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# Ischaemic post-conditioning protects lung from ischaemia–reperfusion injury by up-regulation of haeme oxygenase-1☆

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ABSTRACT

*Objective:* The emergence of ischaemic post-conditioning (IPO) provides a potential method for experimentally and clinically attenuating various types of organ injuries. There has been little work, however, examining its effects in the setting of lung ischaemia reperfusion (IR). Stress protein, haeme oxygenase-1 (HO-1), has been found to exert a potent, protective role in a variety of lung injury models. In this study, we hypothesised that the induction of HO-1 by IPO plays a protective role against the deleterious effects of IR in the lung.

*Methods:* Anaesthetised and mechanically ventilated adult Sprague–Dawley rats were randomly assigned to one of the following groups ($n = 8$ each): the sham-operated control group, the IR group (40 min of left-lung ischaemia and 105 min of reperfusion), the IPO group (three successive cycles of 30-s reperfusion per 30-s occlusion before restoring full perfusion) and the ZnPPIX + IPO group (ZnPPIX, an inhibitor of HO-1, was injected intra-peritoneally at 20 mg $kg^{-1}$ 24 h prior to the experiment and the rest of the procedures were similar to that of the IPO group). Lung injury was assessed by arterial blood gas analysis, wet-to-dry weight ratio and tissue histological changes. The extent of lipid peroxidation was determined by measuring plasma levels of malondialdehyde (MDA) production. Expression of HO-1 was determined by immunohistochemistry.

*Results:* Lung IR resulted in a significant reduction of $PaO_2$ (data in IR, $P < 0.05$ vs. data in sham) and increase of lung wet-to-dry weight ratio, accompanied with increased MDA production and severe lung pathological morphological changes as well as a compensatory increase in HO-1 protein expression, as compared with sham (All $P < 0.05$). IPO markedly attenuated all the above pathological changes seen in the IR group and further increased HO-1 expression. Treatment with ZnPPIX abolished all the protective effects of post-conditioning.

*Conclusion:* It may be concluded that IPO protects IR-induced lung injury via induction of HO-1.

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Pulmonary ischaemia–reperfusion (IR) injury occurs after various clinical procedures, including lung transplantation, cardiopulmonary bypass, pulmonary thrombo-endarterectomy and trauma.[3] IR injury of the lung causes significant morbidity and mortality and is characterised by neutrophil extravasation, interstitial oedema, disruption of epithelial integrity and leakage of protein into the alveolar space that are associated with severe alterations in gas exchange.[30] In the past several decades, extensive studies have demonstrated beneficial effects of ischaemic and pharmacological pre-conditioning in reducing the extent of lung injury.[10,31,37] However, the clinical applicability of pre-conditioning has been limited in condition, that is, only when the occurrence of ischaemic event is predictable.

Recent studies of the heart have demonstrated that brief intermittent cycles of ischaemia alternating with reperfusion applied after the prolonged ischaemic event attenuated myocardial injury.[13,41] The novel approach for myocardial protection has been termed 'ischaemic post-conditioning' (IPO). Subsequently, beneficial effects of IPO were shown in a wide range of organs, including the heart, brain, spinal cord, liver, kidney and skeletal muscle.[11,19,28,34,40] Despite the emergence of post-conditioning as a potential alternative method for experimentally and clinically attenuating various types of organ injuries, it remains unknown whether post-conditioning can confer protective effects against IR injury in the lung.

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Haeme oxygenase (HO) belongs to the heat-shock protein family. It is the rate-limiting enzyme that catalyses the NADPH, $O_2$ and cytochrome P450 reductase-dependent oxidation of haeme to carbon monoxide (CO), iron and billiverdin.[5] To date, three isoforms of HO have been identified (HO-1, HO-2 and HO-3). HO-1 is a stress-responsive protein induced by various oxidative agents; HO-2 and HO-3 genes are constitutively expressed.[4] Accumulating data have demonstrated a cytoprotective role of HO-1 in various *in vivo* and *in vitro* pulmonary disease models, including IR injury.[25,38] In this study, we hypothesised that IPO may confer protection against lung IR injury and that induction of HO-1 expression may play an essential role in post-conditioning-mediated lung protection.

