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EFFECTS OF THERMAL PRECONDITIONING ON THE ISCHEMIA-REPERFUSION–INDUCED ACUTE LUNG INJURY IN MINIPIGS

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ABSTRACT—Lung ischemia-reperfusion (I/R) injury plays an important role in many clinical issues. A series of mechanisms after I/R has been uncovered after numerous related studies. Organ preconditioning (PC) is a process whereby a brief antecedent event, such as transient ischemia, oxidative stress, temperature change, or drug administration, bestows on an organ an early or delayed tolerance to further insults by the same or different stressors. In this study, we want to uncover the optimal thermal PC patterns that cause maximal early or delayed protective effect on the subsequent pulmonary I/R with the use of miniature pig model. Twenty-eight 15- to 20-kg weight Lanyu miniature pigs are used and divided into four groups (seven sham operation control [NC], seven PC only [PC], seven I/R [I/R], and seven PC followed by I/R [PC + I/R]). The PC was performed with the animals being anesthetized and, using an alternative hyperthermic (40°C) and normothermic moist air to ventilate their lungs for 15 min, respectively, for 2 cycles, followed by I/R, which consists of 90 min of blocking the perfusion and ventilation of the left lung followed by 240 min of reperfusion. Control animals had a thoracotomy with hilar dissection only. Indicators of lung injury included hemodynamic parameters, blood gas analysis, histopathological (lung pathology, wet/dry weight ratio, myeloperoxidase assay), and molecular biological profiles (interleukin-1β [IL-1β], IL-6, tumor necrosis factor-α by enzyme-linked immunosorbent assay analysis). Lung tissue heat shock protein 70 (HSP-70) expression was also detected by Western blotting. This model of lung I/R induced significant lung injury with pulmonary hypertension, increased pulmonary vascular resistance, and pulmonary venous hypoxemia at the ischemia side, increased pulmonary tissue injury score and neutrophil infiltration, increased wet/dry ratio, myeloperoxidase assay, tumor necrosis factor-α, IL-1β, and IL-6 assay. This type of thermal PC would not injure the lung parenchyma or tracheal epithelium. Moreover, it could attenuate the I/R-related lung injury, with some of these parameters improved significantly. Increased expression of HSP-70 was also found in the group of PC plus I/R than the I/R only. Less prominent and transient increase in expression of HSP-70 was found in the PC group. We concluded that the intratracheal thermal PC can effectively attenuate I/R-induced lung injury through various mechanisms, including the decrease of various proinflammatory cytokines. The mechanism of its protective effect might be related to the increased expression of HSP-70.

KEYWORDS—Preconditioning, ischemia-reperfusion, hyperthermia

ABBREVIATIONS—IL—Interleukin; I/R—ischemia-reperfusion; MPAP—mean pulmonary arterial pressure; MPO—myeloperoxidase; PC—preconditioning; TNF—tissue necrosis factor; W/D ratio—wet-to-dry weight ratio

INTRODUCTION

Lung ischemia-reperfusion (I/R) injury plays an important role in many clinical issues, including thromboembolism, trauma, thermal injury, hypovolemic and endotoxin shock, organ transplantation, and many respiratory diseases in the critical care (1–6). Organ ischemia or hypoperfusion caused adenosine triphosphate exhaustion in mitochondria, with subsequent cell membrane permeability change, intracellular osmolality change, cytoskeletal and mitochondrial damage, or even cell apoptosis or necrosis. Organ reperfusion caused further degree of injury, which is related to the interaction between neutrophil and dysfunctional endothelial cells (1–4). Subsequent changes, such as the organ free radical production, activation of the coagulation system, or further inflammatory cell adhesion and other types of tissue injuries occurred in the reperfusion stage (4–6). Besides, tissues under the I/R stimuli can induce the production of proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, or tumor necrosis factor-α (TNF-α) (6, 7). Interleukin-1β, a 17-kd polypeptide mainly produced by monocytes or macrophages, has been proved to play the pivot role in the pulmonary I/R injury in animal studies (8, 9). Interleukin 6, a pleiotropic cytokine with both proinflammatory and anti-inflammatory functions, can be released by various types of cells into the plasma in response to various stimuli, including I/R (10, 11). Tumor necrosis factor-α is also a potent early proinflammatory mediator released from a variety of cell types in response to injury (12, 13).

