行政院國家科學委員會專題研究計畫　成果報告

血基質氧化酵素在缺氧前置訓練的保護角色

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國科會91年度研究計畫成果報告

一、基本資料　　計畫編號：NSC91-2314-B002-348

計畫種類	新進人員研究計畫			
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計畫名稱	中文	血基質氧化酵素在缺氧前置訓練的保護角色		
	英文	The Protective Role of Heme Oxgenase in Ischemic Preconditioning		
研究性質	□基礎研究			

本研究計畫發表情形:	□ 論文已發表	V 投稿中	□ 學會發表	□ 未發表

計畫成果摘要:

關鍵詞: (英文)

Background: Hypoxic preconditioning (HP) confers cytoprotection against ischemia/reperfusion （I/R）injury, this effect is in part due to the induction of heme oxygenase-1. This experiment evaluates liver cell damage after I/R injury in HP rats.

Methods： HP rats were prepared by exposure (15hours day^{-1}) to an altitude chamber (5500m) for 2 weeks. Partial hepatic ischemia was produced in the left lobes for 45 minutes followed by 180 minutes of reperfusion. Zinc-protoporphyrin IX(ZnPP), a specific inhibitor of HO enzymatic activity, was subcutaneously injected 1 hour before the I/R injury in separate groups of sea-level （SL）control and HP rat. Serum alanine transaminase (ALT) levels, liver HO-1 mRNA and protein, and HO enzymatic activity were measured.

Results：Heme oxygenase-1 (HO-1) was induced in the livers of rats exposed to HP. The levels of HO-1 mRNA and protein were obviously overexpressed after two weeks of hypoxic preconditioning. HP diminished the elevation of serum ALT levels after I/R injury （83.7±4.9 U L^{-1}）when compared with SL controls （280.8±19.4 U L^{-1}） and HP+ ZnPP pre-treated groups（151.3±4.4 U L^{-1}）. The heme oxygenase activity in treated rats also correlated these results （237.9±19.8 pmol mg^{-1} protein hr^{-1} for the HP group, 164.3±12.7 pmol mg^{-1} protein hr^{-1} for the HP+ ZnPP, and 182.6±8.9 pmol mg^{-1} protein hr^{-1} for the SL controls.

Conclusions：Our results indicated that the induction of HO-1 in hypoxic

preconditioning played a protective role against hepatic I/R injury.

計畫成果

I. 簡介（Introduction）與背景說明

Altitude training, used by endurance athletes to improve sea-level performance, is an exercise under exposure of chronic hypoxia （1）. Physiological adaptation to chronic hypoxia includes the induction of erythropoietin and the increase of red cell mass with the resultant increase of oxygen-carrying capacity（1）. Cytoprotective effect of chronic hypoxia was demonstrated in the studies of cardiac preconditioning （2）. Exposure of the rat to a period of hypoxia increases the cardiac tolerance to subsequent ischemic insults. The mechanism of hypoxic preconditioning remains to be elucidated. Only limited studies including the involvement of K_{ATP} channel （2,3） were postulated.

Heme oxygenase belongs to the heat-shock protein families. It is the rate-limiting enzyme to oxidize heme to biliverdin and carbon monoxide （4）. Biliverdin is further metabolized to bilirubin that is a strong anti-oxidant（5）. Through the Guanylyl cyclase, the carbon monoxide works as an intracellular messenger like the nitric oxide does. The isoform Heme oxygenase-1 (HO-1) was found to be inducible in stress conditions including hypoxia （6）, heat shock （7）, free radicals （8）and so on. The induction is considered to be a cellular adaptive protection （9）. It was found that glutamine-induced heme oxygenase-1 protects small intestines from warm ischemic injury （10）. Also, it was demonstrated that HO-1 had a protective effect on liver injury in the cecum-ligated sepsis model（11）. However, whether HO-1 involved in the hypoxic preconditioning is not studied before. We conducted this experiment to elucidate the role of HO-1 in the protective effect of hypoxic preconditioning on ischemic liver insult.

II.關鍵材料及方法 (Subjects and Methods)

Animal Care and Preparation.

Female Wistar rats weighing 200-250 g were used. All animal experiments and animal care were performed in accordance with the “Guides for the Care and Use of Laboratory Animals” (published by National Academy Press, Washington DC, 1996).

Experimental protocol

Hypoxic preconditioning (HP) was induced by exposing female Wistar rats to an altitude chamber set at 5500m 15 hours per day for two weeks, as described previously （16）. The age-matched sea-level (SL) control rats were similarly treated and studied at the same time.