## Materials and methods

### *Animals*

The experimental procedures and protocols used in this investigation were approved by the Animal Use Committee at Wuhan University. Specific pathogen-free Sprague–Dawley (SD) rats of either sex, weighing between 190 and 230 g, were housed under constant temperature (23 ± 1 °C) with 12-h light/dark cycles. All rats were fed with water and rodent chows *ad libitum*.

### *Surgical procedure and experimental protocol*

The animals were anaesthetised with 7% chloral hydrate (5 ml $kg^{-1}$, i.p.). A 14-gauge angiocatheter was inserted into the trachea through a midline neck incision. The animals were then connected to a volume-controlled ventilator (DW-2000, Jiapeng Keji, Shanghai, China) with room air at a breath rate of 40 $min^{-1}$, a tidal volume of 12 ml $kg^{-1}$ and a positive end-expiratory pressure of 2 cm $H_2O$. The left femoral vein was catheterised and 3:1 crystalloid to colloidal fluid mixture was infused intravenously. The right femoral artery was catheterised for continuous monitoring of mean arterial pressure (MAP) and for blood sampling. A heating pad was applied during anaesthesia in order to keep the body temperature between 36.5 °C and 37.5 °C.

The animals were randomly assigned to one of the four groups. Under aseptic conditions, an *in situ* unilateral lung warm ischaemia model was used. In brief, a left anterolateral thoracotomy in the fifth intercostal space was made. The left lung was mobilised, the pulmonary hilum was dissected and perivascular and peribronchial tissues were removed. Then, all animals received 500 U $kg^{-1}$ of heparin intravenously in saline (total volume 500 μl). In group 1 (sham), animals underwent a sham thoracotomy and hilar dissection, but the lungs were not rendered ischaemic. In group 2 (IR), 5 min after heparin administration, the left pulmonary artery, bronchus and pulmonary vein were occluded with a non-crushing microvascular clamp, maintaining the lung in a partially inflated state. Lungs were kept moist with periodic applications of warm, sterile saline, and the incision was covered to minimise evaporative losses. The period of ischaemia was held constant at 40 min, after which the clamp was removed and the lung re-perfused for up to 105 min. In group 3 (IPO), post-conditioning was performed by three successive cycles of 30-s reperfusion per 30-s occlusion, starting immediately after release of the index ischaemia. In group 4 (zinc protoporphyrin IX + IPO group, ZnPPIX + IPO), rats were intra-peritoneally injected with zinc protoporphyrin IX (Sigma, USA), a specific HO-1 inhibitor, at a dose of 20 mg $kg^{-1}$ 24 h prior to the experiment and the rest of the procedures were similar to that of the IPO group. The rats, which were not administered with any preoperative treatment of ZnPPIX, were injected with an isovolume of normal saline.

### *Arterial blood gas analysis*

Arterial blood sample for blood gas analysis were taken at 20 min of mechanical ventilation (baseline) and 105 min after reperfusion (postoperative). Arterial blood specimens were analysed for $PaO_2$ and $PaCO_2$ using blood gas analyser.

### *Lung wet-to-dry weight ratio*

At the end of the experiments, the left lower lobe of the lung was dissected and dried at a constant temperature of 80 °C for 24 h to obtain a dehydrated consistency. The ratio of wet weight to dry weight (W/D) was calculated to assess tissue oedema, as described previously.[36]

### *Lung histopathological analysis*

At the end of the experiments, the left upper lobe of lung was fixed in 10% buffered formalin and 4-μm sections were prepared from paraffin-embedded tissues. The level of histological tissue injury was assessed by haematoxylin–eosin (H&E) staining using light microscopy. For each animal, three random tissue sections (eight fields per section) were examined. The severity of lung injury was graded by an investigator who was initially blinded to research groups, using a four-point scale according to combined assessments of amount of alveolar congestion, haemorrhage, infiltration or aggregation of neutrophils in the airspace or vessel wall, and thickness of alveolar wall/hyaline membrane formation.[29] The following criteria were considered: 0 = no damage, l = mild damage, 2 = moderate damage and 3 = severe damage.

