prognosis of ischemic stroke (IS), the leading disease in global disability and fatality rates. Recent studies have shown that endoplasmic reticulum stress (ERS) may be a potential target against CIRI and leptin, a peptide hormone, has neuroprotective activity to mitigate CIRI. In present study, an in vitro CIRI model was induced in primary cortical neurons by oxygen-glucose deprivation and reoxygenation (OGD/R) after pre-treatment with LY294002 (10 umol/L) or (and) leptin (0.4 mg/L), and then cell viability, neuronal morphology as well as endoplasmic reticulum dysfunction were evaluated. An in vivo CIRI model was established in rats by middle cerebral artery occlusion and reperfusion (MCAO/R) after injecting LY294002 (10 µmol/L) or (and) leptin (1 mg/kg), and studies for neurological function, infarct volume, cerebral pathological changes, the expression of ERS-related proteins along with cell apoptosis. In vitro, leptin treatment improved cell survival rate, ameliorated pathological morphology of neurons and alleviated OGD/R-induced ERS. In vivo, administration of leptin showed a significant reduction in infarct volume, neurological deficits scores and neuronal apoptosis as well as pathological alterations. In addition, leptin suppressed MCAO/R-induced ERS and have anti-apoptotic potential by inhibiting the ERS-related death and caspase 3 activation. It also regulates anti-apoptotic, Bcl-2 and pro-apoptotic, Bax proteins in cortex region. Furthermore, the inhibitory of leptin on ERS was significantly weakened by the specific PI3K inhibitor LY294002. Our findings elucidate the neuroprotective mechanism of leptin, and reinforce its role as a potential agent for treatment of CIRI.

Introduction

Stroke was the second largest contributor to globally all-age disability-adjusted life years, and remains one of the greatest health challenges for the foreseeable future (Collaborators, 2019). With the intensification of population aging and the transformation of life style, the incidence and mortality of stroke are increasing annually, and became the first contributor to lead to death for Chinese residents (Wang et al., 2017). Stroke mainly comprises of two types, hemorrhagic stroke and ischemic stroke, and the later approximately accounted for 80–87% of all strokes. IS characterized by interrupted cerebral blood flow, leading to insufficient energy supply that initiates metabolic disorders and culminating cerebral injury. To date, the key to save patients with ischemic stroke is believe to be recanalization of vessel within a limited therapeutic window after IS (Matsumoto et al., 2018). However, reperfusion therapy may triggers complex pathological reactions, such as a surge in oxidative stress, an inflammatory cascade and excessive apoptosis (El Khashab et al., 2019; Liu et al., 2020).

Recent studies have demonstrated that CIRI has been associated with ERS and ERS-related death (Yang and Hu, 2015; Zhao et al., 2019; Zhao et al., 2022). Endoplasmic reticulum (ER), the organelle of protein synthesis and folding, post-translational modification and Ca2 + homeostasis, is sensitive to stress stimuli (Groenendyk et al., 2021; Roussel et al., 2013). Under ischemic conditions, abnormal accumulation of unfold or misfold proteins in the ER lumen triggers on a pathological state called ERS, which induces a cellular self-protective reactivity termed unfold protein response (UPR). During UPR, cell can reinforce
protein folding, improve degradation of ER-associated protein, and attenuate the translation of nascent protein by activating inositol-requiring enzyme 1 (IRE1), protein kinase receptor-like endoplasmic reticulum kinase (PERK) and ATF6 signaling pathways (Li and Yang, 2021). To a certain extent, ERS is an adaptive programme of damaged cells, which helps to restore cellular homeostasis, but excessive and persistent ERS activates pro-apoptotic signaling pathways such as cysteiny1 aspartate specific proteinase (Caspase) 12 and C/EBP homologous protein (CHOP), leading to apoptosis, and then aggravating CIRI (Iurlaro and Munoz-Pinedo, 2016). Recent studies have revealed that targeting ER dysfunction has beneficial outcomes in CIRI (Pan et al., 2021; Wang et al., 2021; Zhong et al., 2019).

