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Short Communication

Preconditioning effect mediated by heme oxygenase-1 (HO-1) is lung-protective in a rat model of limb ischemia and reperfusion

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The preconditioning with short limb ischemia has been shown to be protective to prevent tissue damage caused by ischemia. Numerous underlying factors have been suggested to be important in these processes, including heme oxygenase-1 (HO-1). This study investigated the potential protective mechanism mediated by HO-1 for lung protection in a rat model of limb ischemia and reperfusion. The results showed that HO-1 was upregulated by pre-conditioning treatment (transient ischemia), and was associated with lung protection, which could be reversed with HO-1 inhibitor administration. Moreover, the upstream regulator of HO-1, nuclear factor kappa beta (NF-kB) was increased with pre-conditioning treatment, suggesting the role of inflammatory signaling in protective mechanism of limb ischemia pre-conditioning.

Key words: Lung injury, limb ischemia, reperfusion, heme oxygenase-1 (HO-1), nuclear factor kappa beta (NF-kB), inflammation.

INTRODUCTION

Ischemia and reperfusion often lead to serious local tissue injuries. In recent years it has been shown that the preconditioning induced by limb ischemia was protective not only for the local organ - the limb, but also for remote organs, such as the lung. The heme oxygenase-1 (HO-1) is a part of the oxidative stress response system and plays important role in preventing cells and tissues against injury in many disease settings (Aztatzi-Santillan et al., 2010; Raval and Lee, 2010). However, whether and how HO-1 is involved in preconditioning is left uninvestigated. The present study was, therefore, designed to examine the function of HO-1 in remote organ protection with the limb ischemia preconditioning.

MATERIALS AND METHODS

Eighty male Sprague-Dawley (SD) rats (230 - 260g) were used for all the experiments. The day before the experiment, they were given only water without food. The rats were anesthetized with an intraperitoneal injection of 4% chloral hydrate (0.6 ml/kg). Anesthesia was maintained through the experiment with additional dose of intraperitoneal chloral hydrate (0.2 - 0.3 ml/kg). The left jugular vein was cannulated (catheter 24G, Belgium) for drug administration and continuous perfusion of physiological saline (8 ml/kg/h), heparin was administered intravenously (500 IU/kg) immediately after cannulation. The central temperature was maintained between 36 to 38°C by a heating lamp placed above the

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Table 1. The W/D ratio changes of lung tissue (mean ± SD; n = 8).

Group	W/D	Group	W/D
S2	3.8 ± 0.6#	S4	3.2 ± 0.1##
IR2	4.4 ± 0.7	IR4	4.4 ± 0.7
IP2	3.5 ± 0.6#	IP4	2.7 ± 0.3##
HI2	4.2 ± 0.7	HI4	4.7 ± 0.6Δ

*In comparison to IR2, # suggests for P<0.05; in comparison to IR4, ## P<0.05; in comparison to IP4, ΔP<0.05.

animal. After the equilibration period of 30 min, the bilateral femoral aorta were dissected and clamped by noninvasive microarterial clip (HC-X018, HC-X023 Cheng-He Microsurgical instruments factory, Shanghai, China). The study was approved by the Wenzhou Medical Institution Animal Care and Use Committee, and all animals were treated according to the Zhejiang regulation for animal experimentation.

The animals were divided into 8 groups: First and second groups: (1) S2 and S4 groups: sham operated, remained anesthetized for the whole duration of the study without undergoing any surgical intervention but the bilateral femoral aorta were dissected. S2 (n=10) for 4 h, S4 (n=10) for 6 h as the control for following groups; Third and fourth groups: (2) I2R and I4R groups: 1 ml saline was injected intravenously after 30 min underwent 2 h of ischemia followed by 2 h (n=10) and 4 h (n=10) of reperfusion; Fifth and sixth groups: (3) IP2R and IP4R groups (n=10 each): the bilateral femoral artery were exposed to three cycles of ischemic preconditioning (5 min of ischemia followed by 5 min of reperfusion), then treated as I2R and I4R groups; Seventh and eighth groups: (4) HI2 and HI4 groups (n=10 each): Zinc protoporphyrin IX, a heme oxygenase-1 inhibitor, was injected i.p. (10 mg/kg) 1 day in prior to the surgery, then treated as IP2R and IP4R groups.

At the end of the experiment the rats were sacrificed by exsanguination. A median sternotomy was performed and the left atrium was excised allowing free pulmonary drainage. The pulmonary circulation was slowly injected with 20 ml of physiological saline at 4℃ into the right ventricle. The left lung was harvested and stored at -80℃ and used for determination of HO-1expression (Western blotting). The right superior and median lobes of the lungs were immersed in 10% formalin for 24 h for histology, to understand the morphological expression of HO-1. The inferior lobe was used for the calculation of Wet/Dry ratio.