### *Immunohistochemical staining for HO-1*

The expression of HO-1 was determined by immunohistochemistry. After deparaffinisation, endogenous peroxidase was quenched with 0.3% $H_2O_2$ in 60% methanol for 30 min. The sections were permeabilised with 0.1% Triton X-100 in phosphate-buffered saline for 20 min. Nonspecific absorption was minimised by incubating the section in 2% normal goat serum in phosphate-buffered saline for 20 min. The sections were then incubated overnight with 1:500 dilution of primary rabbit anti-HO-1 polyclonal antibody (Boster Bio-Tech, Wuhan, China), followed by biotin-conjugated secondary antibody at 1:1000 dilutions. Finally, the sections were incubated with avidin–biotin complex kit (Boster Bio-Tech, Wuhan, China) and detected by using a diaminobenzidine (DAB) reagent (Boster Bio-Tech, Wuhan, China).

The slides were examined in 400-fold magnification by light microscopy (Olympus BX50 microphotographic system, Japan) by an investigator who was blinded to research groups. For each animal, three random tissue sections (eight fields per section) were examined. Quantitative immunohistochemical assessments for lung HO-1 expression were performed as previously described.[36] A mean optical density (OD), which relates to immunohistochemical staining intensity, was measured by image cytometry with HIPAS-2000 image analysis software (Qianli Technical Imaging, Wuhan, China).

### *Determination of lipid peroxidation*

The plasma lipid peroxidation contents were assayed by the measurement of MDA, an end product of fatty-acid peroxidation. At 105 min after reperfusion, plasma was isolated from fresh blood samples by centrifugation at 4000 rpm for 10 min at 4 °C. Plasma MDA content was determined by the thiobarbituric acid reaction using a commercial kit (Jiancheng Biological, Nanjing, China), as

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described previously.[18] The OD was measured at 532 nm. The values of MDA level were expressed as nanomoles per millilitre.

*Statistical analysis*

Parametric data were expressed as means ± S.D. Statistical comparisons within groups were analysed by using paired Student's *t*-test. Comparisons for multiple groups were analysed by using one-way analysis of variance (ANOVA) followed by the Bonferroni's multiple *t*-test. Lung injury score was presented as median (range) and analysed with Kruskal–Wallis rank test. $P < 0.05$ was considered statistically significant.

## Results

*Changes in haemodynamics and blood gas analysis*

All animals were haemodynamically stable during the experimental procedure (data not shown). The effect of IPO on lung function as measured by $PaO_2$ is shown in Fig. 1A. No group differences in the values of $PaO_2$ were observed at baseline ($P > 0.05$). No substantial changes in $PaO_2$ were observed in the sham group ($P > 0.05$). At 105 min of reperfusion, $PaO_2$ significantly decreased in the IR, the IPO and the ZnPPIX + IPO ($P < 0.05$ or $P < 0.001$ vs. respective baseline values) groups. However, the IPO group had significantly higher $PaO_2$ compared with the IR ($P < 0.01$) and the ZnPPIX + IPO groups ($P < 0.01$), respectively. There was no significant difference in the value of $PaO_2$ between the IR group and the ZnPPIX + IPO group ($P > 0.05$). $PaCO_2$ level (Fig. 1B) at 105 min of reperfusion was significantly higher in the IR ($P < 0.01$) and the ZnPPIX + IPO ($P < 0.01$) groups compared with the sham group, but there was no statistical difference between the IPO group and the sham group ($P > 0.05$).

*Lung wet-to-dry weight ratio*

The effects of IR on the lung wet-to-dry weight ratio are illustrated in Fig. 2. Lungs exposed to IR (group IR) had significantly higher lung wet-to-dry weight ratio compared with the sham group ($P < 0.001$). IPO significantly prevented the marked increase in wet-to-dry weight ratio in response to exposure to IR ($P < 0.001$ IPO vs. IR group). However, administration of ZnPPIX prior to the induction of IPO did not alter the tissue wet-to-dry weight ratio levels when compared to the IR group ($P > 0.05$, ZnPPIX + IPO vs. IR group). The IPO group had significantly lower wet-to-dry weight ratio compared with the IR ($P < 0.001$) and the ZnPPIX + IPO ($P < 0.01$) groups, respectively.