Obesity is well-known to be a crucial risk factor for IS in all race-ethnic groups and significantly increases the risk of atherosclerosis, which is a vital pathological mechanism involved in IS (Herrington et al., 2016; Zahn et al., 2018). Leptin, one of the most common adipocytokine, encoded by the obese gene and principally synthesized and secreted by adipocytes, and it not only has a decisive role in metabolic homeostasis, but also involved in myriad of pathophysiological processes, including inflammation regulation, proliferation and apoptosis (Gairolla et al., 2017; Tang, 2008; Zhang et al., 2019). Accumulating studies have demonstrated that exogenous leptin can bind to its receptors in the cerebral tissue after crossing the blood-brain barrier; exert an anti-apoptotic effect in CIRI; and increase neuron survival, neurogenesis and angiogenesis (Avraham et al., 2013; Calió et al., 2021; Yook et al., 2019). The neuroprotective mechanism of leptin involves multiple anti-apoptotic pathways, including the PI3K/Akt signaling pathway (Avraham et al., 2013). Akt is an important survival factor related to brain development, aging and diseases and involved in a myriad of cellular processes, such as glucose metabolism, protein synthesis and ERS (Bi et al., 2018; Cui et al., 2017; Shen et al., 2019). However, the neuroprotective properties of leptin on alleviating ERS has not been reported.

Based on this background, current research should aim to explore the effects of leptin on intracellular ERS and ERS-associated apoptosis after MCAO/R and OGD/R. Moreover, the PI3K inhibitor LY294002 was used to investigate whether the PI3K/Akt signaling pathway is involved in these processes.

**Material And Methods**

**Animals, chemicals and reagents**

Adult male Sprague-Dawley (SD) rats (250-280 g) and newborn SD rats (within 24 h) were supplied by the Experimental Animal Center of Guangxi Medical University. All experimental procedures and animal usage were performed in accordance with Ethical Review of Animal Welfare administered by The Animal Care & Welfare Committee of Guangxi Medical University (Project Proposal number 2020006014).

Recombinant rat leptin and Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) were purchased from Roche Pharmaceuticals, Switzerland. Primary antibodies against p-Akt, Akt, GRP78, p-PERK, PERK, p-IRE1α, IRE1α, ATF6, CHOP, Caspase12, Caspase3, Bcl-2 and Bax were obtained Abcam and Cell Signaling Technology, USA. Anti-β-actin antibody was purchased from Servicebio Technology, China. PI3K inhibitor LY294002 was obtained from MedChemExpress, USA.
Preparation of rat primary neurons

Cortical neurons were extracted from newborn SD rats. Briefly, dissected cortical tissues were sheared separately into small fragments, digesting in 0.25% trypsin for 10 min, and then adding DMEM/F-12 with 10% fetal bovine serum for neutralization. Neurons were centrifuged for 5 min at 1000 rpm at 4°C, and subsequently re-suspended in DMEM/F-12 serum followed by plated in poly-D-lysine pre-coated plates. After 4 h, the original medium were replaced with neurobasal-A medium supplemented with 2% B27 and 1% GlutaMAX. The purity of neurons was identified with NeuN marker on the 7th day.

OGD/R model and leptin pre-treatment

OGD/R model was performed on the 7th day of neuronal culture. In brief, the neurobasal-A medium was removed and replaced with EBSS without glucose. Later, neurons were placed in a modular incubator filled with 5% CO2/95% N2 for 1 h, 2 h and 4 h respectively at 37°C. After that, EBSS was replaced with the neurobasal-A medium, and then neurons were cultured for another 24 h in a normal incubator filled with 5% CO2 at 37°C.

For this study, recombinant rat leptin, at the dose of 0, 0.1, 0.2, 0.4 and 0.8 mg/L, were chosen to pretreat the neurons for 24 h followed by OGD/R. Furthermore, LY294002, at the dose of 10 μmol/L was applied for 30 min before intervention with leptin in LY+Leptin group.

Cell viability assay

Cell Counting Kit-8 (CCK-8) assay was done to assess the viability of neurons. The neurons were plated in 96-well plates at 2 × 10^5 cells/cm². The blank group refers to an equal volume of medium without cells. After reoxygenation for 24 h, 10 μL of CCK-8 solution was applied to each well and incubated with cells for 3 h in the dark at 37°C. The absorbance was detected with enzyme-labeled instrument (Thermo Scientific, USA) at a wavelength of 450 nm. The cell viability (%) = (experimental group-blank group) / (control group-blank group) × 100%.

