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Original articles

Protective effect of ischemic postconditioning on lung ischemia-reperfusion injury in rats and the role of heme oxygenase-1

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Objective: To investigate the effect of ischemic postconditioning (IPO) on acute lung ischemia-reperfusion (I/R) injury and the protein expression of haeme oxygenase-1 (HO-1), a cytoprotective defense against oxidative injury.

Methods: After being anesthetized with chloralhydrate, forty-eight healthy SD rats were randomly divided into 6 groups (8 in each): sham operation group (S group); I/R group: left lung hilum was clamped for 40 minutes followed by 105 minutes of reperfusion; IPO group: left lung hilum was clamped for 40 minutes and postconditioned by 3 cycles of 30 seconds of reperfusion and 30 seconds of reocclusion; Hemin (HM)+ I/R group: hemin, an inducer of HO-1 was injected intraperitoneally at 40 μmol·kg^{-1}·day^{-1} for two consecutive days prior to 40 minutes clamping of left lung hilum; ZnPPIX+IPO group: zinc protoporphyrin IX, an inhibitor of HO-1 was injected intraperitoneally at 20 mg·kg^{-1} 24 hours prior to 40 minutes clamping of left lung hilum; and HM+S group: HM was administered as in the HM+I/R group without inducing lung I/R. Arterial partial pressure of oxygen (PaO_2) and malondialdehyde (MDA) content in serum were assessed. The left lung was removed for determination of wet/dry lung weight ratio and expression of HO-1 protein by immuno-histochemical technique and for light microscopic examination.

Results: The PaO_2 was significantly lower in all the experimental groups compared with sham group (90 mm Hg ±11 mm Hg). However, the values of PaO_2 in IPO (81 mm Hg ±7 mm Hg) and HM+I/R (80 mm Hg±9 mm Hg) were higher than that in I/R (63 mm Hg±9 mm Hg) and ZnPPIX+IPO (65 mm Hg±8 mm Hg) groups (P<0.01). The protein expression of HO-1 in lung tissue was significantly increased in I/R group compared with S group (P<0.01). While the HO-1 protein expression was higher in IPO and HM+I/R groups as compared with I/R group (P<0.05, P<0.01). The lung wet/dry (W/D) weight ratio and MDA content in serum were significantly increased in I/R group as compared with S or HM+S groups (P<0.01), accompanied by severe lung tissue histological damage, which was attenuated either by IPO or by HM pretreatment (P<0.01, IPO or HM+I/R *vs*. I/R). The protective effect of IPO was abolished by ZnPPIX.

Conclusion: Ischemic postconditioning can attenuate the lung ischemia-reperfusion injury through upregulating the protein expression of HO-1 that leads to reduced post-ischemic oxidative damage.

Key words: ***Ischemic postconditioning; Reperfusion injury; Lung; Heme oxygenase-1; Malondialdehyde***

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Ischemia-reperfusion injury (IRI) of the lung is one of the common pathophysiological phenomena, encountered in clinical cases of trauma, shock, cardiopulmonary bypass, lung transplant, bronchial sleeve resection, etc. Ischemic postconditioning (IPO) is the process whereby the blood flow to an ischemic part is resumed by immediate single or multiple brief reperfusion/re-occlusion cycles, thus making that organ more resistant to the previous long standing ischemia.[1] Heme oxygenase (HO) is a rate-limiting enzyme in the degradation of heme and under condition of stress. HO especially HO-1 has a protective effect, which limits ischemia-reperfusion injury to organs.[2] Our earlier study has already confirmed the protective effect of HO-1 protein against endotoxin-induced acute lung

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injury.[3] Other experiments have shown that IPO can decrease reperfusion injury to heart muscle, liver[4] and kidney[5].But the literature about the protective effect of IPO against lung IRI is still scarce. The participation of HO-1 in the protective mechanism of IPO is still unknown. This research is mainly concerned about the expression of HO-1 protein, the effect and mechanism of IPO during ischemia-reperfusion injury of the lung.

