Japanese Journal of Gastroenterology and Hepatology

Research Article

HO-1 as a Dynamic Sensor in the Livers of Chronic Cerebral Hypoperfusion Rats

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Received: 01 May 2020

Accepted: 16 May 2020

Published: 21 May 2020

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1. Abstract

Heme oxygenases, mainly including heme oxygenase-1(HO-1) and heme oxygenase-1(HO-2), play a key role in protecting from oxidative stress and inflammation associated with vascular disease. Little is known, however, about the roles of HO-1 and HO-2 in the livers of rats with chronic cerebral hypo perfusion. The study aims to evaluate the expression and roles of HO-1 and HO-2 in the livers of cerebral hypo perfusion rat. Adult male SD rats aged 4 months and weighing 250-300g, were enrolled and performed by permanent bilateral common carotid arteries occlusion (2 vessel occlusion, 2VO), then randomly divided into 6 groups (sham-operated, postischemia 1d, 7d, 14d, 21d and 28d). HE staining showed hydropic degeneration happened after 2VO operation. RT-PCR and Western blot results showed that HO-1 was increased in the livers in postischemia 1d group and peaking in 7d group ($p<0.05$), compared with sham-operated group. Nevertheless, the expression of HO-2 was not changed with time gradient. Meanwhile, immunohistochemistry results were consistent with RT-PCR and Western blot results. Exposure of liver to 2VO was resulted into a time-dependent increase of heme oxygenase activity. The increase was evident at 1 day after 2VO compared to sham-operated rats, and heme oxygenase activity continued to rise at 7 days after hypoperfusion ($P<0.05$). Then it gradually decreased at 14 days, 21 days and 28 days respectively. This trend was corresponding with the mRNA and protein expression levels of HO-1. **T**herefore, our findings suggested that the expression of HO-1 mRNA and protein were increased in a time-dependent manner in the livers of chronic cerebral hypo perfusion rats, and HO-1 might be a new target of drug treatment for the livers of cerebral hypo perfusion injury.

2. Keywords: HO-1; HO-2; Hypoperfusion; Liver

3. Introduction

Heme oxygenases (HOs), as dynamic sensors of cellular oxidative stress, catalyze the cleavage of the heme to form Carbon Monoxide (CO), ferrous iron and biliverdin [1-2]. Biliverdin rarely accumulates in tissues and is rapidly reduced by biliverdin reductase to bilirubin in intact tissues. It suggested that the physiologic product is generally thought to be main bilirubin [3]. And ferrous iron is immediately oxidized to ferric iron in normal bodies. Besides, as a putative neurotransmitter, CO is best supported by studies of endogenous neurotransmission in the intestine [4-5]. The two principal forms of HOs (HO-1 and HO-2) have been distinguished and molecularly cloned [6]. HO-1 is an inducible enzyme, which can be elicited by multiple stimulants especially those associated with red blood cell damage, such as heme and other porphyrins [7]. Also it is designated as one of the heat shock proteins, and mainly concentrated in the

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spleen and liver. By contrast, HO-2 is constitutive and most concentrated in the brain and testes, accounting for the great majority of HO activity in the normal tissues [8].

Numerous researchers confirmed that HO-1 can exert anti-inflammatory, antiproliferative, anti-apoptotic and anti-oxidant effects in the body [9-11]. Hyun H and Aztatzi-Santillán E demonstrated that HO-1 played a protective role in cerebral ischemia [12-13]. While the role and expression of HO-2 is vague after injury. Over decades ago, there were lots of articles to describe the role of HOs in hepatic diseases [14-16], but few of research to depict the expression of HOs in liver after chronic cerebral hypo perfusion. So, the purpose of our study is to determine whether chronic cerebral hypo perfusion alters hepatic HOs expression and HO activity at different time points, which performed by permanent bilateral common carotid arteries occlusion (2 vessel occlusion, 2VO).

4. Materials and Methods

4.1. Rat Model

All experiments on animals were approved by the Chongqing Medical University Institutional Animal Care and Use Committee (animal certificate number: SCXK2007-0001). For- ty-two Sixty Sprague-Dawley rats, aged 4 months and weighing 250g-300g, were enrolled and randomly divided into a sham-operated group (n=7) and a model group (n=35). All rats were allowed free access to chow and water before and after surgery, and housed at 24 - 25°C, humidity 40% - 70% , under 12 h-dark and 12 h-light illumination. Rats were anesthetized by 3.5% chloralic hydras (w/v) in accordance with 0.35mg/g undergoing intraperitoneal injection. Bilateral common carotid arteries of sham operation group were severally separated without ligation. Rats of model group were performed by permanent bilateral common carotid arteries occlusion (2 vessel occlusion, 2VO), which was in accordance with those described above [17]. The model rats (n = 35) were randomly subdivided into five groups: 1, 7, 14, 21, 28 days after 2VO.