Middle cerebral artery occlusion and reperfusion and experimental groups

Prior to the surgery, the rats were fasted overnight but given sterile water. Rats were anaesthetized with pentobarbital, 40 mg/kg, intraperitonially (i.p). The surgical region was shaved and sterilized with iodophor. The surgery was performed on an animal operating table with an insulated pad, and the rectal temperature of the rats maintained at 37.0±0.5°C. As previous described (Ashafaq et al., 2017; Longa et al., 1989), the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were discovered through a midline neck incision and isolated with the vagus nerve and the carotid bifurcations. The right CCA near the heart and ECA were ligated, and an oblique incision, about 5 mm from right ICA, was cut at right CCA. An intraluminal monofilament was introduced into ICA about 18 mm. After 2 h, the intraluminal monofilament was retracted. The incision was sutured in layers and disinfected with iodophor. After that, the rats were placed in cages, where animals can diet and drink freely, and
continued 24 h for reperfusion. The sham-operation group did not insert a monofilament but performed the same surgery.

80 male SD rats (89 underwent surgery, 80 survived) were divided into 4 groups randomly. The first group was sham-operated group (Sham), second group served as MCAO/R surgery group (Vehicle), both the sham-operated and vehicle groups were immediately administered 0.9% saline via tail vein after surgery. Refer to Zhang et al. (Zhang et al., 2013), the third group (Leptin) was were immediately given recombinant rat leptin, at a dose of 1 mg/kg, after MCAO surgery. The forth group (LY+Leptin), in addition to leptin administration, was pretreated with LY294002 (10 μmol/L via tail vein) 30 min before surgery. In the present study, the mortality rate of rats after surgery was 10-20%.

Evaluation of neurologic deficits

As described previously (Longa et al., 1989), Longa’s 5-point scale was used to evaluate neurologic function as follows: 0 = no neurologic deficit; 1= paralysis and inability to fully extend the anterior or hind limbs; 2 = circling to the paralyzed side; 3 = falling to the paralyzed side and crawling; and 4 = an inability spontaneously to walk and loss of consciousness.

Measurement of the percentage of the infarct area

Cerebral infarct volume was measured after neurological examination. Briefly, the rats were anaesthetized deeply with 4% pentobarbital, and the brains were rapidly dissected out and then refrigeration for 20 min. After that, coronal segments of 3 mm thickness were cut. Further, the segments were incubated with 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution for 20 min at 37°C. ImageJ software was used to analyze the total infract area of each segments, and the cerebral infarct volume data are presented as the percentage of [(Contralateral volume)-(Ipsilateral undamaged volume)]/ Contralateral volume*100% (Zhang et al., 2020).

Histopathological evaluation

Hematoxylin-eosin staining was used to evaluate histological study. After perfusion with 0.9% saline, brains of rats were isolated and fixed with 4% paraformaldehyde solution overnight at 4°C, which embedded in paraffin subsequently. Afterwards, coronal sections of 5 um thickness were cut and placed at -20°C for reservation. Prepared sections were soaked in xylene followed by gradient ethyl alcohol, and then stained with hematoxylin and eosin. Further, the pathological morphology of cerebral cortex were observed under fluorescence microscope (OLYMPUS, Japan).

TUNEL staining

Prepared sections were de-waxed with xylene and then washed in graded alcohol. After incubated with 0.25% Triton X-100 solution for 20 min, sections were covered with prepared TUNEL reaction mixture and incubated for 1 h at dark room. Finally, each section was mounted with fluorescence decay resistant
medium and visualized under fluorescence microscope. The data are presented as the apoptotic index (apoptotic index = the number of positive cells in each field/all cells in the field×100%).

**Transmission electron microscopy**

Morphological alteration of endoplasmic reticulum using transmission electron microscopy (TEM). Briefly, primary neurons were fixed with 2.5% glutaraldehyde overnight and then embedded with 100% epoxy resin. Further, ultrathin sections of 70~80 nm thickness were cut using an ultramicrotome. Subsequently, sections were stained with 2% uranyl acetate and 2% lead acetate. Finally, the sections were observed under transmission electron microscope (GOM, Germany).