METHODS

Reagents and instruments

Hemin and zinc protoporphyrin IX were purchased from Sigma Company (USA), and HO-1 polyclonal antibody was from Boster Biological Technology Ltd (Wuhan,China), MDA detection kit from Nanjing Jiancheng Shengwu Company Limited (Nanjing, China), DW-2000 animal mechanical ventilator from Shanghai Jiapeng Technical Company (Shanghai, China), I-STAT handheld blood gas analyzer from Hong Kong Lixin Yiqi Company Limited (Hong Kong, China), Olympus BX50 microphotographic system and HPIAS-2000 model image analysis software from Wuhan Qianli Technical Imaging Company Limited (Wuhan, China).

Animal grouping and model preparation

Sprague-Dawley (SD) rats were provided by Wuhan University Medical College Animal Lab Centre. Forty-eight specific-pathogen-free (SPF) rats, of either sex with body weight of 210 g±20 g, were randomly assigned to 6 groups (8 in each): sham operation group (S group), ischemia/reperfusion group (I/R group), ischemic postconditioning group (IPO group), hemin+ischemia/reperfusion group (HM+I/R group), zinc protoporphyrin IX+ischemic postconditioning group (ZnPPIX+IPO group) and hemin + sham operation group (HM+S group). Before the experiment, the rats were abstained from food for 12 hours but free to drink water at any time. Anesthesia was given by injection of 7% chloral hydrate 5 ml/kg intraperitoneally. Tracheotomy and intubation were performed, and the animals were ventilated with room air *via* the ventilator. The left femoral vein was catheterized and 3:1 crystalloid to colloidal fluid mixture was infused intravenously. The right femoral artery was catheterized and connected to a monitoring unit to continuously monitor mean arterial pressure (MAP) and collect blood samples. Thoracotomy was performed at the level of the 5th intercostal space, exposing the hilum of the left lung. A dose of 500 U/kg of heparin was given intravenously for anticoagulation. After 5 minutes, at the end of inspiratory phase, the hilum of the left lung was clamped using a non-traumatic clamp (without collapsing the lobes).

Rats in S group were allowed to a continuous perfusion for 150 minutes. I/R group rats were kept ischemic for 40 minutes by clamping the left hilum and reperfused for 105 minutes. IPO group rats were maintained ischemia for 40 minutes, then postconditioning was performed by three cycles of 30-second reperfusion and 30-second ischemia, and finally reperfused for 102 minutes. HM+I/R group rats was intraperitoneally injected with hemin at a rate of 40 μmol·kg^{-1}·day^{-1} for 2 consecutive days prior to the experiment and then the same procedure was performed as group I/R. ZnPPIX group+IPO group rats were intraperitoneally injected with zinc protoporphyrin IX (20 mg·kg^{-1}·day^{-1}) 24 hours prior to the experiment and the rest of the procedures were the same as IPO group. HM+S group rats were handled preoperatively as HM+I/R group and intraoperatively as S group. The rats that did not have any preoperative intraperitoneal injection with ZnPPIX or HM, were injected with an identical dose of normal saline.

Measurement

Blood samples were drawn from the femoral artery to determine PaO_2 level at 20 minutes (T_1) after mechanical ventilation and at the end of the experiment (T_2). Immediately after the experiment, 2 ml of blood was collected and centrifuged for 10 minutes at a temperature of 4°C and rotational speed of 4000 r/min. Then the serum was extracted and stored at -70°C. According to the instruction of the MDA kit, thiobarbituric acid method was used to determine the concentration of malondialdehyde (MDA) level. Part of the left lung was dissected and dried at a constant temperature of 80°C for 24 hours to obtain a dehydrated consistency. The ratio of wet weight to dry weight (W/D) was calculated. A section of 0.5 cm×0.5 cm in size was dissected from the left lung and fixed in 10% formalin for 24 hours. It was dehydrated in gradient alcohol and then in xylene. The sample was embedded in paraffin and then slices of 4 μm thick were made on slides. The slices were stained by SABC method. For the determination of HO-1, at a magnification of 400×, the buffy granules were set as a positive unit product of HO-1 protein. Eight visual fields (not overlapping with each other) were randomly selected from each slide. A semiquantitative analysis

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of expression of HO-1 was performed by measuring the positive granule average optical density value (OD value), using HPIAS-2000 high resolution color picture analysis system. The HE stained lung tissue slides were viewed under the light microscope to observe the pathological changes.