Western Blot Analysis.

For detection of HO-1 immunoreactive proteins, liver tissue was homogenized in ice-cold lysis buffer. The enhanced chemiluminescence Western blotting system was used for detection. Quantification of protein signals was performed using computer-assisted densitometry.

RT-PCR analysis.

The procedure was performed as reported （12）. Total RNA was extracted from freshly isolated liver cell fractions using a commercially available kit (Ultraspec RNA; Biotecx Laboratories, Houston, TX).

The hepatic Heme oxygenase (HO) acitivity

HO activity in liver cells was measured by the generation of bilirubin（13）. The HO activity was expressed as pmol h^{-1} mg^{-1} protein.

General Surgical Procedures.

Rats were anesthetized with sodium pentobarbital (35 mg/kg, i.p.). The left carotid artery was catheterized for continuous measurement of systemic blood pressure and blood withdraw.

Induction of Hepatic Ischemia-Reperfusion Injury and Rat Grouping.

The left hepatic artery was isolated and partial hepatic ischemia-reperfusion (I/R) injury is produced by placing an atraumatic microvascular clip across the origin of left hepatic artery for 45 min (ischemia period), and by removing the clip for 180 min (reperfusion period). Some rats were treated with intra-peritoneal injection of the HO-1 inhibitor zinc-protoporphyrin (ZnPP) (25ì mol Kg^{-1} body weight; Prophyrin products, Logan, UT) one hour before experiment. Four groups were assessed for data: 1) rats raised at sea-level (SL) subjected to partial hepatic ischemia-reperfusion injury (SL + I/R)；2) hypoxic preconditioning (HP) rats subjected to partial hepatic I/R injury (HP+ I/R)；3) rats raised at sea-level received ZnPP injection and then were

subjected to partial hepatic ischemia-reperfusion injury (SL+ZnPP+I/R)；4) HP rats received ZnPP injection and were then subjected to partial hepatic ischemia-reperfusion injury (HP+ZnPP+I/R). In each of the group, 6 rats were used for measurement. Serum samples and hepatic tissues were harvested at predetermined time intervals and prepared for assay.

Quantitation of IIR Injured Marker

We quantitated the expression of ALT in arterial blood (each 60 min) in each group (n=6) to ascertain the I/R injury.

Data Treatment.

Statistical analysis was performed using the Newman-Keuls test of ANOVA for multiple comparisons and Student's *t* test for paired comparisons between groups. A significance level of 5% was chosen.

III.重要之結果 (Results)

1.The effect of hypoxic preconditioning on hepatic HO-1 protein and mRNA expression.

A time-dependant increase of the expression of hepatic HO-1 protein in livers of rats experiencing different durations of HP was shown in Figure 1. Significant overexpression of HO-1 protein was observed after one week's preconditioning by hypoxia.

The RT-PCR was used in this study to detect the mRNA expression of HO-1 in liver cells isolated from hypoxia-preconditioned rats. Representative autoradiography is shown in Figure 2. After more than one week's exposure to hypoxia, HO-1 mRNA expression were substantially upregulated as compared to that of the control.

2. The effect of hypoxic preconditioning on hepatic Heme Oxygenase activity

In rats preconditioned to 2 weeks' of hypoxic environment, the mean heme oxygenase activity (237.9±19.8 pmol mg^{-1} protein hr^{-1}) was significantly higher than the other three groups (Figure 3). Pretreated with ZnPP, the mean HO activity in the HP-ZnPP group（164.3±12.7 pmol mg^{-1} protein hr^{-1}） was similar to those of control groups（182.6±8.9 pmol mg^{-1} protein hr^{-1} for the SL group and 142.7±3.7 pmol mg^{-1} protein hr^{-1} for the HP-ZnPP group）.

3. The effects of hypoxic preconditioning on liver cell injury experiencing I/R.

The basal serum levels of ALT of four groups were all within normal range (Figure 4). After 45 minutes of ischemia, the ALT levels of the two groups pre-treated with ZnPP were slightly higher than those of the control and HP

group, but were not statistically significant. After 180 minutes' reperfusion, marked elevation of ALT levels were noted in the SL+I/R（280.8±19.4 U L^{-1}）, SL+ ZnPP + I/R（349.6±10.6 U L^{-1}）and HP+ ZnPP+ I/R （151.3±4.4 U L^{-1}）groups, while the HP+ I/R group had the least elevation of ALT （83.7±4.9 U L^{-1}）. Our results showed that hypoxic preconditioning could diminish the plasma ALT elevation in I/R injury of liver, and could lessen the degree of liver injury. After inhibition of HO activity by ZnPP, the protective effect of hypoxic preconditioning was lessened, suggesting the role of heme oxygenase played in this protective effect.