4.2. RT-PCR

Livers RNA were isolated using Biozol (BioFlux, Osaka, Japan) according to the manufacturer's instructions after homogenate. Total RNA(1 ug) was reverse transcribed into single-stranded cDNA using Moloney murine leukemia virus reverse transcriptase and random hexamers (Promega, Madison, WI, USA). PCR amplification of target cDNAs and an internal control(-beta-actin) cDNA were carried out using the following primer pairs: HO-1 sense, 5'- TGG CCC ACG CAT ATA CCC GCT A-3', antisense, 5'-TGG GAT TTT CCT CGG GGC GTC T-3'; HO-2 sense, 5'-TAG CAC CTC TGC CTG CCA GCA T-3', antisense, 5'-ACA AAA CCT GGG TGT GGG CAG C-3'; beta-actin sense, 5'-AAG ATC CTG ACC GAG CGT GGC T-3', antisense 5'-ACG CAG CTC AGT AAC AGT CCG C-3'. Reaction mixtures were first denatured at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds over 35 cycles, followed by a final extension step at 72°C for 10 minutes. PCR products were visualized by ethidium bromide staining after 2% (w/v) agarose gel electrophoresis. The lengths of the amplifications for HO-1, HO-2 and beta-actin were 385, 226 and bp, respectively. The relative absorbance was expressed as the ratio of the target gene mRNA absorbance value to the beta-actin absorbance value, and quantified by Quantity One software (Bio-Rad, Hercules, CA, USA). The tissue lysates were analyzed by RT-PCR three times.

4.3. Western Blot Analysis

For protein analysis, liver tissues were lysed with PRO-PREP™ liquid (0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Triton X-100, 0.15 mol/L NaCl, 0.05 mol/L Tris-HCl, PH 7.2) in the presence of a protease inhibitor cocktail (Roche, Welwyn Garden, Hertfordshire, UK). Tissue lysates were clarified by centrifugation (12 000 × g, 10 minutes, 4°C), and protein concentration determined by the Bradford method using a Universal Microplate Reader (Bio-Rad) at 595 nm. After gel electrophoresis (12% tris-glycine) in the presence of sodium dodecyl sulfate, proteins were transferred to polyvinylidene fluoride membranes (Millipore Corporation, Billerica, MA, USA). After blocking, membranes were incubated with primary antibodies directed against rabbit anti-rat HO-1 antibody, anti-rat HO-2 antibody and rabbit anti-rat beta-actin antibody (Beijing Biosynthesis Biotechnology CO., LTD; 1:500), diluted in 0.1% (w/v) non-fat dry milk and incubated overnight at 4°C. After washing, bound antibodies were detected by incubation for 2 hours at room temperature with peroxidase-conjugated mouse anti-rabbit antibodies (Zhongshan Goldenbridge biotechnology Company, Beijing, China; 1:1000). Membranes were then developed using a commercial ECL system (Bio-Rad). The tissue lysates were analyzed by Western blot three times.

4.4. HO Activity Measurement

HO activity was measured by the rate of generation of bilirubin in liver homogenate isolated from the strips of liver tissue [18]. The tissues were homogenized in 5 vol of 0.1 M potassium phosphate (pH 7.4) buffer on ice for 30 minutes. The

homogenate was centrifuged at 1,000×g at 4°C for 10 min, and the supernatant subsequently was centrifuged at 12,000×g at 4°C for 10 min. the resulting supernatant was seperated and frozen at -80°C Then the supernatant (0.2 mg of protein) was added to a reaction mixture (0.4 ml total volume) containing fresh rat liver cytosol (2 mg of cytosolic protein), hemin (20 uM), glucose 6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 unit), and NADPH (0.8 mM) and incubated for 1 h at 37°C in the dark. The formed bilirubin was extracted with chloroform (0.5 ml) and (ΔA464-A530) was measured (extinction coefficient, 40 $mM^{-1}.cm^{-1}$ for bilirubin). HO activity is expressed as picomoles of bilirubin formed per 60 min per milligram of protein, determined by the Lowry method.