**Double immunofluorescence staining**

4% paraformaldehyde solution were used to fix sections of primary neurons or brain tissues for 30 min. Then, sections were incubated with 0.25% Triton100-X solution for 15 min at room temperature. After blocking with 10% goat serum, sections were incubated with primary antibodies (anti-p-Akt, anti-GRP78, anti-CHOP and anti-NeuN) at 4°C. After overnight incubation, sections were incubated with secondary antibodies for 1 h. Finally, sections were mounted with fluorescence decay resistant medium and visualized under with fluorescence microscope.

**Western blot analysis**

Primary cortical neurons or brain tissues were lysed with RIPA buffer which were added phosphatase and protease inhibitor. After quantitated by BCA kits, proteins were denatured by boiling for 10 min, and then loaded on 8-12% SDS-PAGE, followed by transferred from gels to activated PVDF membranes. The membranes, after blocking with 5% nonfat dry milk, were incubated with primary antibodies (anti-p-Akt, anti-Akt, anti-GRP78, anti-p-PERK, anti-PERK, anti-p-IRE1, anti-IRE1, anti-ATF6, anti-Caspase12, anti-CHOP, anti-Caspase3, anti-Bcl2, anti-Bax and anti-β-actin). Membranes, after washing 3 times with TBST, were incubated with secondary antibody. Enhanced chemi-luminescence reaction was used to visualize membranes, and densitometric analysis was done using ImageJ software.

**RT-PCR**

TRIzol reagent was applied to isolate Total RNA from primary cortical neurons. The content and purity of RNA were detected by a NanoDrop spectrophotometer (Thermo Scientific, USA) at 260/280. Further, the PrimeScript® RT Reagent Kit was used for reverse transcription. The mRNA levels of GRP78, Caspase12 and CHOP were evaluated by RT-PCR, which was performed on RT-PCR instrument (Biometra, Germany). The specific primers for GRP78, Caspase12, CHOP and β-actin are given below.

**GRP78:** Forward primer: TCTGTGAGACACCTGACCGACC

Reverse primer: TGAATAACCGACGCAGGAATAG

**Caspase12:** Forward primer: TTGGATACTCAGTGTTGATAAAGGA
Reverse primer: GGATGCCGTGGGACATAAAGA

CHOP: Forward primer: GCGGCTCAAGCAGGAAATC

Reverse primer: TTGGCACTGGCGTGATGGT

β-actin: Forward primer: TCAGGTCATCACTATCGGCAAT

Reverse primer: AAAGAAAGGGTGTAAAACGCA

β-actin was used to standardize the quantity and quality of RNA among different samples.

Statistical analysis

All statistical analyses were performed using SPSS 22.0 software, and the data are shown as the means ± SEMs if normally distributed. In vivo experiments are repeated 3 times, and in vitro experimental results were represented at least 3 independent experiments. One-way analysis of variance was used to analyze measurement data for multiple comparison. Nonparametric tests were conducted for skewed data, for example the analyses of neurological deficit score. P<0.05 was considered statistically significance.

Results

Leptin exerts a neuroprotective effect against OGD/R injury Identify of neurons, determination of OGD time and confirmation of leptin concentration

Immunofluorescence staining was used to ensure the purity of neurons for the accuracy of experiments in vitro. All nuclei were stain blue by DAPI, and neuronal nuclei were marked red by NeuN. The ratio of neurons was more than 90% (Figure 1a).

To select the optimal time to establish OGD model, neurons were deprived of glucose and oxygen for respectively 1 h, 2 h and 4 h, and then reoxygenation for 24 h. After that, the viability of neurons at different time points was determine using CCK-8 assay. Cell viability gradually decreased as the OGD time increased. After subjecting to OGD 2h/R 24h, the viability of cells dramatically decreased to 42.06%. Considering the injury process of reperfusion, cells subjected to OGD for 2 h and reoxygenation for 24 h to model CIRI in vitro (Figure 1b).

To study the neuroprotective effect of leptin, neurons were pre-treatment with leptin, at dose of 0.1, 0.2, 0.4, 0.8 mg/L. Afterwards, the viability of cells were examined using CCK-8 assay. The results showed that cell viability in the addition of leptin at a concentration of 0.4mg/L was significantly higher than that in the vehicle group (P<0.001). After considered comprehensively, the optimal concentration of leptin was determined as 0.4 mg/L (Figure 1c).