Preconditioning (PC) is a process where cells or tissues exposed to a sublethal stimulus are transiently protected from a subsequent normal lethal stress, including I/R (14–19). Many forms of PC have been investigated, such as ischemic, thermal, pharmacologic, or gas inhalation (particular in lung injury) (20–27). Preconditioning can attenuate the subsequent prolonged or lethal tissue injury by increasing the cell

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tolerance to the stress. Preconditioning can reduce the I/R injury in solid organs, such as the heart, liver, kidney, and bones. However, there are relatively limited data about the similar effects on lungs (28–33).

There are several types of PC for lung published in previous literature. Besides the ischemia, hyperthermia has been applied as a common type of PC. The hyperthermal PC can attenuate pulmonary dysfunction after various animal models of injuries mimicking clinical conditions, such as organ transplantation, pulmonary air emboli, hemorrhagic shock, or abdominal surgery (34–36). The detailed molecular mechanism of hyperthermic PC is still unclear, but it has been reported that the heat shock proteins (HSPs), specially the HSP-72, played an important role (26, 34–36). These HSPs act as molecular chaperones, maintaining normal protein folding in adverse cellular environments (36).

Most of previous literature about the hyperthermic PC were performed by water immersing topically or changing core temperature (26, 34–36). In this article, we developed a new model of PC by hyperthermic gas inhalation. We will measure its effects in attenuating the subsequent I/R by measuring related hemodynamic, histopathological, and molecular biological profiles.

MATERIALS AND METHODS

Development of the model

In the first pilot investigation, we found that unilateral obstruction of the miniature pigs' pulmonary artery, veins, and bronchus by mass snaring with a rubber tube for 90 min followed by restoration of the perfusion and ventilation caused overt pulmonary injuries. The injuries would not be so significant if the pulmonary perfusion and ventilation were blocked for 60 min only. We cut holes over the distal sides of their snared pulmonary artery, veins, and bronchus to test any persistent hemorrhage or air leakage from the proximal heart or trachea by water immersion. We proved that this type of snaring can totally block the blood flow and nearly totally block the ventilation. In the second pilot investigation, we compared the subsequent vascular injuries by mass hilar clamping, hilar dissection and snaring, and mass snaring. We found that mass snaring caused significantly less degree of injuries over the vascular walls than other two groups by histopathological examination. In the third pilot investigation, we used hilar dissection only (sham operation) and found that this manipulation still caused minor subsequent lung injuries due to compromised perfusion. In this experiment, we found that miniature pigs' pulmonary vessels were much more fragile than the canine ones. In the fourth pilot investigation, we tested the effects of hyperthermic gas ventilation in the subsequent lung and tracheal epithelial injuries. We challenged four animals with three episodes of 15 min, 40°C hyperthermic gas ventilation. A transient increase in neutrophil infiltration of the tracheal epithelium and lung parenchyma was noted 30 min after the completion of PC, but these recovered within 2 h.

Model of PC preparation of hyperthermic gas transtracheal delivery

In this experimental design, we used the transtracheal delivery of hyperthermic gas. An MR850-heated humidifier with dual heated wire (Fisher & Paykel, Auckland, New Zealand) was connected on the circuit between the ventilator and the airway (Fig. 1). Temperature of the chamber and airway can be selected and displayed on the machine (37).