4.5. HE Staining

The liver tissues were removed immediately, and washed with iced saline, fixed in 10% formalin, embedded in paraffin, sectioned (about 5 μm thick), stained with hematoxylin and eosin, dehydrated in 95% , 90% and 70% ethanol, cleared in xylene, mounted in neutral gum, and observed under a microscopy.

4.6. Immunohistochemistry

The staining protocol employed a modified streptavidin-HRP immunohistochemistry procedure (CoWin Century Biotechnology, Inc, Beijing, China). Briefly, the slides were incubated overnight with the rabbit anti-rat HO-1 polyclonal antibody (1:100) or rabbit anti-rat HO-2 polyclonal antibody (1:200). The slides were treated with peroxidase- conjugated streptavidin and visualized by the diaminobenzidine (DAB) Kit (CoWin Century Biotechnology, Inc, Beijing, China), and sections were counterstained with haematoxylin. PBS was used as a negative control, and sections which were presented by company known to express HO-1 and HO-2 protein were used as positive control.

4.7. Statistical Analysis

All data are presented as the mean ± the Standard Deviation (SD). Homogeneity test for variance was performed using SPSS 11.5 software (SPSS, Chicago, IL, USA). The parametric variables were analyzed by using one-way analysis of variance (ANOVA) in conjunction with a SNK-q post hoc test. A two-tailed value of *P* less than 0.05 was considered to indicate a statistically significant difference.

5. Results

5.1. Expression of HO-1 was Dynamic But HO-2 was not Changed at Mrna Expression after Cerebral Hypoperfusion

The mRNA expression levels of HO-1 after cerebral hypoperfusion were increased at different time points, compared to the sham-operated group. While it was markedly increased at 7 days after 2VO ($P<0.05$), and reached a peak at 7 day. Then it was gradually decreased at 14 days, 21 days, and 28 days respectively, compared to 7 days after 2VO. On the contrary, there was not any change at the expression of HO-2 mRNA after 2VO (Figure 1).

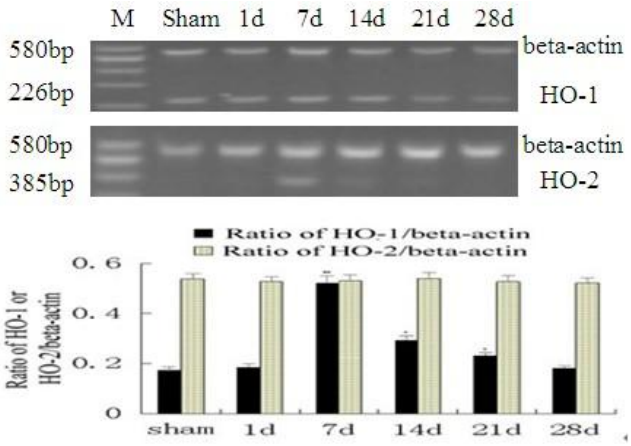

Figure 1: Expression of HO-1 and HO-2 mRNA in rats' liver after chronic cerebral hypoperfusion. HO-1 and HO-2 mRNA expression were measured at different time points after chronic cerebral hypoperfusion and compared with sham-operated group. Each bar represents the mean (±S.E.) of three experiments performed independently. M: marker, sham: sham-operated group, 1d: 1 day after 2VO, 7d: 7 days after 2VO, 14d: 14 days after 2VO, 21d: 21 days after 2VO, 28d: 28 days after 2VO. * P<0.05 versus sham, ** P<0.01 versus sham

5.2. Expression of HO-1 was Dynamic but HO-2 was not Changed at Protein Levels after Cerebral Hypoperfusion

The results showed that the expression of HO-1 protein after cerebral hypo perfusion was increased compared to the sham-operated group. And it was reached a peak at 7 days after 2VO ($P<0.05$). However it was gradually decreased at 14 days, 21 days and 28 days respectively compared to 7 days after 2VO accompanied by recovery of cerebral blood flow. It indicated that up-regulation of the HO-1 gene by hypo perfusion furthered protein expression. Compared to HO-1, there were not significant differences of HO-2 between the model groups and the sham-operated group (Figure 2).