Leptin improves the cell morphology and viability
Cell morphology was observed using phase-contrast inverted microscope. The results showed that normal neurons had clearly defined round and bright nuclei with abundant cytoplasm as well as integrated neuronal network. After OGD/R, neurons shrank, cytoplasm reduced and neuronal network gradually disappeared (Figure 1d). As compared with vehicle group, there were fewer pathological morphological alterations in leptin group. Furthermore, the results of CCK-8 assay were consistent with the microscopic observation showed that the cell viability in leptin group was significantly improved in comparison of the vehicle group. However, the neuroprotective effect of leptin was weakened in LY+leptin group, as demonstrated by greater damage in cell morphology and failed to improve cell viability (Figure 1d, e).

**Leptin activates the PI3K/Akt signaling pathway during OGD/R**

To explore the effect of the PI3K/Akt signaling pathway on leptin-mediated neuroprotection during OGD/R, we examined the level of Akt phosphorylation in neurons. The change of p-Akt was found an increased significantly in vehicle group in comparison to the control group. With leptin pre-treatment, the p-Akt expression was further increased in leptin group when compared with vehicle group (P<0.001). After pre-treatment with LY294002, we found a significant decrease in the p-Akt expression in LY+Leptin group as compared with leptin group, indicating Leptin activated the PI3K/Akt pathway in vitro (Figure 2).

**Leptin inhibits OGD/R-induced ERS via the PI3K/Akt signaling pathway**

To study whether leptin alleviates OGD/R-induced ERS, cell morphology and ER structure was observed. In TEM analysis, neurons in control group were observed to have normal cell morphology with completed cell membranes, nuclear membranes, and normal ER structure which manifested as a flattened and interconnected tubular network surrounded by massive ribosomes. In the vehicle group, ER was dilated and partly fragmented with fewer studded ribosomes. The pathological alterations of ER structure were greatly prevented by leptin pre-treatment as reflected in leptin group, whereas LY294002 pre-treatment interfered with the protective effect of leptin on ER structure (Figure 3).

Furthermore, we investigated the impact of leptin on the expression of ERS markers. Results of RT-PCR showed that GRP78, CHOP and Caspase12 mRNA levels were significantly increased in vehicle group in comparison of control group. Leptin pre-treatment significantly reduced the mRNA levels of pro-apoptotic proteins CHOP and Caspase12 and further enhanced pro-survival protein GRP78 in leptin group as compared with vehicle group. Whereas, LY+leptin group showed an increase in mRNA levels of Caspase12 and CHOP, and a decrease in the expression of GRP78 in comparison of leptin group (Figure 4).

**Leptin exerts neuroprotective effect against MCAO/R injury**

In TTC staining, compared with sham group, the prominent infarct volume (white area) was observed in the vehicle group. The infarct lesions was significantly decreased in leptin group when compared with vehicle group, and this effect of leptin was reversed by LY294002 (Figure 5a-b).
As HE staining indicated, rats in sham group showed normal corticocerebral structure, round or cone-shaped nerve cells with obvious nucleus, continuous cell membrane and rich cytoplasm. Rats in the vehicle group showed loose tissue structure, irregular nerve cells with solidified and fragmented nucleus, and a large number of vacuoles in the cytoplasm. Damage of cerebral cortex in the leptin group was reduced to varying degrees compared with the vehicle group, and other pathological changes were also alleviated. But pre-treatment with LY294002 markedly abolished the protective effect of leptin (Figure 5c).

After MCAO surgery, neurological deficit score was evaluated after 24 h of reperfusion to investigate the neuroprotective efficacy of leptin. No neurological deficit symptoms were observed in the sham group, while rats presented varying degrees of neurological deficits after MCAO/R. The neurological deficit score in the leptin group was lower than that in the vehicle group. However, the neurological deficit score in the LY+leptin group was significantly higher than that in the leptin group (Figure 5d).