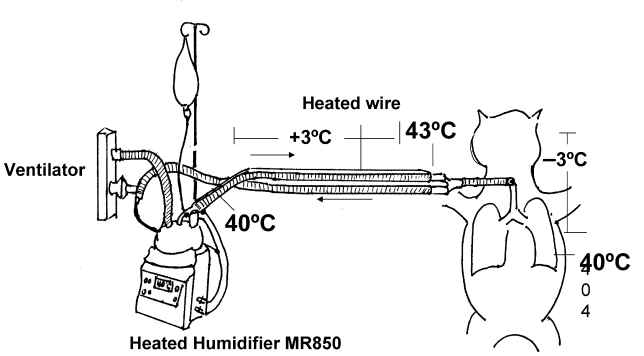

FIG. 1. **Design for hyperthermic gas inhalation as the mode of PC.**

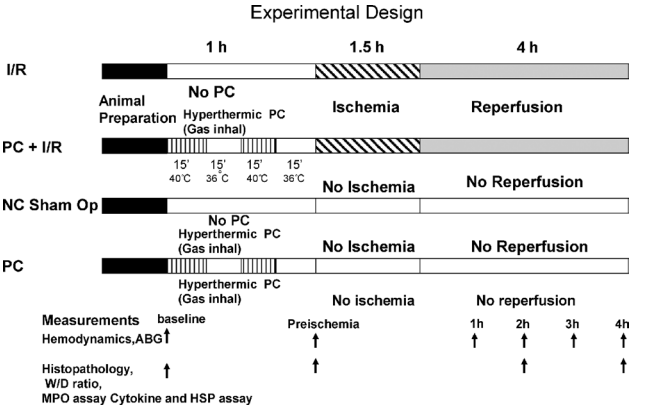

FIG. 2. **Experimental design.**

Surgical preparation and experimental protocol

Twenty-eight Lanyu miniature pigs (about 5 to 6 months of age) weighing from 15 to 20 kg body weight were used in this study. The protocol was approved by the National Science Committee in Taiwan and Experimental Animal Care Committee in the research institute. All animals received humane care in compliance with the *Principles of Laboratory Animal Care* and *Guide for the Care and Use of Animal Resources* published by the National Institutes of Health. After fasting for 8 to 12 h, the pigs were i.m. injected with atropine (atropine sulfate, Tai yu, Taiwan), followed by tiletamine and zolezepam 1:1 mixture (Zoletil, Virbac, France) 0.55 to 0.80 mg/kg i.m. injection to achieve the initial stage of anesthesia. Gas anesthesia with isoflurane 2.5% to 4.5% was used for induction, followed by 1% to 3% in maintenance after completion of intubation through the tracheotomy. The animals were placed in supine position, intubated through cervical tracheostomy, and mechanically ventilated with a tidal volume of 20 mL/kg at a rate of 15 to 20 per min using an ADS 1000 ventilator (Engler Engineering, Hialeah, Fla). An arterial line and a Swan-Ganz catheter were inserted through the left carotid artery and jugular vein. Then the miniature pig was placed on right decubitus position for surgical procedure.

These animals were divided into four groups. In animals in the I/R control group (n = 7, I/R group), a left lateral thoracotomy was made over the fifth intercostal space after heparinization (initially 200 IU/kg, then 100 IU/kg every 2 h). The inferior pulmonary ligament was dissected, and then the pulmonary hilum was snared with a no. 8 negaton rubber catheter to block the blood and gas flow. The left lung was kept in normothermic ischemia for 90 min, followed by restoration of ventilation and perfusion for 6 h. Animals in the hyperthermic PC before I/R group (n = 7, PC + I/R group) underwent an alternative hyperthermic (40°C) and normothermic (36°C) moist air to ventilate their lungs for 15 min, respectively (the delivery of hyperthermic gas has been described in the previous paragraph), for two cycles, followed by I/R as in the I/R group. Animals in the PC only group (n = 7, PC group) underwent the hyperthermic PC as in the PC plus I/R group, but without the subsequent I/R. In animals in the negative sham operation control group (n = 7, NC group), the thoracotomy and hilum was dissected only, without subsequent I/R. The protocol is summarized in Figure 2.

