Research Report

HO-1 attenuates hippocampal neurons injury via the activation of BDNF-TrkB-PI3K/Akt signaling pathway in stroke

Dashi Qi, Changjie Ouyang, Yulan Wang, Shichun Zhang, Xijuan Ma, Yuansong Wang, Hongli Yu, Jiali Tang, Wei Fu, Lei Sheng, Lihua Yang, Mei Wang, Weihao Zhang, Lei Miao, Tengteng Li, Xiaojing Huang, Hongyan Dong

Research Center for Neurobiology and Department of Neurobiology, Xuzhou Medical College, 209 Tongshan Road, Xuzhou, Jiangsu 221004, PR China
Department of Human anatomy, Xuzhou Medical College, PR China
School of Public Health, Xuzhou Medical College, PR China
Department of Radiology, Xuzhou Central Hospital, PR China
School of Life Science and Technology, China Pharmaceutical University, PR China
Department of General Surgery, Affiliated Hospital of Xuzhou Medical College, PR China
Department of Clinical Pharmacology, Zhong Shan Hospital, Fudan University, PR China

Article history:
Accepted 25 June 2014
Available online 2 July 2014

Keywords:
HO-1
Stroke
Brain-derived neurotrophic factor (BDNF)
TrkB
PI3K/Akt
Apoptosis

Abstract

Although recent studies have found that HO-1 plays an important role in neuronal survival, little is known about the precise mechanisms occurring during cerebral ischemia/reperfusion (I/R). Therefore, the aim of this study was to investigate the neuroprotective mechanisms of HO-1 against ischemic brain injury induced by cerebral I/R and to explore whether the BDNF-TrkB-PI3K/Akt signaling pathway contributed to the protection provided by HO-1. Over-expressed HO-1 plasmids were employed to induce the over-expression of HO-1 through hippocampi CA1 injection 5 days before the cerebral I/R animal model was induced by four-vessel occlusion for 15 min transient ischemia and followed by reperfusion in Sprague-Dawley rats. Immunoblotting was carried out to examine the expression of the related proteins, and HE-staining was used to detect the percentage of living neurons in the hippocampal CA1 region. The results showed that over-expressed HO-1 could significantly protect neurons against cerebral I/R. Furthermore, the protein expression of BDNF, TrkB, and p-Akt also increased in the rats treated with over-expressed HO-1 plasmids. However, treatment with tropomyosin receptor kinase B (TrkB) receptor antagonist (K252a) reversed the HO-1-induced increase in BDNF and p-Akt protein levels and decreased the level of cleaved caspase-3 protein in I/R rats. In summary, our results...
imply that HO-1 can decrease cell apoptosis in the I/R rat brain and that the mechanism may be related to the activation of the BDNF–TrkB–PI3K/Akt signaling pathway.

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1. Introduction

The risk of ischemic stroke, a major cause of disability and mortality worldwide, occurs when a cerebral blood vessel is ruptured or occluded, contributing to a variety of acute and chronic diseases of the brain (Brima et al., 2013; Hankey, 2006). The World Health Organization has estimated that approximately 15 million people worldwide suffer from stroke annually (Hoffmann et al., 2012). Therefore, a safe and effective therapy that can target multiple pathways for neuroprotection is urgently needed.

Heme oxygenase (HO) can catalyze the first and rate-limiting enzymatic step of heme degradation and produces carbon monoxide (CO), iron and biliverdin (BVD), which are converted into bilirubin (BR) via biliverdin reductase (Banerjee et al., 2011). HO can be classified as three genetically distinct HO isoforms: HO-1, HO-2, and HO-3 (Lin et al., 2010). Among the three known oxygenases, HO-1 is the only inducible isoform, whereas HO-2 and HO-3 are constitutively expressed (de Sousa et al., 2011). HO-1 is the major inducible low molecular weight stress protein of mammalian cells and tissues, and its expression is increased several folds in response to a variety of cellular stresses and stimuli, including ischemia, hypoxia, oxidative stress and inflammatory cytokines, suggesting an important role of this enzyme in tissue protection (Morse et al., 2009). Accumulating evidence indicates that HO-1 over-expression protects against injury in a variety of tissues, while reduced HO-1 levels increase susceptibility to injury in a variety of stress conditions (Fachor et al., 2007). For example, cerebral ischemic injury is amplified in HO-1 knockout mice (Choi and Kim, 2008). In addition, HO-1 is an important protective protein in many clinically relevant disease states, such as Alzheimer’s disease, hypertension, atherosclerosis, and diabetes (Calabrese et al., 2005).