**Leptin activates the PI3K/Akt signaling pathway during MCAO/R**

LY294002 was applied to explore whether PI3K/Akt signaling pathway related to effect of leptin on suppressing ERS in vivo. Consistent with the results in vitro, the expression of p-Akt was significantly increased in leptin group when compared with vehicle group. Nevertheless, the expression of p-Akt was distinctly decreased in LY+leptin group in comparison in leptin group (Figure 6a, b).

**Leptin inhibits MCAO/R-induced ERS via the PI3K/Akt signaling pathway**

Result of immunofluorescence staining and western blot reflected that MCAO/R mediated the occurrence of ERS as demonstrated by an increase in the expression of GRP78, p-PERK, p-IRE1, ATF6, Casspase12 and CHOP significantly in vehicle group as compared to sham group. Leptin group showed considerable decrease in the levels of p-PERK, p-IRE1, ATF6, Casspase12 and CHOP, whereas further increase in GRP78 expression in comparison to vehicle group. However, LY294002 pre-treatment significantly aggravated ERS as showed that in LY+leptin group, the protein levels of ERS-related proteins p-IRE1, Caspase12 and CHOP was increased, while the expression of GRP78 was decreased significantly compared with leptin group. These demonstrated that blocking the PI3K/Akt signaling pathway affected the inhibitory of leptin on ERS (Figure 6-8).

**Leptin reduces apoptotic rate and apoptosis-related proteins in vivo**

TUNEL staining was done to reflect the neuronal apoptosis in cerebral cortex of rats. In contrast to the sham group, the TUNEL-positive cells were increased significantly in vehicle group. Interestingly, Leptin treatment was obviously decreased the rate of neuronal apoptosis in leptin group in comparison of vehicle group. However, LY+leptin group showed a distinct increase in the percentage of TUNEL-positive cells when compared with leptin group (Figure 9a, b).

With the deterioration of ERS, it will result in apoptosis. In this study, pro-apoptotic, Bax, anti-apoptotic, Bcl2 and Caspase3 proteins were examined. In vehicle group, the expression of Bax and cleaved-Caspase3 was increased while the expression of Bcl2 was decreased when compared with sham group.
Leptin treatment reduced the Bax/Bcl2 ratio and cleaved-Caspase3 expression in leptin group as compared to vehicle group. But LY294002 pre-treatment significantly reversed the expression of Bax, Bcl2 and cleaved-Caspase3 in leptin group (Figure 9c, f), suggesting that leptin alleviates ERS-mediated apoptosis through the PI3K/Akt signaling pathway.

**Discussion**

In present study, the primary neuronal injury model of OGD/R and a rodent surgical model of MCAO/R were established to mimic the clinical features of CIRI in vitro and in vivo, respectively. Our results showed that in vitro, pre-treatment with leptin improved the survival rate of neurons and maintained neuronal morphology; in vivo, administration of leptin reduces cerebral infarct volume, morphological damage and neuronal apoptosis; revealing that leptin reduces the secondary events of brain injury after ischemia. Furthermore, we found that leptin therapy significantly alleviated CIRI-induced ultrastructural damage to the endoplasmic reticulum, and reduced the expression of ERS-related proteins and pro-apoptotic factors, indicating that leptin may inhibits ERS and ERS-induced death to exert its neuroprotective efficacy. In addition, we explored the potential mechanism of leptin by using the PI3K inhibitor, and found that the inhibitory of leptin on ERS may be related to the PI3K/Akt signaling pathway.

