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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 PaO2 (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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Haeme oxygenase (HO) belongs to the heat-shock protein family. It is the rate-limiting enzyme that catalyses the NADPH, O2 and cytochrome P450 reductase-dependent oxidation of haeme to carbon monoxide (CO), iron and biliverdin. 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. Accumulating data have demonstrated a cytoprotective role of HO-1 in various in vivo and in vitro pulmonary disease models, including IR injury. 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 H₂O. The left femoral vein was catheterised and 3:1 tidal volume of 12 ml kg⁻¹ crystalloid to colloidal fluid mixture was infused intravenously. Under aseptic conditions, an animal was exposed through a left thoracotomy in the fifth intercostal space. 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 PaO₂ and PaCO₂ 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 to dry weight (W/D) was calculated to assess tissue oedema, as described previously.

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. The following criteria were considered: 0 = no damage, 1 = 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% H₂O₂ in 60% methanol for 30 min. The sections were permeabilised with 0.1% Triton X-100 in phosphate-buffered saline for 20 min. Non-specific 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 1:1000 dilution of primary rabbit antihuman HO-1 polyclonal antibody (Boster Bio-Tech, Wuhan, China), followed by biotin-conjugated secondary antibody at 1:100 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. 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 (Jiangcheng Biological, Nanjing, China), as...
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 paired Student’s t-test. Comparisons for multiple groups were analysed by 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 PaO2 is shown in Fig. 1A. No group differences in the values of PaO2 were observed at baseline (P > 0.05). No substantial changes in PaO2 were observed in the sham group (P > 0.05). At 105 min of reperfusion, PaO2 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 PaO2 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 PaO2 between the IR group and the ZnPPIX + IPO group (P > 0.05). PaCO2 level (Fig. 1B) at 105 min of reperfusion was significantly higher in the IR (P < 0.01) and the ZnPPIX + IPO groups (P < 0.01), respectively. 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 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 significantly 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
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. In the current study, post-conditioned lungs showed only slight damage after a sustained IR.

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.

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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. 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. In the studies of pulmonary ischaemic pre-conditioning, receptor-mediated signalling pathways, including bradykinin, adenosine, peroxynitrite as well as ATP-sensitive potassium channels, were found to be involved in the mechanism of lung protection. The protective effect of pulmonary IPO may foster further extensive studies exploring the underlying 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.

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 ZnPP+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, **P < 0.05 vs. group I/R, #P < 0.001 vs. group IPO.

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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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