Brain-derived neurotrophic factor (BDNF), a member of neurotrophin family protein, exerts a neuroprotective effect against ischemic brain injury in vivo via its effects on anti-excitatory amino acids, inhibiting inflammatory factor and decreasing apoptosis (Fanai et al., 2014; Harada et al., 2012). Interventions that increased BDNF levels in the perilesional areas are most often associated with improved recovery of function (Rejot et al., 2011). However, attenuating BDNF levels or its effects following cerebral ischemia reduces neuropsychological changes or recovery of function, either spontaneously or induced by rehabilitation (Ploughman et al., 2009). Various studies have shown that BDNF represents one of the most important signaling molecules for adaptive brain plasticity after stroke (Cowansage et al., 2010). Based on these data, pharmacological strategies for post-ischemic cerebral BDNF production provide a new experimental foundation for treatment, which appears to be a promising option in the treatment of stroke.

Cell apoptosis is generally thought to play a crucial role in the process of neuronal death after cerebral ischemia (Broughton et al., 2009; Vakili et al., 2014). Increasing experimental studies have shown that the oxidative load in mitochondria leads to the release of cytochrome c and the translocation of Bax from cytosol to mitochondria, which is controlled by the Bcl-2 family proteins (Kuwana et al., 2002). The release of cytochrome c into the cytosol causes the formation of apoptosome, which permits the autoactivation of procaspase-9 (Ferraro et al., 2008). After the autoactivation of procaspase-9, procaspase-3 is activated and results in DNA fragmentation (Li et al., 1997). In addition, it has been shown that apoptosis contributes to the development of ischemic infarction with DNA fragmentation (Yao et al., 2001). Thus, the ideal preventive or therapeutic approach would target apoptosis after cerebral ischemia.

Some researchers have demonstrated that HO-1 overexpression induces upregulation of BDNF mRNA levels in Rat C6 Glioma Cells (Morita et al., 2009) and that HO-1 exerts neuroprotection through BDNF induction. Therefore, we investigated the relations between the neuroprotective effects of HO-1 and BDNF in the rat brain after stroke. In this study, we hypothesized that the overexpression of HO-1 may protect neurons against cerebral I/R injury through the BDNF–TrkB–PI3K/Akt signaling pathway and can suppress downstream pro-apoptosis signaling molecules in the rat brain after stroke.

2. Results

2.1. The neuroprotective effects of HO-1 against I/R-induced neuronal loss

To determine whether HO-1 has a neuroprotective effect against neuronal injury induced by reperfusion following ischemia, we investigated the effect of HO-1 overexpression on the survival of CA1 pyramidal neurons in the rat hippocampus, where neurons were particularly vulnerable to ischemic injury, at 5 days of reperfusion. HE-staining was used to examine the surviving neurons after 5 days of reperfusion following 15 min ischemia. Shrunken cells with pyknotic nuclei after ischemia were counted as dead cells. As shown in Fig. 1, transient cerebral ischemia followed by 5 days of reperfusion induces severe neuronal death. However, pretreatment with HO-1 overexpression plasmids significantly decreased the neuronal degeneration, whereas in the controls, nonsense oligonucleotides had no effect on cell death during cerebral I/R in the hippocampal CA1 region. The results suggest that HO-1 may protect neurons against ischemic brain injury.

2.2. HO-1 increased the protein levels of BDNF and TrkB and p-Akt

To confirm the effects of HO-1 overexpression plasmids and HO-1-NON on the protein levels of HO-1, we examined the
expression of HO-1 at I/R 5 days after the treatments detailed above. Fig. 2 illustrates that treatment with HO-1 overexpression plasmids significantly increases the expression of HO-1, while nonsense oligonucleotides have no effect on the expression of HO-1.