*Lung histopathological changes*

The histopathological changes in the left upper lobe of lung tissues at the end of reperfusion were assessed by standard H&E staining. Representative pictures of lung sections from each group are shown in Fig. 3. No histological alteration was observed in the lung sections from sham-operated rats (Fig. 3A). The IR group showed acute lung injury characterised by areas of necrosis, neutrophilic inflammation and intra-alveolar and interstitial oedema (Fig. 3B). The IPO group revealed markedly reduced neutrophilic inflammation and interstitial oedema with preservation of alveoli compared with the IR group (Fig. 3C). However, when ZnPPIX, a specific inhibitor of HO-1 activity, was administered prior to IPO, the destruction of lung tissue was more severe and neutrophilic inflammation was higher as compared to the IPO group (Fig. 3D). The lung injury scores were 2.3(1.0), 8.0(2.3), 5.5(1.6) and 9.9(2.0) in the sham, IR, IPO and ZnPPIX + IPO groups, respectively (Fig. 3E). The lung injury score in the IR group was

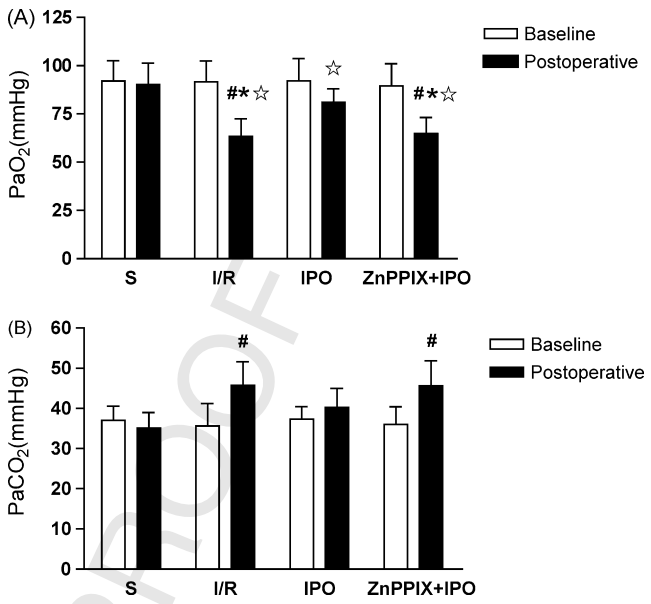


**Fig. 1.** Effect of ischaemic post-conditioning on $PaO_2$ (A) and $PaCO_2$ (B). Arterial blood sample was taken at 20 min of mechanical ventilation (baseline) and 105 min after reperfusion (postoperative). Results are expressed as mean ± S.D. of eight experiments.; $^{\#}P < 0.01$, vs. sham group, $^{*}P < 0.01$, vs. IPO group; $^{\star}P < 0.05$ or $P < 0.001$ vs. baseline value.

higher than that in the sham control group ($P < 0.001$, IR vs. sham group) and was reduced by IPO ($P < 0.05$, IPO vs. IR group). The difference in lung injury score between the IR and ZnPPIX + IPO groups was not significant ($P > 0.05$).

*Expression of HO-1 protein in the lungs*

As shown in Fig. 4, a very small amount of HO-1 was detected in alveolar macrophage cells of sham group. Significantly increased expressions of HO-1 protein were observed in group IR ($P < 0.001$, vs. sham) and group IPO ($P < 0.001$, vs. sham), respectively. When compared to group IR, group IPO had significant higher expression of HO-1 ($P < 0.05$). Pre-administration with ZnPPIX almost completely abolished the induction of the expression of HO-1 ($P < 0.001$, ZnPPIX + IPO vs. IPO group).