CIRI is a complex pathophysiological process and regulated by a variety of factors, including the ERS. Endoplasmic reticulum acts as a multifunctional organelle with scores of ER chaperones, enzymes, and cofactors that regulate the precise folding of nascent proteins and assist polypeptides in obtaining final functional conformations (Yang and Paschen, 2016). In the physiological environment, protein folding efficiency of ER is balance with the rate of mRNA translation, and ER-membrane proteins PERK, IRE1, and ATF6 are inactivate by the binding of the glucose-regulated protein 78 (GRP78) (Calfon et al., 2002; Glembotski et al., 2019; Wang et al., 2020). Under ischemic condition, misfolded or unfolded proteins stay in ER lumen and competitively bind to GRP78, which results in the autophosphorylation of PERK and IRE1, the cleavage of ATF6 and thus the initiation of UPR (Fei et al., 2021; Luo et al., 2008; Yu et al., 2017). Extensive studies have revealed that ERS induced by CIRI could be a key factor in the pathological mechanism involving endothelial cells, glial cells and neurons loss after cerebral ischemia, and a various of experiments in vivo and in vitro have demonstrated that targeted the inhibition of ERS can effectively mitigate experimental CIRI (Haupt et al., 2020; Rissanen et al., 2006; Wang et al., 2019). The results of the present study showed that the expression of GRP78, p-PERK, p-IRE1 and ATF6 was significantly increased after MCAO/R, which were consistent with previous studies, suggesting PERK, IRE1 and ATF6 signaling pathways were activated during CIRI. In pathological state, abnormal endoplasmic reticulum ultrastructure are associated with ER function and signal transduction. TEM revealed that in the damaged cortical neurons, ER structure was swelled and partly fragmented when compared to its counterpart in normal cortical neurons, indicating that intense ERS in the in vitro model of CIRI, might have shifted UPR from the early adaptive protection to cell programmed death. The activation of Caspase12 and the induction of CHOP are considered to be two specific apoptotic signaling pathways during prolonged ERS (García de la Cadena and Massieu, 2016; Hu et al., 2018). Activated Caspase12 activates downstream Caspase families such as Caspase3, induced DNA fragmentation, and then resultes in a series of
complex reactions that ultimately lead to apoptosis (Shimoke et al., 2011). CHOP is low expressed in the physiological states, but its expression will be significantly increased if cells encounter intense stimulation, that also induces cell death by up-regulating protein levels of the Bcl2 family and Caspase12 (Su and Li, 2016). Therefore, in the current study, we measured the expression of pro-apoptotic factors induced by ERS, such as CHOP and Caspase12, and the protein levels of downstream apoptotic-related factors cleaved-Caspase3, Bax and Bcl2. The results showed a distinct increase in the expression of CHOP, Caspase12, Bax and Caspase3 after OGD/R or MCAO/R, while protein levels of Bcl2 was significantly down-regulated, further indicating that the occurrence of ERS-related apoptosis in damaged neurons.