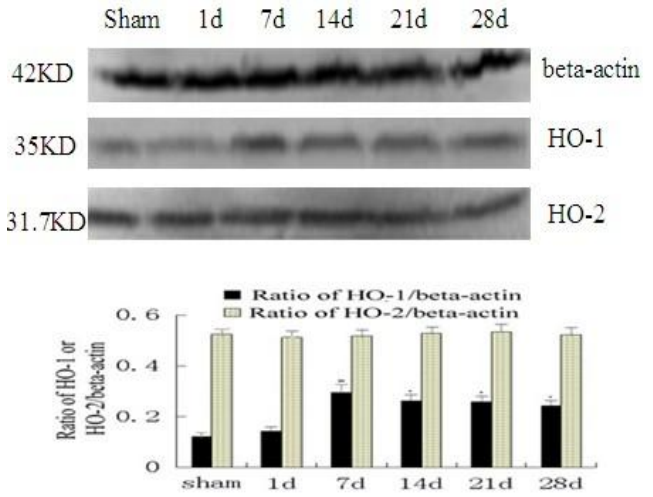

Figure 2: Expression HO-1 protein and HO-2 protein in rats' liver after chronic cerebral hypoperfusion. HO-1 and HO-2 protein expression were measured at different time points after chronic cerebral hypoperfusion and compared with sham-operated group. Each bar represents the mean (±S.E.) of three experiments performed independently. sham: sham-operated group, 1d: 1 day after 2VO, 7d: 7 days after 2VO, 14d: 14 days after 2VO, 21d: 21 days after 2VO, 28d: 28 days after 2VO. * P<0.05 versus sham, ** P<0.01 versus sham.

5.3. Effect of Hypoperfusion on Heme Oxygenase Activity was Dynamic

Exposure of liver to 2VO was resulted into a time-dependent increase of heme oxygenase activity. The increase was evident at 1 day after 2VO compared to sham-operated rats, and heme oxygenase activity continued to rise at 7 days after hypo perfusion (P<0.05). Then it gradually decreased at 14 days, 21 days and 28 days respectively (Figure 3). This trend was corresponding with the mRNA and protein expression levels of HO-1.

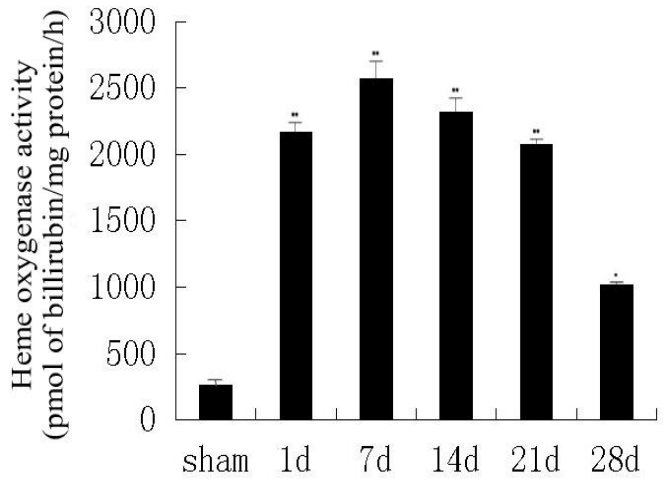

Figure 3: Effect of chronic cerebral hypo perfusion on heme oxygenase activity was changed in rats' liver. Heme oxygenase activity was measured at different time points after chronic cerebral hypo perfusion and compared with sham-operated group. Each bar represents the mean (±S.E.) of five experiments performed independently. sham: sham-operated group, 1d: 1 day after 2VO, 7d: 7 days after 2VO, 14d: 14 days after 2VO, 21d: 21 days after 2VO, 28d: 28 days after 2VO. * P<0.05 versus sham, ** P<0.01 versus sham.

5.4. The Pathological Changes were Dynamic in the Liver Tissues of Rat Models

Compared to the sham-operated group, 2VO provoked many morphological changes in regions of the liver tissues, mainly including swelling of cells, that was volume of cells was becoming bigger, the cytoplasm was becoming more transparent, and these changes were called hydropic degeneration. From the (Figure 4), we could see that the hydropic degeneration was the most significant at 7 d after 2VO, and it gradually decreased, and the cells were almost recovered to normal at 28d.