VI.討論 (Discussion)與成果之貢獻

We reported that HO-1 overexpression induced by hypoxic preconditioning against ischemia-reperfusion injury of liver in a rat model. The main findings of this work are as follows: （a）Increase level of HO-1 mRNA and protein are observed one week following hypoxic preconditioning. The induction of HO-1 indicates that it may participate in the cellular response to hypoxia；（b）hypoxic preconditioning protects the liver from ischemia-reperfusion injury；（c）the protective effects induced by hypoxic preconditioning are reduced by inhibiting HO-1 enzyme activity with pretreated ZnPP, suggesting that the effects are mediated by HO-1.

It was first shown in 1958 that ischemic tolerance of the heart could be increased by long-term exposure of animals to intermittent high altitude hypoxia(hypoxic preconditioning) （14）. The cardiac protection by hypoxic preconditioning may persist longer after other hypoxia-induced adaptive responses, such as polycythemia and pulmonary hypertension （15）. The cytoprotective effects of hypoxic preconditioning are not purely adaptive. Hypoxic preconditioning, as well as ischemic preconditioning, could reduce IIR injury in heart（2, 22）and kidney（16）. In studies of ischemic preconditioning, receptor-mediated triggers including adenosine （17） and catecholamines （18）, post-receptor PKC signaling pathway （19） or end-effector such as ATP sensitive potassium channels（20） were found to be involved in the mechanism of protection. However, previous studies demonstrated that cardiac protection induced by hypoxic preconditioning does not involve the activation of adenosine receptors （21） or PKC pathway （22）, suggesting

a different signaling from well-known mechanisms of ischemic preconditioning. Recently, the mechanisms underlying hypoxic preconditioning are more addressed. Asemu et al （2） found that mitochodrial K_{ATP} channel play a role in the protection afforded by hypoxic preconditioning. Ladilov et al（22）found hypoxic preconditioning exerts PKC-independent protection and protein phosphatase 1 is a possible mediator. Sasaki et al proposed that angiogenesis triggered by hypoxic preconditioning enhance function reserve of ischemic heart （23）. Herein we find that HO-1 is an important mediator in hypoxic preconditioning.

Hypoxic stress is known to induce a nuclear factor, hypoxia-inducible factor-1 (HIF-1), which is required for hypoxic activation of gene transcriptions including erythropoeitin, inducible nitric oxide synthase and vascular endothelial growth factor （4）. In chronic hypoxia, HIF-1 has been demonstrated to regulate the HO-1 gene expression by binding to the hypoxia response elements on the enhancer sequences （4）. Our report was the first in vivo study to show the hepatic enhancement of HO-1 expression after chronic hypoxia.

The beneficial effects of HO-1 induction have been shown to confer protection against liver injury in a variety of experimental models, including sepsis （11） and liver ischemia-reperfusion （24）. Ischemia-reperfusion injury remains a major problem in clinical transplantation. Using a genetic approach, Ke et al （25） have shown HO-1induction significantly prolongs allogeneic liver graft survival. However, the *in vivo* studies of HO-1 induction in hypoxia against liver injury were seldom reported. We therefore employed the ischemia-reperfusion model of rats to distinguish the effect of HO-1 induced by hypoxic preconditioning. The present study demonstrates a direct protective role for HO-1 expression in the setting of hypoxic preconditioning.

The mechanisms by which HO-1 induce cytoprotection against I/R injury of liver are under investigation. It was shown that cells overexpressing HO-1 exhibit low levels of free iron because of the upregulation of ferritin and the extraction of iron into the extracellular space （26）. Cellular iron contributes to the formation of free radicals with resultant DNA, proteins and lipid damages （27）. The elimination of pro-oxidant iron from the cell is considered to be an important mechanism of HO-1 mediated protection against oxidative stress. Besides, CO, a byproduct of HO-1 mediated metabolism, has been demonstrated to confer protection against cellular injury. It was postulated that CO, like NO, modulates intrahepatic sinusoid tone and improves microcirculatory dysfunction（28）, leading to increased blood flow to liver.