During the experiment, pentobarbital (0.1 mg/kg per min i.v. infusion) was administered for anesthesia, whereas pancuronium (0.3 mg/kg) or succinylcholine (0.5 mg/kg) bolus injection was given intermittently to induce muscle relaxation. Intravenous fluid was administered to keep the energy, fluid, and electrolytes in balance, which were monitored by hemodynamic measurements, urine output, sugar, electrolytes, and hemogram at the time of arterial blood sampling for gas analysis.

Hemodynamic measurements

Systolic/diastolic/mean pulmonary arterial pressure (MPAP), pulmonary capillary wedge pressure (PCWP), and central venous pressure are recorded with pressure transducers on electronic recorder (M1205A monitor, Hewlett-Packard). Cardiac output (CO) is measured with a Swan-Ganz catheter using thermal dilution techniques. Injection of saline at 4°C is given as a bolus, and the CO is computed 1 per min. A minimum of three values are averaged. The pulmonary vascular resistance (PVR) per 20 kg is also calculated. The PVR was calculated from the formula: PVR (dyne s cm^{-5}) = (MPAP − PCWP)/CO × 80. The above data were measured before thoracotomy (baseline data), before ischemia, and 1, 2, 3, and 4 h after restoration of perfusion and ventilation.

Blood gas analysis

Arterial and mixed venous blood samples are collected from the arterial line and pulmonary arterial catheter (Swan-Ganz), while the animals are mechanically ventilated with 100% O_2, and 5 cm H_2O positive end-expiratory pressure for 5 min. These samples are heparinized and used for pH and blood gas analysis with an analyzer (178, Corining, Medfield, Mass). Time points of measurements are the same as in the hemodynamic studies.

Lung histology and lung wet-to-dry weight ratio

The left upper and lower lobes of lung were harvested after thoracotomy (baseline), before ischemia, and 2 and 4 h after reperfusion. The cut margins of the resected specimens were oversewn by 3-0 silk running sutures. Specimens for pathological examination were fixed in 10% formalin and examined in blinded fashion. Neutrophil counts and alveolar profiles were evaluated using a ×10-eyepiece reticule, and they were counted in 10 consecutive fields containing only the alveoli. The severity of lung injury was scored from 0 (minimal) to 4 (severe) by the examination of the degrees of alveolar edema/hemorrhage, infiltration of neutrophils, alveolar wall thickness, and vascular wall changes, which included the intravascular thrombi formation. Specimens for the wet-to-dry weight (W/D) ratio calculation were weighed with a tin foil and then placed in 80°C for 24 hr and weighed with a tin foil again. The W/D ratio = (total wet weight-weight of tin foil)/(total dry weight-weight of the tin foil).

Lung tissue myeloperoxidase assay

Myeloperoxidase (MPO) activity in the lung tissues, which were harvested after thoracotomy (baseline), before ischemia, and 2 and 4 h after reperfusion, were measured according to the previously reported method. Samples of lung tissue were stored at −70°C until the assay. Frozen lung tissue was weight and homogenized in 1 cm^3 (per 100 mg lung tissue) 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical Co, St Louis, Mo) in 50 mM of potassium phosphate buffer (pH 6.0, 5.0 mL of 0.5% hexadecyltrimethylammonium bromide per gram tissue) on ice for 20 s. The homogenate was put through a freeze-thaw cycle three times, sonicated for 15 s on ice, and ultracentrifuged at 40,000*g* and 4°C for 30 min. The supernatant was decanted, and 0.1 mL was mixed with 2.9 mL of 50 mM potassium phosphate buffer (pH 6.0) containing 0.167 mg/mL of *O*-dianiside hydrochloride (Sigma Chemical) and 0.0005% hydrogen peroxide. The absorbance change at 460 nm was recorded at 5 min and 20 min at room temperature. The color development from 5 to 20 min was linear. The MPO activity was expressed in terms of absorbance change of optical density unit at 460 nm/min per 100 mg of lung tissues.