*MDA level*

The production of MDA is an indicator for lipid peroxidation and development of oxidative stress. As shown in Fig. 5, at 105 min of reperfusion, MDA level in IR group was significantly higher than that in sham group ($P < 0.001$). IPO significantly prevented the

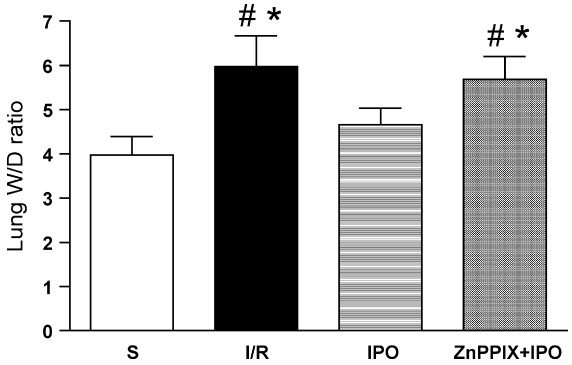


**Fig. 2.** Effect of ischaemic post-conditioning on lung wet-to-dry weight ratio. Results are expressed as mean ± S.D. of eight experiments. $^{\#}P < 0.001$ vs. Sham group, $^{*}P < 0.01$ or $P < 0.001$ vs. IPO group.

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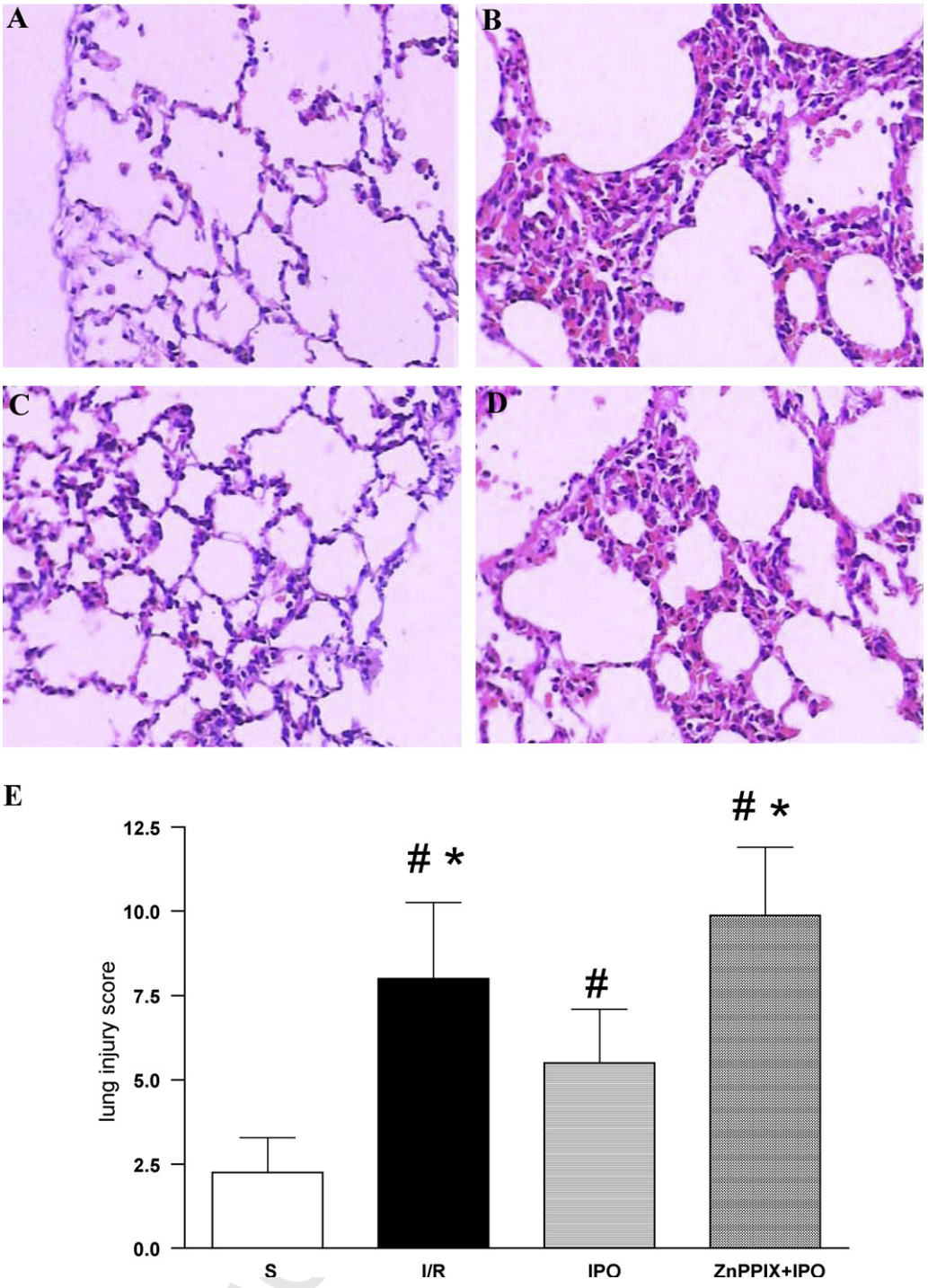