It has been demonstrated that BDNF exerts a neuroprotective effect against ischemic brain injury in vivo via its effects on anti-excitatory amino acids, inhibiting inflammatory factor and decreasing apoptosis. Moreover, HO-1 could induce up-regulation of BDNF mRNA levels. Therefore, to further explore the possible role of the BDNF pathway mediated by HO-1, we examined the expression of BDNF and its receptors TrkB and p-Akt in the rat hippocampi that were transfected with HO-1 overexpression plasmids. I/R significantly decreased the protein expression of BDNF and TrkB compared to that of the sham group (P < 0.05; Fig. 3). In contrast, the protein expression of BDNF and TrkB significantly increased during treatment with HO-1 overexpression plasmids, whereas HO-1-NON had no effects on the proteins expression of BDNF and TrkB compared to that of the I/R group.

Studies have shown that BDNF can attenuate cell apoptosis in Focal Cerebral Ischemia Rat Brain via activation of Akt signaling pathway. Thence, we also examined the expression of BDNF. The examination of p-Akt showed that I/R significantly decreased the expression of p-Akt protein (P < 0.05; Fig. 4). The treatment with HO-1 overexpression plasmids...
after I/R-induced injury of the hippocampal cells significantly reversed the decrease of p-Akt protein levels. TrkB and p-Akt protein levels in the over-expressed HO-1 group restored to almost the same levels found in the control group.

2.3. HO-1 regulates apoptosis-related protein expression

Apoptosis after cerebral ischemia is one of the major pathways that lead to the process of cell death. Because Bcl-2/Bax ratio and cleaved-caspase-3 protein level are widely accepted as apoptosis markers, we detected their protein levels. Figs. 5 and 6 illustrate the results of western blotting with the monoclonal anti-Bcl-2 antibody, the anti-Bax antibody and the anticleaved-caspase-3 antibody in hippocampal cells after I/R injury. Compared with that of the I/R group, treatment with HO-1 overexpression plasmids significantly increased the relative ratio of Bcl-2/Bax protein level ($P < 0.05$) but decreased the expression of the cleaved-caspase-3 protein ($P < 0.05$).

2.4. HO-1 protects neuronal apoptosis through the BDNF–TrkB–p-Akt signaling pathway

To further verify whether TrkB activation was required for the neuroprotective effect of HO-1, we examined the effect of K252a, an inhibitor of Trk family members, on neuronal injury induced by reperfusion following ischemia. In the presence of K252a, HO-1 failed to show neuroprotection against I/R-induced apoptosis. The protein expression of BDNF and p-Akt significantly decreased in the HO-1/K252a group compared to that of the HO-1 group. However, the protein expression of cleaved caspase-3 increased in the presence of K252a ($P < 0.05$; Fig. 7).

3. Discussion

Previous studies have demonstrated that I/R injury can lead to an extensive range of neurological impairments, including motor disabilities, autonomic dysfunction, epilepsy, memory and attention disorders (Carty et al., 2011; Cengiz et al., 2011). The apoptosis of CA1 pyramidal neurons in the rat hippocampus is a major characteristic of I/R injury, a common cause of neurological deficits and cognitive impairment in survivors of birth. In this research, we showed that HO-1 could remarkably improve the survival of CA1 pyramidal neurons in the context of I/R induced brain injury by down-regulation of apoptosis-related protein expression through the activation of the BDNF–TrkB–p-Akt signaling pathway.

HO-1 is the major inducible low molecular weight stress protein of mammalian cells and tissues whose expression is increased several folds in response to a variety of cellular stresses and stimuli (Morse et al., 2009). BDNF is a member of the neurotrophic factor family, which was originally reported to provide trophic support to neurons and to protect neurons from injuries caused by hypoglycemia, ischemia, hypoxia and neurotoxicity (Sun et al., 2008). To elucidate the possible...
mechanism of BDNF mediated by HO-1, we directly injected specific HO-1 oligonucleotides into hippocampi CA1 to up-regulate HO-1 expression in I/R mice. In the current study, we found that BDNF was remarkably up-regulated and HO-1 could protect hippocampal neurons from ischemic injury. Although the exact mechanism of the activation of BDNF mediated by HO-1 remains still unclear, we speculate that HO-1 increases the protein levels of BDNF maybe through MEK, Akt and NF-κB activation. Previous studies have demonstrated that bilirubin, a downstream product of HO-1, enhanced BDNF expression via ERK and Akt phosphorylation in cortical neuron cultures (Hung et al., 2010). Fluoxetine has also been reported to induce BDNF and GDNF mRNA expression in rat astrocytes via the activation of MEK-ERK pathway (Mercier et al., 2004). Furthermore, NF-κB inhibitor PDTC antagonized bilirubin-induced BDNF expression in glia cultures. In addition, NF-κB could promote the expression of BDNF by directly binding to BDNF promoter (Saha et al., 2006). According to these reports and our present study, further studies are aimed to investigate whether HO-1 has active effect on BDNF by ERK or NF-κB.