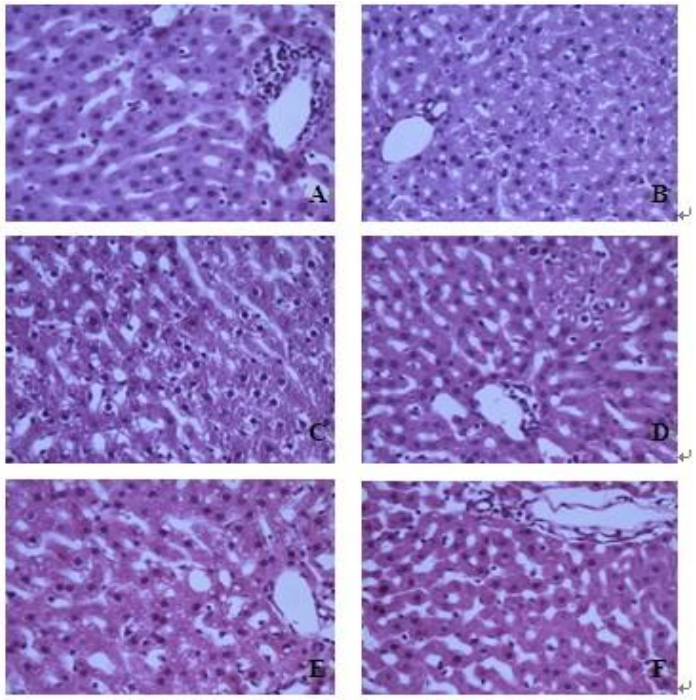

Figure 4: Morphological changes in the liver tissues of rat models. In order to observe the pathological changes after ischemia, we applied HE staining. Compared to the sham-operated group, 2VO provoked many morphological changes in regions of the liver tissues, mainly including swelling of cells, that was volume of cells was becoming bigger, the cytoplasm was becoming more transparent, these changes were called hydropic degeneration. Furthermore, the hydropic degeneration was the most significant at 7 d after 2VO, then, the cells were gradually returned to the normal (A: sham-operated group, B: 1 day after 2VO, C: 7 days after 2VO, D: 14 days after 2VO, E: 21 days after 2VO, F: 28 days after 2VO).

5.5. Distribution of HO-1 and HO-2 Positive Cells in Liver of Rat Models

The positive expression of HO-1 and HO-2 were mainly located in the cytoplasm. The HO-1 staining intensity was significantly increased at 1 day after 2VO, and there was a peak at 7 days (P<0.001), subsequently, it was gradually becoming more and more weak at 14 days, 21 days and 28 days (Figure 5). On the contrary, the positive staining intensity of cytochrome HO-2 was no any significant changes between the sham group and model groups (P>0.05) (Figure 6).

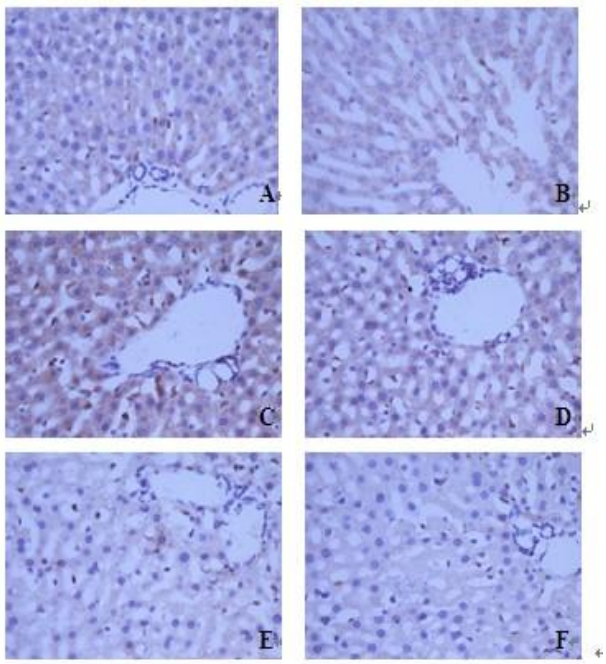

Figure 5: Dynamic positive expression of HO-1 in liver tissue of rat models The positive staining of HO-1 was mainly in the liver cells of periportal. Compared with the sham-operated group, the positive staining of HO-1 was gradually increased after 2VO, and it was reached a peak at 7 d ($P < 0.01$). Then, it was becoming more and more weaker at 14 d, 21 d and 28 d after 2 VO (A: sham-operated group, B: 1 day after 2VO, C: 7 days after 2VO, D: 14 days after 2VO, E: 21 days after 2VO, F: 28 days after2VO).