**Fig. 3.** Effect of ischaemic post-conditioning on lung histology. (A) sham operation group (group S), (B)ischaemia/reperfusion group (group I/R), (C) ischaemic post-conditioning group (group IPO), (D) zinc protoporphyrin IX + ischaemic post-conditioning group (group ZnPPIX + IPO), (E) Lung injury score in each group. Results are expressed as median (range) of eight experiments. $^{\#}P < 0.05$ or $P < 0.001$ vs. sham group, $^{*}P < 0.05$ vs. group IPO.

marked increase in MDA formation in response to exposure to IR ($P < 0.001$, IPO vs. I/R group). HO-1 inhibitor, ZnPPIX, reversed the reductions of MDA in IPO ($P < 0.001$, ZnPPIX + IPO vs. IPO group).

## Discussion

In the present study, IPO improved pulmonary oxygenation, reduced lung wet-to-dry weight ratio, MDA concentrations and histological damage. The protective effects induced by post-conditioning were accompanied by a specific, marked lung expression of HO-1. These protective effects were blocked by an HO-1 inhibitor (ZnPPIX), suggesting that HO-1 mediated the protective effects of lung IPO.

Post-conditioning is a recently described novel approach to attenuate IR injury and may have greater clinical potential than pre-conditioning. The concept of IPO was originally described by Zhao et al., who showed that brief intermittent episodes of myocardial IR performed at the onset of reperfusion, reduced infarct size in the canine heart.[41] In the current study, post-conditioned lungs showed only slight damage after a sustained IR