Serum TNF-α, IL-1β, and IL-6 assay

In all pigs, peripheral venous blood samples were collected. Serum was separated from blood cells by centrifugation at 4,000 cycles/min. All samples were stored at −70°C until analyzed. Serum levels of IL-1β, IL-6, and TNF-α were measured in duplicate using commercially available enzyme-linked immunosorbent assay kits (IL-1β (no. PLB00), IL-6 (no. P6000), and TNF-α (no. PTA00) for porcine cell culture supernates, serum, and plasma. Quantikine P-PTA00, R&D Systems, Minneapolis, Minn). The dynamic ranges of the TNF-α, IL-1β, and IL-6 assay were 38.4 to 1,500 pg/mL, 39 to 2,500 pg/mL, and 39.1 to 2,500 pg/mL, respectively, with the sensitivities of 6.1, 10, and 10 pg/mL, respectively. The interassay and intra-assay CV was 5.2 and 7.4% (35). The above data were measured before thoracotomy (baseline data), before ischemia, and 1, 2, 3, and 4 h after restoration of perfusion and ventilation.

Tissue heat shock protein 70 expression

Tissue samples from the lungs were removed at the time after thoracotomy (baseline), preischemia (post-PC on group 2), and 4 h after reperfusion, homogenized in phosphate-buffered saline (1 mL), and centrifuged at 4,100*g* for 30 min at 4°C. The supernatants were collected, and protein concentration was quantified using a Coomasie protein assay reagent (Rockford, Ill). The final protein concentration of the samples was diluted for 50 ug/10 μL. The protein was denatured at 100°C for 10 min, and aliquots containing equal amounts of proteins were then suspended in sodium dodecyl sulfate–glycerol loading buffer, and proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (ExcelGel, Pharmacia, Sweden). Proteins were then transferred to a nitrocellulose membrane (Sigma Chemical) and labeled with a primary monoclonal antibody, mouse anti–heat shock protein 70 (HSP-70) monoclonal antibody (SPA-810, Stressgen Bioreagents Co, Ann Arbor, Mich), which can be cross-reacted with the human beings and porcine HSP-70. After the secondary monoclonal Ab, the goat

TABLE 1. **Hemodynamic parameters**

	Baseline		Preischemia		Postreperfusion	
			1 h	2 h	3 h	4 h
Mean arterial pressure (mmHg)						
I/R	92.4 ± 3.2	92.0 ± 4.2	81.7 ± 4.0*†	83.1 ± 4.3*†	84.6 ± 2.9*†	86.6 ± 4.2
PC + I/R	91.1 ± 4.5	90.4 ± 4.0	81.1 ± 2.5*†	83.7 ± 3.7*†	87.4 ± 2.4	89.1 ± 2.3
NC	92.1 ± 4.6	92.0 ± 3.9	91.7 ± 3.2	90.1 ± 4.5	91.0 ± 3.4	91.1 ± 4.9
PC	91.1 ± 5.1	92.6 ± 4.3	91.9 ± 3.5	91.1 ± 4.7	92.1 ± 4.4	91.1 ± 4.9
MPAP (mmHg)						
I/R	11.1 ± 1.5	11.3 ± 1.2	14.9 ± 1.2*†	18.6 ± 1.6*†	23.1 ± 2.2*†	26.0 ± 2.6*†
PC + I/R	11.4 ± 1.6	11.7 ± 1.4	15.6 ± 1.6*†	17.3 ± 1.0*†	18.0 ± 1.3*†‡	17.7 ± 1.0*†‡
NC	11.4 ± 1.4	11.1 ± 1.1	11.6 ± 1.4	11.4 ± 1.0	11.3 ± 0.9	11.1 ± 0.8
PC	11.2 ± 1.6	11.3 ± 1.5	11.9 ± 1.6	11.2 ± 1.2	11.4 ± 0.7	11.0 ± 0.7
PCWP (mmHg)						
I/R	4.7 ± 0.9	4.1 ± 0.6	4.0 ± 0.5	4.6 ± 0.7	5.4 ± 0.7†	5.6 ± 0.9†
PC + I/R	4.6 ± 0.9	4.4 ± 0.9	3.6 ± 0.6	4.0 ± 0.8	4.1 ± 0.6‡	4.3 ± 0.7‡
NC	4.7 ± 0.7	4.3 ± 1.0	4.4 ± 0.7	4.1 ± 0.6	3.9 ± 0.6*	4.0 ± 0.8
PC	4.8 ± 0.8	4.4 ± 1.2	4.6 ± 0.8	4.4 ± 0.7	4.1 ± 0.7	4.0 ± 0.9
Cardiac index (L/min per 20-kg body weight)						
I/R	1.3 ± 0.1	1.1 ± 0.1*	1.0 ± 0.1*	1.0 ± 0.1*†	0.9 ± 0.1*†	0.8 ± 0.0*†
PC + I/R	1.3 ± 0.1	1.1 ± 0.1*	1.0 ± 0.1*	1.0 ± 0.1*†	1.0 ± 0.1*†‡	1.0 ± 0.1*†‡
NC	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
PC	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1