In the current study, we also examined the expression of TrkB and p-Akt in I/R mice. TrkB, a high-affinity receptor for BDNF, is widely expressed in neurons throughout the CNS, and the activation of TrkB has been shown to be essential for the survival-promoting actions of BDNF (Patapoutian and Reichardt, 2001). It is reported that upon binding to the TrkB receptor, BDNF can activate various intracellular signaling cascades, including PI3K/Akt, thereby affecting both the development and function of the nervous system. Phosphorylation of Akt (pAkt) promotes cell survival and prevents apoptosis by regulating several downstream targets, including the pro-apoptotic Bcl-2 family members and caspases (Bejot et al., 2011). The present results show that TrkB and p-Akt are remarkably up-regulated in I/R mice. However, these results were reversed in the presence of K252a, a TrkB receptor antagonist. These results indicate that HO-1 exerts neuroprotective effects via the BDNF–TrkB–PI3K/Akt signaling pathway to a certain extent.

There are three different death signaling pathways leading to apoptosis, including the intrinsic mitochondrion-dependent pathway, the extrinsic death receptor pathway, and the intrinsic endoplasmic reticulum (ER) stress pathway (Majewski et al., 2004). In these three different death signaling pathways, apoptosis mediated by activation of the mitochondria-dependent apoptotic pathway represents the dominant cause of programmed cell death, which is well known to be involved in I/R-induced cellular damage. As important mitochondrial membrane-associated proteins, the Bcl-2 family proteins play a vital role in the regulation of the mitochondrial pathway (Fontenay et al., 2006). Increasing evidence suggest that the Bcl-2 family proteins exert the anti-apoptotic effect through inhibition of Bax expression and activation of caspase-3 and that the
Bcl-2/Bax ratio can regulate the caspase-3 apoptotic pathway (Albamonte et al., 2008). Therefore, we further examined the expression of apoptosis-related proteins in I/R mice. The present results showed that I/R significantly decreased Bcl-2 expression and dramatically increased Bax and cleaved-caspase-3 expression. HO-1 over-expression significantly decreased Bax and caspase-3 activation, while Bcl-2 was remarkably up-regulated. These results suggest that HO-1 over-expression can inhibit caspase-3-mediated apoptosis. However, these results were reversed in the presence of the TrkB receptor antagonist K252a. We confirmed that preconditioning with over-expressed HO-1 could remarkably inhibit the activation of caspase-3 during reperfusion after lethal ischemia through HO-1-mediated activation of the BDNF–TrkB–PI3K/Akt signaling pathway. These results might provide some clues to understand the mechanism underlying ischemia tolerance and to find clinical therapies for stroke using endogenous neuroprotection.

In summary, this work demonstrated, for the first time, that HO-1 could protect neurons against brain ischemia followed by reperfusion through up-regulating the activation of BDNF and PI3K/Akt anti-apoptosis signaling pathway. In addition, HO-1 could protect against delayed neuronal death by suppressing elevated caspase-3 activation. We investigated the neuroprotective effect of HO-1 and shed light on the underlying signaling mechanism. However, the exact mechanism of the activation of BDNF mediated by HO-1 remains unclear.

4. Experimental procedures

4.1. Antibodies and reagents

The following antibodies were selected: rabbit polyclonal anti-HO-1 (Santa Cruz, USA), rabbit polyclonal-BDNF (Santa Cruz, USA), polyclonal-TrkB (Santa Cruz, USA), mouse monoclonal phospho-Akt (Chemicon, USA), rabbit polyclonal cleaved-caspase-3 (Cell Signaling, USA), and rabbit polyclonal Bax (Sigma, USA). The secondary antibodies used in our experiment were obtained from Sigma, and K252a, the Trk family member antagonist used in our experiment, was obtained from Cell Signaling.