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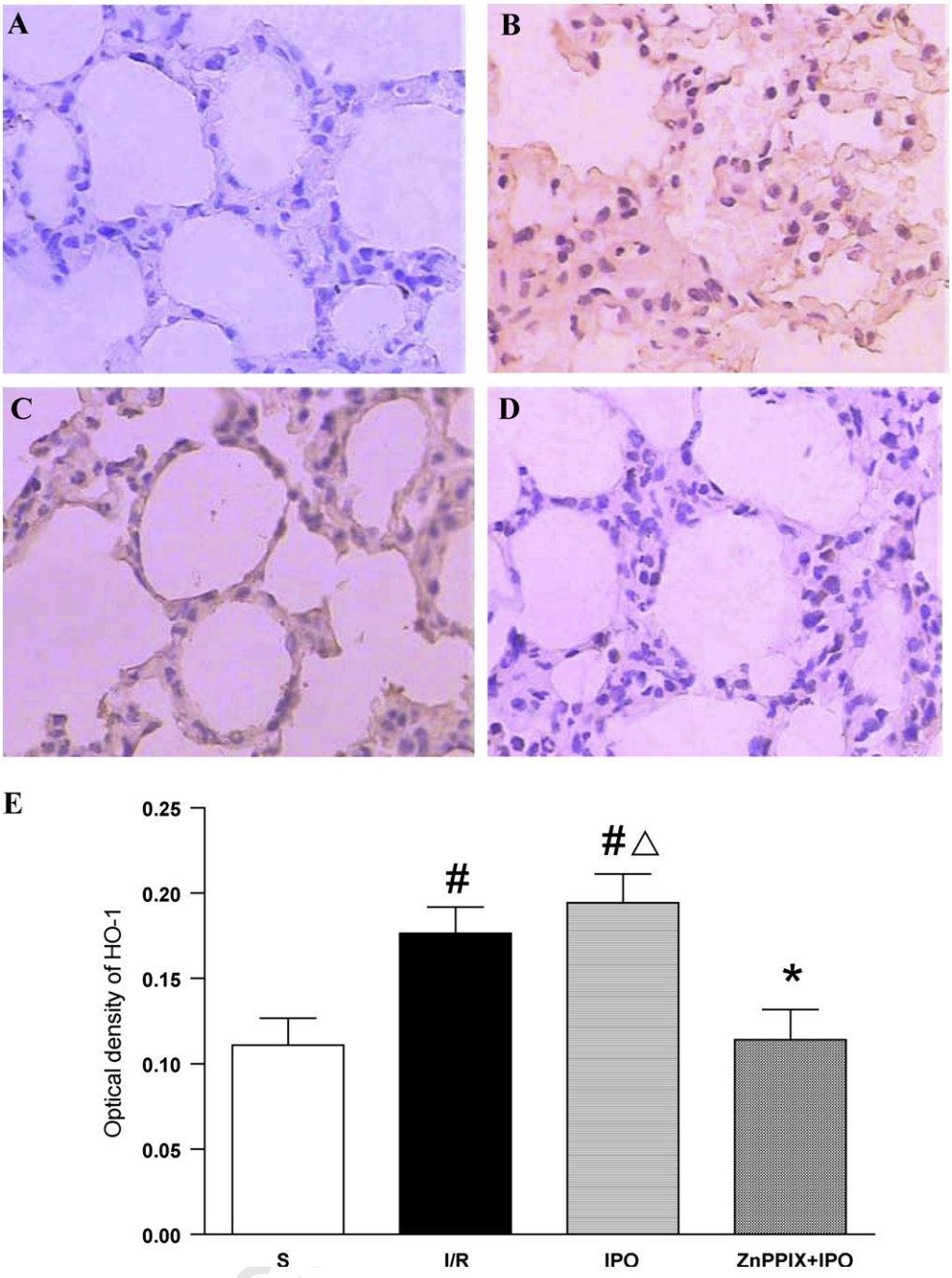


**Fig. 4.** Effect of ischaemic post-conditioning on lung HO-1 expression. (A) Sham operation group (group S), (B) ischaemia/reperfusion group (group I/R), (C) ischaemic post-conditioning group (group IPO), (D) zinc protoporphyrin IX + ischaemic post-conditioning group (group ZnPPIX + IPO). (E) Quantitative densitometric data of HO-1 expression in each group. Results are expressed as mean ± S.D. of eight experiments. $^{\#}P < 0.001$ vs. sham group, $^{\triangle}P < 0.05$ vs. group I/R, $^{*}P < 0.001$ vs. group IPO.

injury. IPO was implemented by three cycles of 30-s reperfusion per 30-s re-occlusion by the action of clamping and unclamping of the hilum of the left lung. However, it is unclear whether the number of cycles imposed affects the pulmonary protection effects of post-conditioning. Previous studies in the myocardium, from different species, revealed that post-conditioning lost its protection when it is initiated after 60–90 s of full reperfusion.[13,24] Therefore, it seems important that the post-conditioning stimulus must be applied immediately upon relief of sustained ischaemia.

Although the protective methods differ in timing and adaptive changes between pre-conditioning and post-conditioning, a number of studies have suggested that both protective manoeuvres share some, but not all, mechanisms.[23,33] In the studies of pulmonary ischaemic pre-conditioning, receptor-mediated signalling pathways, including bradykinin,[20] adenosine,[37] peroxynitrite[31] as well as ATP-sensitive potassium channels,[7] were found to be involved in the mechanism of lung protection. The protective effect of pulmonary IPO may foster further extensive studies exploring the underling mechanisms.