Leptin is a peptide hormone that binds to its receptors to lead pleiotropic effects on biological functions. Leptin has a universal role in promoting survival and proliferation, which has been confirmed in a variety of tissue and cell types (Ekraminasab et al., 2022; McGregor and Harvey, 2018). Accumulating evidence emphasizes that leptin protects mitochondria, increases anti-inflammatory factor levels, and reduces excitatory neurotransmitter levels and apoptotic protein levels (Gairolla et al., 2017; Hu et al., 2019). The results of current study revealed that leptin effectively reduced in vivo and in vitro experimental CIRI, which are consistent with previous studies. Many researchers have demonstrated that exogenous leptin has a protective effect on the brain damage, but its underlying mechanisms remain to be explored. Previous studies have shown that enhanced ERS and activation of UPR pathways have a pathogenic role in the occurrence of leptin resistance (Ye et al., 2018); later scholars reported that in non-small cell lung cancer, leptin down-regulated protein level of CHOP through the PERK and ATF6 signaling pathways and thus inhibited apoptosis induced by ERS, revealing that ERS plays an important role on biological effects of leptin (Lai and Sun, 2013). Therefore, we assumed that leptin exerts its neuroprotective effect by inhibiting ERS induced by CIRI to promote neuronal survival. Results of this study provided evidence for our assumption that leptin treatment further increased the expression of GRP78 which is considered as a pro-survival protein during ERS, while significantly reduced the expression of pro-apoptotic factor CHOP and Caspase12 by activating PERK, IRE1 and ATF6 pathways. TEM showed that OGD/R disrupted the integrity of neuronal morphology and induced ultrastructural abnormalities in the ER, whereas leptin pre-treatment reduced pathological changes of cell morphology and ER structure, suggesting the neuroprotection offered by leptin may be attributed by inhibition in ERS and promotion of cell survival. Upon ischemic damage, the imbalance between the pro-apoptosis and anti-apoptosis proteins which lead to the activation of caspase cascade, eventually results in cell apoptosis. Studies have shown that in penumbral area, concealing the apoptotic process may be a potential therapeutic target to improve secondary events of brain injury after ischemic stroke (Tabas and Ron, 2011; Uzdensky, 2019). Our findings revealed that treatment with leptin protects neurons from CIRI which is related to its suppression of ERS, which plays significant role in neuronal apoptosis via the activation of CHOP and Caspase12 pathways in ischemic stroke. Furthermore, the Bax/Bcl2 ratio and the expression of cleaved-Caspase3 reflected that administration of leptin was able to facilitate the activity of Bcl2, and limit the expression of Bax.
Extensive studies have highlighted the neuroprotective effect of leptin in anti-apoptotic mechanisms, which related to a variety of pathways including PI3K/Akt (Zhang et al., 2019). In consensus with previous findings, the results of this study showed a significant enhancement in p-Akt protein levels under ischemia/reperfusion condition, which was further strengthened after treatment of leptin, suggesting that leptin may activate the PI3K/Akt signaling pathway during ischemia/reperfusion. However, after blocking the PI3K/Akt pathway by using LY294002, the neuroprotective effect of leptin was significantly weakened, as demonstrated by increased in infarct volume, pathological changes and neurological deficit score, suggesting that leptin probably alleviated CIRI by regulating the PI3K/Akt signaling pathway. Studies in recent years have revealed that the PI3K/Akt signaling pathway is related to ERS closely; Bi et al. found that the ERS marker GRP78 migrate to the cell surface and interact with PI3K under ischemic conditions (Bi et al., 2018); Liu et al. demonstrated that GRP78 and PI3K/Akt signaling pathways have bidirectional regulation, which promote the survival and growth of post-OGD/R neural stem cells (Liu et al., 2018). Therefore, PI3K inhibitor was used to explore the alteration in the expression of ERS-associated proteins. Our results revealed an increased significantly in protein levels of ERS markers p-IRE1, CHOP and Caspase12, as well as downstream pro-apoptotic protein Bax and Caspase3 in LY + leptin group when compared with leptin group, while an apparent decrease in GRP78 and Bcl2 protein levels, suggesting that leptin inhibits CIRI-induced ERS, and which may related to PI3K/Akt signaling pathway. However, the expression of p-PERK and ATF6 in the LY + Leptin group was increased as compared with the Leptin group, but there was no statistical difference, suggesting that leptin may affect ERS through other signaling pathways, which needs to be further explored in the future.

How to improve CIRI is a challenge in the treatment of ischemic stroke. This study preliminarily verified that leptin attenuates ERS and ERS-related death during reperfusion, and PI3K/Akt signaling pathway may be participated in its suppression of ERS. However, considering the differences between clinical practice and animal models, and leptin mediated effect on multiple tissues, it is necessary to measure the efficacy and safety of leptin in patients with CIRI through high-quality and multicenter prospective studies. In addition, more inhibitors or agonists were not applied in this study to block or activate related signaling pathways for further verification. Therefore, the exact neuroprotective mechanism of leptin needs to be studied in the future.

Conclusion

In summary, this study revealed that CIRI induces ERS in damaged cells, whereas leptin attenuates ERS and ERS-related death during reperfusion, and PI3K/Akt signaling pathway may be related in its inhibitory on ERS. These findings further elucidate the neuroprotective mechanism of leptin and provide a good experimental and theoretical basis for its clinical application for the treatment of CIRI.

Declarations

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Authors' Contributions

Yan Zhang: conceptualization, methodology, writing; Chunxiao Jie: visualization, data curation; Daobin Cheng and Shixiong Huang: investigation, visualization, and validation; Shijun Hu: writing—reviewing and editing, supervision. All authors read and approved the final manuscript.

Data Availability

All the data that supports this study are available from the corresponding author on reasonable request.

Ethics Approval

The research protocol of animals was approved by The Animal Care & Welfare Committee of Guangxi Medical University (Project Proposal number 2020006014).

Consent for Publication

Not applicable.

Disclosure

All the authors declares that there is no conflicts of interest in this work.

References


**Figures**

A. Cortical Neurons

B. Survival rate of neurons (%)

C. Survival rate of neurons (%)

D. Control, Vehicle, Leptin, LY+Leptin

E. Survival rate of neurons (%)

![Image of figures](image-url)
Figure 1

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Figure 2

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Figure 7

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Figure 8

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Figure 9

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