Statistical method

Measured data are expressed as mean ± standard deviation. Intergroup comparison analysis was done using a single factor variance and intragroup by Student's *t* test. All calculations were done using SPSS 14.0 statistics software. Variance for $P<0.05$ was set to have statistical significance.

RESULTS

Within each group, the body weight, gender and baseline of MAP did not have any statistical significance ($P>0.05$).

Effect of ischemic postconditioning on arterial PaO_2 level

The baseline of PaO_2 between each group did not have statistical difference ($P>0.05$), but there was a considerable decrease in the values of PaO_2 of all the groups ($P<0.01$) except HM+S group. When compared with S group, there were significantly decreased values of postoperative PaO_2 level in I/R, IPO, HM+I/R and ZnPPIX+IPO groups. I/R group had lower PaO_2 value than IPO, HM+I/R, HM+S groups ($P<0.05$ or 0.01, Table 1).

Table 1. Changes in arterial partial pressure of oxygen in different groups (n=8, mean±SD, mmHg)

Groups	Basic values (T_1)	Postoperative values (T_2)
S	92 ± 10	90 ± 11
I/R	91 ± 10	63 ± 9 *##
IPO	92 ± 13	81 ± 7 *#☆
HM+I/R	91 ± 9	79 ± 10 *# ☆
ZnPPIX+IPO	89 ± 12	65 ± 8 *##
HM+S	89 ± 12	89 ± 10☆

*$P<0.01$ compared with the basic value within groups; # $P<0.05$, ## $P<0.01$ compared with S group; ☆ $P<0.01$ compared with I/R group.

Effect on lung tissue W/D, serum MDA level and expression of HO-1 protein

I/R, IPO, HM+I/R and ZnppIX+IPO groups showed elevated values of lung tissue W/D when compared with S group ($P<0.05$ or 0.01); as for HM+S group, the differences of this parameter did not have any statistical significance ($P>0.05$). The lower values of lung tissue W/D ratio and MDA level were observed in I/R, IPO, HM+I/R and HM+S groups in comparison with I/R group ($P<0.01$). An obviously higher lung tissue expression of HO-1 protein was seen in I/R, IPO, HM+I/R, and HM+S groups than that in S group ($P<0.01$). As compared with I/R group, there was a stronger expression of HO-1 protein in IPO and HM+I/R groups and a weaker expression in HM+S group ($P<0.05$ or 0.01). (Table 2 and Fig. 1)

Table 2. Serum MDA, lung tissue W/D and expression of HO-1 protein in different groups (n=8, $\bar{x} \pm s$)

Groups	MDA (nmol/ml)	W/D	HO-1
S	3.77 ± 0.57	3.97 ± 0.43	0.111 ± 0.016
I/R	6.46 ± 0.73 **	5.97 ± 0.69 **	0.177 ± 0.015 *
IPO	4.62 ± 0.39 **##	4.66 ± 0.37 **#	0.194 ± 0.017 **#
HM+I/R	4.46 ± 0.42 *##	4.71 ± 0.45 **#	0.209 ± 0.013 **##
ZnPPIX+IPO	6.70 ± 0.46 **	5.69 ± 0.52 **	0.114 ± 0.018 #
HM+S	3.58 ± 0.50 ##	4.05 ± 0.50 ##	0.117 ± 0.019 #

*$P<0.05$, **$P<0.01$ compared with S group; #$P<0.05$, ##$P<0.01$ compared with I/R group.

Effect of ischemic postconditioning on the lung tissue morphology (Fig. 2)

S group: The structure of the alveoli was clear and alveolar wall was thin and intact. There was no hemorrhage or edema.

I/R group: Destruction of the alveolar structure was noted and partial alveolar compensating emphysema was seen. In the alveolar space there was exudate, thickening of the alveolar septum and massive inflammatory cell infiltration with hemorrhage.

IPO and HM+I/R groups: The extent of pathological injury in these two groups was milder than that in I/R group with a narrowing alveolar septum and mild inflammatory cell infiltration and hemorrhage.

ZnPPIX+IPO group: The pathological injury degree was similar to Group I/R.

HM+S group: The alveolar structure was normal.