Another byproduct of heme metabolism, bilirubin, which was known as a potent antioxidant（5）, has also been demonstrated to attenuate inflammatory response by preventing oxidant-induced microvascular leukocyte adhesion（29）.

In a clinical setting, hypoxic preconditioning is more applicable than ischemic preconditioning. People who do altitude training or mountain climbing in daily life are in fact leading a way of hypoxic preconditioning. However, there are several unsolved problems. How long does the effect of hypoxic preconditioning last? Is there other factors induced by hypoxic preconditioning also confer protection? As figure 4 showed, the protective effect of HP was only partially reversed by the inhibition of HO activity. Other mediators including the previously mentioned mitochondrial K_{ATP} channel opener, protein phosphatase-1, and even other endogenous vasodilators such as nitric oxide, may all participate in or interact with each other to provide the protection against ischemia-reperfusion damage. Further studies to elucidate the mechanism of hypoxic preconditioning are mandated.

In conclusion, we have shown that hypoxic preconditioning-induced

成果之貢獻

1. Over-expression of HO activity protects against warm I/R injury of liver in a rat model. Our results suggest that HO-1 play a role in the protective mechanism of hypoxic preconditioning.

2. This is the first *in vivo* study to document the protective role of HO-1 in hypoxic preconditioning.

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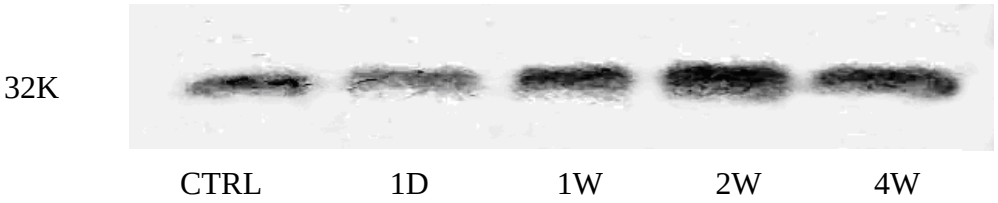
Figure 1（A）. Effects of hypoxic preconditioning on heme-oxygenase-1 (HO-1) expression in rat liver. Representative Western blots for HO-1 protein expression is shown. （B）.Quantity of HO-1 protein levels in livers obtained from rats exposed to different durations of hypoxic preconditioning. * p<0.05 vs. control group （CTRL=control; D= day; W= week; HP=hypoxia）

（A）



（B）.

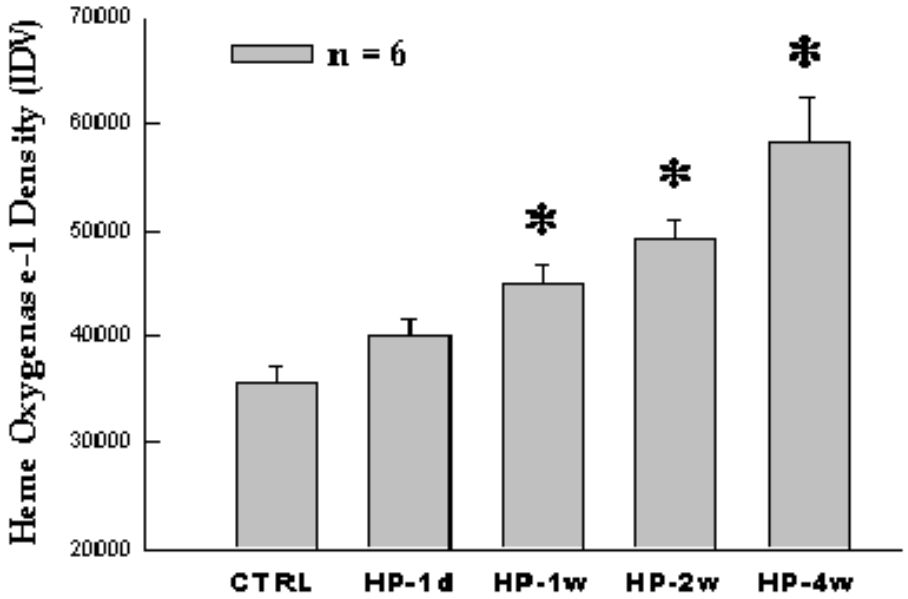


Figure 2. Expression of HO-1 mRNA in liver obtained from rats exposed to different durations of hypoxic preconditioning and sea level (SL) control. GADPH was used as internal controls. 1W= 1 week's hypoxic preconditioning; 2W= 2 weeks' hypoxic preconditioning