In this study, we found that the protective effect of lung IPO is mediated, at least in part, by HO-1. IPO attenuated the increase in necrosis, neutrophilic inflammation, and intra-alveolar and interstitial oedema. Lung tissue wet-to-dry weight ratios and blood gas exchanges were affected by IPO in a pattern similar to the changes

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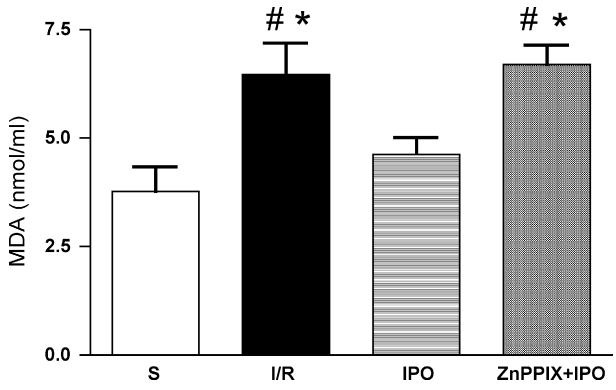


**Fig. 5.** Effect of ischaemic post-conditioning on blood malondialdehyde level expressed as nmol/ml. Blood samples were taken at 105 min at reperfusion. Results are expressed as mean ± S.D. of eight experiments. $^{\#}P < 0.001$ vs. sham group, $^{*}P < 0.001$ vs. group IPO.

of pulmonary histology. Plasma MDA level, reflecting the magnitude of lung oxidative injury following IR,[1,35] was also significantly decreased in IPO group. IPO induced up-regulation of HO-1, while HO-1 inhibitor (ZnPPIX) reversed the protective effects of IPO, associated with down-regulation of HO-1 expression. Therefore, the present study demonstrated that HO-1 might be responsible for the protective effect of IPO against IR-induced lung injury.

The beneficial effects of HO-1 induction have been shown to confer protection against lung injury in a variety of experimental models. Using genetic approaches, previous studies have demonstrated that overexpression of HO-1 can attenuate severe lung injury in mice induced by hyperoxia, lipopolysaccharide and influenza virus infection, and so on.[8,9,22] *In vitro* studies have also shown that the overexpression of HO-1 in rat foetal lung cells or human lung epithelial cells prevents apoptosis in response to increased oxygen tension.[16] In addition, chemical induction of HO-1 was found to protect the lung against the pulmonary injury.[10,17] Conversely, inhibition of HO-1 was suggested to be potentially detrimental. Otterbein et al. first demonstrated that pharmacological inhibition of HO-1 activity enhanced the susceptibility of rats to lung injury from endotoxaemia.[21] Fujita et al. showed that HO-1-deficient (Hmox1−/−) mice exhibit lethal ischaemic lung injury.[6] Zhang et al. showed that specific knockdown of HO-1 expression using small-interfering RNA *in vitro* and *in vivo* significantly increased anoxia–re-oxygenation- and IR-induced apoptosis, respectively.[39] The findings of the current study have added an insight into the association of HO-1 expression with IPO in the lung.

The mechanisms by which HO-1 induces cytoprotection against IR injury of the lung are not completely understood, but appear to involve the protective effects of HO-1 by-products, CO, biliverdin/bilirubin and free iron.[5] CO is produced via haeme catabolism by HO-1 and plays a protective role in lung injury. At low concentrations, CO can confer anti-apoptotic, anti-inflammatory and vasodilatory effects via activation of intracellular signalling pathways, which include soluble guanylate cyclase and/or p38 mitogen-activated protein kinase.[14,15,25] Besides, bilirubin, another end product of haeme catabolism, also contributes to the protective effect of HO-1. Bilirubin has been shown to protect against acute lung injury caused by endotoxaemia.[12,26] The protective effects of bilirubin in IR injury are due to its antioxidant properties.[27] In addition, cytoprotection by HO-1 is attributable to its augmentation of iron efflux. The HO-dependent release of iron results in the up-regulation of ferritin, which in turn limits the capacity of iron to generate reactive oxygen species (ROS) and iron-based free radicals.[2,32]

In summary, we demonstrated for the first time that lung IPO attenuated the severity of lung injury induced by IR in the rat. The protective effect of IPO is mediated in part through the induction of endogenous HO-1, and may suggest a potential target for the development of therapeutic strategies to prevent lung IR injury.

## Conflict of interest

The authors certify that no actual or potential conflict of interest in relation to this article exists.

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