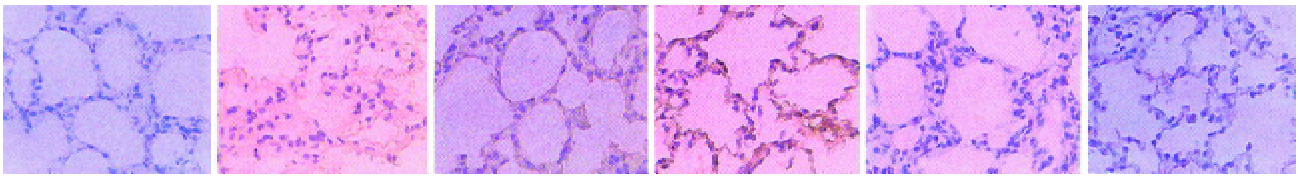

Fig. 1. Lung tissue expression of HO-1 protein in different groups (SABC, 400×).

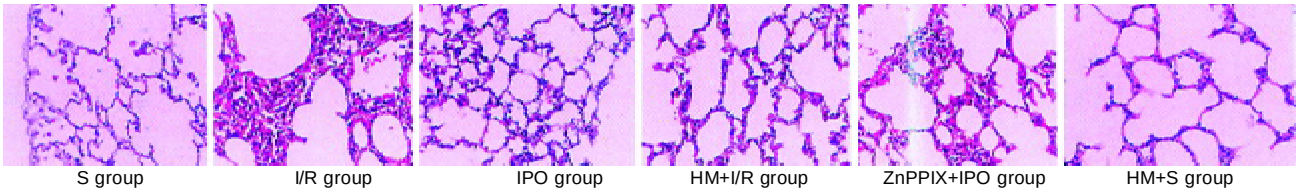

Fig. 2. Histopathology of lung tissue in different groups (HE, 200×).

DISCUSSION

A lung IRI model was established in rats by clamping and releasing the hilum of the left lung through a thoracotomy incision. In light of past studies[6] and many preliminary experiments, ischemic postconditioning was implemented by 3 cycles of 30-second reperfusion/30-second re-occlusion by unclamping and clamping the hilum of the left lung. The dosage and administration method of HM and ZnPPIX were derived from preliminary experiments and the literatre.[7,8] The results show that the lung with ischemia followed by an uninterrupted reperfusion, has an increase in lung tissue W/D and serum MDA level, and a decrease in arterial PaO_2 (P<0.01), thereby indicating that lung tissue ischemia/reperfusion will cause pulmonary edema, lipid peroxidation injury and a decrease in lung oxygenation capacity. Hence, the preparation of IRI model is successful.

Compared with I/R group, there is a net amelioration in lung tissue W/D, arterial PaO_2 and serum MDA levels (P<0.01) in IPO group, indicating that IPO has a certain protective effect against reperfusion injury in lung tissue. It is believed that lung IRI is an injury factor and a pathophysiological process, dynamically developed by the protective mechanism of the organism. And HO-1 acts as a type of stress protein that participates in the injury/anti-injury pathophysiological mechanism of the body and this issue is presently gaining a general interest among scholars. In some recent researches, it has been found that HO-1 has an important effect on lung IRI and most probably it participates in stress counteracting reaction in cells.[9] Under the condition of I/R of the lung, superoxide anions, hydrogen peroxide, hydroxyl radicals, free hemoglobin and heme will induce oxidative stress injury and HO-1/CO pathway activation in the lung. HO-1 catalyses the products of biliverdin, ferrous ions and CO through diverse regulatory effects and complex signal transduction pathways, [10] thus maintain a stable lung homeostasis and reduce tissue injury. There are more and more evidences that CO and bilirubin have a protective role in lung injury and transplantation. From our results, the expression of HO-1 is enhanced in I/R group, suggesting that I/R can induce lung injury and at the same time it triggers the protective mechanism involving HO-1.[10]