4.2. Animals and ischemic model

Adult male Sprague-Dawley rats weighing 200–250 g (Shanghai Experimental Animal Center, Chinese Academy of Science, Shanghai, China) were given free access to food and water before surgery. All rats were divided into the following groups: the sham group, the I/R group, the HO-1-treated group, and the HO-1-NON-treated group. Each group comprised 6 animals. Sham controls were performed using the same surgical procedures, with the exception that the occlusion of carotid arteries was not performed. The surgical procedures were approved by the Shanghai Experimental Animal Center. The surgical procedures were conducted under the guidelines and terms of all relevant local legislations. Our best efforts were made to minimize the number of animals used and the suffering that they experienced. Transient cerebral ischemia (15 min) was induced by four-vessel occlusion (4-VO) as described before (Li et al., 2010; Li et al., 2009; Xu et al., 2010). Briefly, after being anesthetized with chloral hydrate (350 mg/kg, i.p.), both of the vertebral arteries were occluded permanently by electrocautery, and the common carotid arteries were exposed. Then, the rats were recovered for 24 h and fasted overnight. To induce cerebral ischemia, aneurysm clips were used to occlude both of the carotid arteries. After 15 min of occlusion, the aneurysm clips were removed for reperfusion. In this process, three mice died.

4.3. Administration

One-hundred micrograms of specific HO-1 overexpression plasmids were administered to the rats 5 days before ischemia through hippocampi CA1 injection (anteroposterior, 3.6 mm; lateral, 2.0 mm; and depth, 4.0 mm from bregma) (Pei et al., 2005). The same dose of nonsense oligonucleotides and vehicle was used as a control. The locus of HO-1 was BC091164, and its CDS region was over-expressed (Qi et al., 2012).

4.4. Tissue preparation

After reperfusion under anesthesia, the rats were decapitated immediately; then, the hippocampi were removed and quickly frozen in liquid nitrogen. The hippocampi were homogenized in 1:10 (w/v) ice-cold homogenization buffer containing 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS; Sigma, St. Louis, MO; pH 7.4), 100 mM KCl, 320 mM...
sucrose, 50 mM NaF, 0.5 mM MgCl$_2$, 0.2 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA, 1 mM Na$_3$VO$_4$ (Sigma), 20 mM sodium pyrophosphate, 20 mM β-phosphoglycerol, 1 mM p-nitrophenyl phosphate (PNPP), 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 μg/ml each of leupeptin, aprotonin, and pepstatin A. Then, they were centrifuged at 12,000g for 15 min at 4°C. The supernatants, including nuclear parts, were collected, and the protein concentrations were determined by the Lowry method. The samples were stored at –80°C and were thawed only once.

4.5. Immunoblotting

Proteins were separated on polyacrylamide gels and then electrotransferred onto a nitrocellulose membrane (Amer-sham, Buckinghamshire, United Kingdom). The membranes were blocked for 3 h in Tris-buffered saline with 0.1% Tween 20 (TBST) and 3% bovine serum albumin (BSA) and then incubated overnight at 4°C with primary antibodies in TBST containing 1% BSA. The membranes were then washed and incubated with alkaline phosphatase-conjugated secondary antibodies in TBST for 2 h and developed with NBT/BCIP color substrate (Promega, Madison, WI). The densities of the bands on the membrane were scanned and analyzed using an image analyzer (LabWorks Software, Upland, CA).

4.6. Hematoxylin–eosin staining (HE-staining)

The rats subjected to 5 days of reperfusion were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under anesthesia. The paraffin-embedded brain sections (5 μm) were prepared and stained with hematoxylin and eosin. Histological evaluations were performed with HE-staining for the assessment of neuronal damage in the hippocampus. An initial disector frame was positioned randomly in the hippocampal sector and cells in every 10th section throughout the entire hippocampus. The cell numbers in the hippocampus were assessed using previously published unbiased stereological techniques. In brief, cell counts were performed at 400× magnification with the use of an Olympus BH-2 microscope connected to a Sony charge-coupled device video camera, a motorized stage system, and commercial stereology software.

4.7. Statistical evaluation

Six animals were independently selected as samples in all groups for immunoblotting and histology assays. Image J (Version 1.30v) analysis software was used to conduct quantitative analysis of the bands. Values were expressed as mean±SD. Statistical analysis of the results was carried out by one-way ANOVA followed by Newman–Keuls test. P-values of P<0.05 were considered significant.

Acknowledgments

This work was supported by the foundation of Xuzhou Medical College (2011KJ11).

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