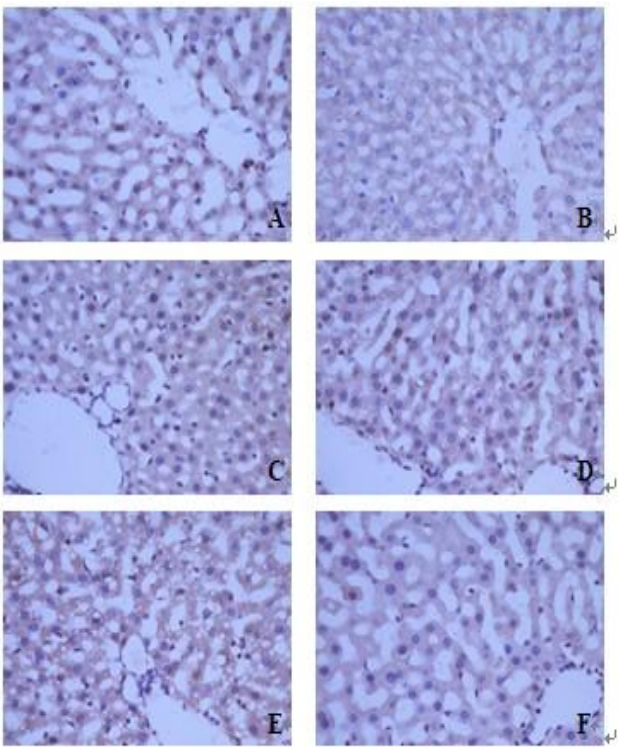

Figure 6: Positive expression of HO-2 in liver tissue of rat models The positive staining of HO-2 was mainly in the cytoplasm in the liver cells. Compared with the sham-operated group, in the 2VO groups, the positive staining of HO-2 was not significantly changed with the time extension ($P > 0.05$) (A: sham-operated group, B: 1 day after 2VO, C: 7 days after 2VO, D: 14 days after 2VO, E: 21 days after 2VO, F: 28 days after 2VO).

6. Discussion

As two main isoenzymes of HOs, HO-1 and HO-2 played important roles in mammal bodies. Both of them can catalyze heme to form Carbon Monoxide (CO), ferrous iron and biliverdin. The expression of HO-1, as one of the heat shock proteins, can be induced by multitudinous stimulants, including ischemia, hypoxia and another damage factors [19-22] discovered that HO-1 can be induced by hypoxia, and the mRNA expression of HO-1 was increased at different time points under severe hypoxic conditions accompanied by the up-regulation of heme oxygenase activity [23]. Some other researchers investigated that HO-1 over expression protects the liver against ischemia and reperfusion injury by modulating oxidative stress and pro inflammatory mediators, such as by modulating expressions of serum TNF-α, iNOS, and COX-2 protein and mRNA [24]. Our previous study also discovered that HO-1 was up-regulated after hypo perfusion in rat brain. All of these data displayed that HO-1 might be play a protective role in different tissues which injuryed by various stimuli. However there was an interesting study that renal HO-1 was induced via Nrf2 to protect the kidney from remote organ injury after hepatic ischemia-reperfusion in rats and mice [25]. And our study discovered that cerebral hypo perfusion can also induce hepatic HO-1 mRNA and protein expression. Both of them were up-regulated at different time points, and reached a peak at 7 days after 2VO.

On the contrary, HO-2 is constitutive and difficult to be induced, accounting for the great majority of HO activity. However, Han F et al demonstrated that the expression of HO-2 protein was increased in the sub-endocardial myocardium of ventricles under hypoxia [26]. HO-2 deficiency can cause endothelial cell activation [27], increase brain swelling and inflammation [28], and change mitochondrial signaling pathway [29] discovered that HO activity was decreased by using HO-2 siRNA, while it was increased by the administration of CoPP [29]. It suggested that the HO activity and the HO-2 expression were parallel confirmed that both of HO activity and HO-1 expression was enhanced by hypoxia [30] demonstrated that HO activity was at its peak on day 5, and had returned to baseline by day 8, accompanied by the expression trends of HO-1 after experimental intracerebral hemorrhage [31]. In our study, the changes of HO-2 mRNA and protein expression were not significant at different time points after cerebral hypo perfusion. And HO activity was increased at different time points and at its peak on day 7 after 2VO, which paralleled with the mRNA and protein expression of HO-1.

In summary, both of HO activity and HO-1 mRNA and protein expression were up-regulated in liver from remote organ injury after chronic cerebral hypo perfusion. While the mRNA and protein expression of HO-2 was not markedly at different time points in liver after 2VO. Thus increased hepatic HO-1 expression may protect liver from injury by up-regulated HO activity after chronic cerebral hypo perfusion. And activation of HO-1 might be a useful therapeutic approach for protection from hepatic dysfunction during brain injury.

7. Acknowledgement

This work was supported by the National Science Foundation of China (NSFC: 30973154 and NSFC 81100948), and by the Science Foundation of Chongqing (by the Science Foundation of Chongqing (CSTC, 2009BB5270).

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