For Western blot, the lung was homogenized in 1:10 (W/V) ice-cold homogenization buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 50 mM NaF, 1%(W/V) Triton X-100,0.1%(W/V) SDS, 1 mM deoxycholate, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride (PMSF). The homogenates were centrifuged at 12,000 ×g for 20 min at 4℃, then the supernatants were collected and protein concentrations were determined using a protein concentration determination kit (P0012s, Beyotime Biotechnology Co, Ltd, Shanghai, China). Samples were stored at -80℃ until use. An aliquot of homogenate was suspended in sodium dodecyl sulphate (SDS) sample buffer (P0015, Beyotime, Shanghai, China). Equal amounts of protein per lane (50 µg and 15µL) were placed on the electrophoresis apparatus in a 10% SDS-polyacrylamide gel. Gels were transferred onto a polyvinylidene fluoride (PVDF) membrane (FFP30, Beyotime, Shanghai, China) by electroblotting. The membranes were blocked by incubation in Tris buffered saline with 0.1% Tween-20 (TBST) and 5% fat-free milk, and then incubated overnight at 4℃ with primary antibodies (1:2000 Abcam,abl3243, Shanghai, China) in TBST containing 5% fat-free milk. Membranes were then washed and incubated with horseradish peroxidase conjugated secondary antibodies (1:10000) in TBST containing 5% fat-free milk for 1.5 h and then developed using BeyoECL Plus color substrate (Byotime, Shanghai, China). The intensity of the bands on the film was scanned and analysed with Quantity One 4.4.0 image analyzer (Bio-Rad Company, USA).

Statistical analysis

The data were analyzed with SPSS 11.0 software (Chicago, USA). Data were expressed as mean ± standard deviation (SD) or as median (lowest, highest). Significance among the four groups were determined by one-way ANOVA or Kruskal-Wallis H test, intergroup comparisons between groups were determined by least significant difference (LSD) test or Dunnett T3 test. Individual comparison between groups was made using the T-test or Mann-Whitney test. P<0.05 was considered statistically significant.

RESULTS

W/D changes of lung tissue

The results suggested that the W/D ratio of lung tissue significantly increased after ischemia and reperfusion injury, both in IR2 and IR4 groups (P<0.05). Preconditioning could prevent the increase, while HO-1 inhibitor blocked the effects of pre-conditioning treatment (P<0.05) (Table 1).

The HO-1 protein expression levels in rat's lung tissue

HO-1 protein expression was found in epithelial cells, endothelial cells, and the macrophages. The Western blot results reflected the changes of HO-1 protein in different groups: IR could significantly upregulate the expression of HO-1, which was further enhanced in IP groups, and these changes were blocked with HO-1 inhibitor treatment (Table 2).

The expression of NF-κB in rat's lung tissue

We further determined the expression level of NF-κB in rat lung tissue, as shown in Table 3. IR2 and IR4 group both showed increase in comparison to S2 and S4 groups; while the IP4 decreased the f-level of NF-κB.

DISCUSSION

The present study proved the protective mechanism of HO-1 underlying the preconditioning-mediated protection of remote organ in a rat model of limb ischemia and reperfusion, which could be blocked by HO-1 inhibitor

Table 2. Expression of HO-1 in rat's lung tissue.

Groups	Mean ± SD	Groups	Mean ± SD
S2	6.3 ± 1.9	S4	13.2 ± 4.2
IR2	12.5 ± 4.3 $^{\#}$	IR4	28.2 ± 1.1$^{\#}$
IP2	35.4 ± 1.5$^{\#*}$	IP4	99.6 ± 2.3$^{\#*}$
HI2	5.8 ± 1.3$^{\Delta}$	HI4	12.0 ± 3.6$^{\Delta}$

In comparison to S2 and S4, $^{\#}$suggest for $P<0.05$; in comparison to IR2 and IR4, *suggests for $P<0.05$; in comparison to IP2 and IP4, $^{\Delta}$suggest for $P<0.05$.

Table 3. The expression of NF-κB in rat's lung tissue.

Groups	Median (5% 95%)
S2	0.278 (0.205 0.601)
IR2	0.529 (0.067 0.148) #
IP2	0.416 (0.307 1.164) #*
HI2	0.498(0.265 1.037) #
S4	0.532 (0.285 0.778)
IR4	1.131 (0.775 1.488)#
IP4	0.662 (0.453 0.871)#*
HI4	0.698 (0.560 0.934)#*

$^{\#}$ In comparison to S2, P<0.05, *in comparison to S2, P<0.01. $^{\#}$ In comparison to S4, P<0.05, *in comparison to IR4, P<0.05.

zinc protoporphyrin IX. The HO-1 expression might be regulated by its upstream factor, NF-kB, suggesting for a potential signaling pathway controlled by inflammatory factors. HO-1 was induced by oxidative stressful stimuli, and could be protective in many cases including ischemia, oxidation and immune rejection in transplantation (Aztatzi-Santillanet al., 2010; Immenschuh et al., 2010; Li et al., 2010; Ollinger and Pratschke, 2010; Raval and Lee, 2010). The HO-1 deficiency leads to enhanced injury upon oxidative stress, as well as other inflammatory events (Koizumi, 2007).

The increased level of HO-1 expression was considered as reactive protection response to tissue injury, and with pre-conditioning the HO-1 expression further increased in several hours, contributing to the lung protection in the present case. The level of HO-1 was also closely related to the inflammation level (Paine, et al., 2010; Vijayan, et al., 2010), and many pro-inflammatory factors such as NF-kB could regulate the expression of HO-1 (Paine et al., 2010). Our study showed that this is also the case in pre-conditioning treatment.

In conclusion, the present study proved the protective effects of HO-1 for lung in limb ischemia and reperfusion injuries, which could be maximized with pre-conditioning manipulation. In the future, it will be important to further characterize the upstream signaling pathways other than NF-kB.

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