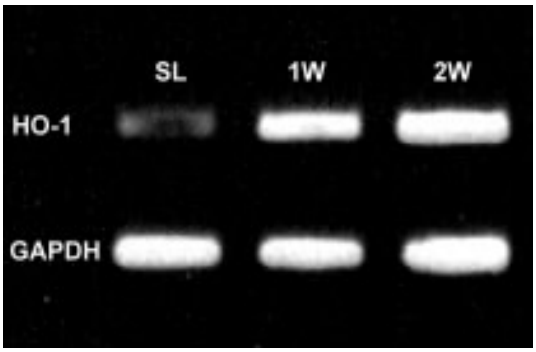


Figure 3. HO enzyme activity (pmol bilirubin mg^{-1} $hour^{-1}$) measured in liver homogenate before I/R injury. * $P<0.05$ as compared with other three groups. （SL= sea level control; HP= 2 weeks' hypoxic preconditioning; ZnPP= pre-treated with zinc-protoporphyrin ）

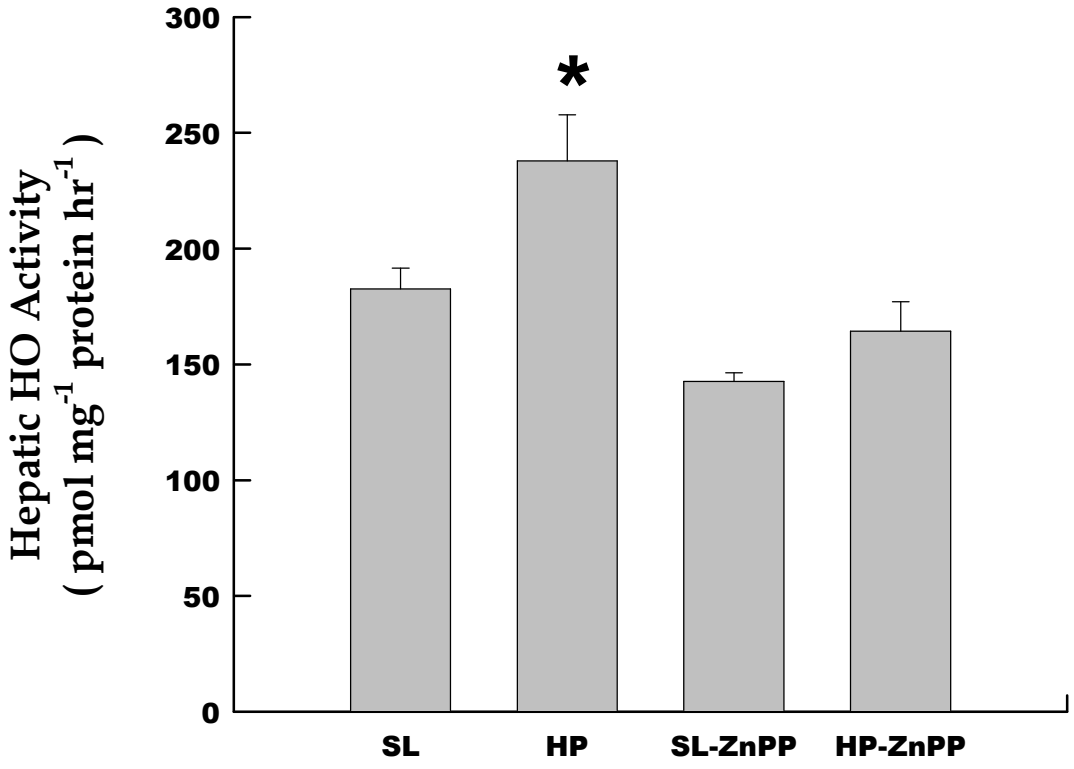


Figure 4. Effects of Hypoxic preconditioning on Serum ALT Level in Rats experiencing I/R injury. Addition of ZnPP lessened the HP-induced protection of the liver from I/R damage. The difference of serum GPT levels between ZnPP+HP v.s SL (P=0.102) and ZnPP+HP v.s ZnPP+SL (P=0.052) is not statistically significant. * P=0.0251, 0.006, and 0.007 for HP group vs. ZnPP+HP , SL, and ZnPP+SL groups separately.（SL= sea level control; HP= 2 weeks' hypoxic preconditioning; ZnPP= pre-treated with zinc-protoporphyrin ）