Moreover, the results suggest that the administration of a specific HO-1 inducer, hemin, for 2 consecutive days prior to IRI will lead to an increase of lung tissue HO-1 protein expression, and a decrease of W/D and serum MDA. PaO_2 is close to the normal level. Thus it is evident that the induced expression of HO-1 protein may decrease oxygen radical assault to lung parenchyma. Pretreatment by an HO-1 inhibitor, ZnPPIX did not show significant difference between ZnPPIX+IPO group and I/R group (P>0.05) in spite of using IPO before reperfusion. Therefore, IPO fails to show protective effect when HO-1 protein expression is suppressed. In the lung, the protective effect of IPO against IRI probably involves the induction of high expression of HO-1 protein. Some researches have confirmed that the activation of HO-1 limits the production of NO and the synthesis of peroxynitrite in the process of ischemia. High expression of HO-1 provides protective effect against ischemia/reperfusion injury.[11] Stimuli triggering HO-1 gene activation has a common feature, e.g. to initiate

oxidative stress (which destroys cellular redox equilibrium). In other words, the expression of HO-1 protein will not be elevated if there is no oxidative stress injury. Hemin does not have effect on the organism at normal condition even if hemin is administered as a premedication, which explains why HM+S group has a weaker expression of HO-1 than I/R group (P<0.05).

Based on past and present researches, we can assume that the protective effect of IPO against IRI of the lung is probably related to the following factors:(1) brief repetitive intermittent reperfusion and re-occlusion limits regional blood flow, decreases the production of oxygen radical in the reperfused area and stimulates the release of intracellular antioxidase and free radical scavengers, thus attenuates the injury due to lipid peroxidation;(2) IPO is given as a slow and dose-gradually increased way, which may decrease vascular distention and exosmosis due to the sudden increase in reperfusion pressure, thereby reduce lung tissue injury and edema;(3) IPO, by inducing HO-1, indirectly attenuates lung tissue injury, but the mechanism is to be clarified.

In summary, early transient ischemic postconditioning can attenuate IRI of the lung and this process involves the expression of HO-1 protein and inhibition of lipid peroxidation.

REFERENCES

1. Zhao ZQ, Corvera JS, Halkos ME, et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 2003;285(2): H579-H588.

2. Jia XM, Zhou ZX, Huang JJ, et al. Protective effects of the induction of heme oxygenase-1 on ischemia reperfusion lung injury: in vivo experiment with rats. Natl Med J China 2007;87(17): 1211-1213.

3. Xia ZY, Chen C, Wang XY. Effect of radix paeoniae rubra on expression of heme oxygenase and inducible nitric oxide synthesis in lipopolysaccharide-induced acute lung injury in rats. Chin J Trauma 2005 ;21(9) :675-678.

4. Wu BQ, Chu WW, He GY,et al. The protective role of ischemic postconditioning against ischemia-reperfusion injury in rat liver**.** J Kunming Med Coll 2005;26(3): 64-67.

5. Tang TL, Lu YP, Zhou L. Model construction of ischemic postconditioning on renal warm ischemia-reperfusion injury in rat. West Chin Med J 2006;21(3):540-542.

6. Li WL, Liu XY, Ke W, et al. Effects of ischemic postconditioning on renal ischemia-reperfusion injury in rats. Chin J Anesthesiol 2007; 27(11): 1045-1047

7. Christou H, Morita T, Hsieh CM, et al. Prevention of hypoxia-induced pulmonary hypertension by enhancement of endogenous heme oxygenase-1 in the rat. Circ Res 2000; 86(12): 1224-1229.

8. Zhou JL, Zhu XG, Zhang GS, et al. Protective effect of hemoglobin-induced heme oxygenase-1 on injured lungs caused by limb ischemia-reperfusion in rats. Chin J Traumatol 2002; 5(2): 86-91.

9. Yachie A, Toma T, Mizuno K, et al. Heme oxygenase-1 production by peripheral blood monocytes during acute inflammatory illnesses of children. Exp Biol Med 2003;228(5): 550-556.

10. Ryter SW, Morse D, Choi AM, et al. Carbon monoxide and bilirubin: potential therapies for pulmonary/vascular injury and disease. Am J Respir Cell Biol 2007; 36(2):175-182.

11. Salom MG, Ceron SN, Rodriguez F, et al. Heme oxygenase-1 induction improves ischemic renal failure: role of nitric oxide and peroxynitrite. Am J Physiol Heart Circ Physiol 2007; 293(6): H3542-H3549.

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