Values are expressed as mean ± SD.
*$P < 0.05$ vs. its own baseline data; †$P < 0.05$ vs. NC data; ‡$P < 0.05$ vs. the I/R data.

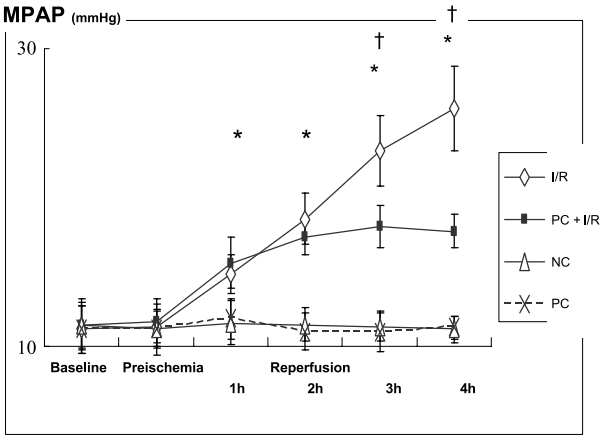

FIG. 3. **The MPAP response.** *$P < 0.05$ between the I/R group and the NC or PC group. †$P < 0.05$ between the I/R group and the PC plus I/R group.

antimouse IgG horseradish peroxidase (AP124P, Chemicon Co) was added, the protein was visualized using the 5-bromo-4-chloro-indolyl-phosphatase/nitroblue tetrazolium (Sigma Chemical) (36).

Statistical analysis

All values are expressed as mean ± SD. To compare the means of various outcome indicators, a repeated-measures analysis of variance was used using the general linear model repeated measures of SPSS version 11.0 for Windows XP. The intervention effect was tested by examining the interaction effects between the four groups (I/R, PC + I/R, NC, PC) and time which was composed of six levels (baseline, preischemia, postreperfusion 1, 2, 3, and 4 h). After global testing, *post hoc* Tukey procedures were applied; $P < 0.05$ was considered statistically significant.

RESULTS

Hemodynamic measurements

The mean arterial pressure, MPAP, cardiac index, and PCWP of these four groups are shown in Table 1. The MPAP and PVR index (PVRI) are shown in Figures 3 and 4, respectively. There was no statistical difference in the baseline data among these groups. All of above parameters were unchanged during the experiment course in the NC and PC groups. For I/R and PC plus I/R groups, the MPAP and PVRI increased in proportion to the reperfusion time. However, the mean arterial pressure decreased significantly after reperfusion. For the PC plus I/R group, the MPAP and PVRI values were significantly lower than those of the I/R group after 2 h of reperfusion.

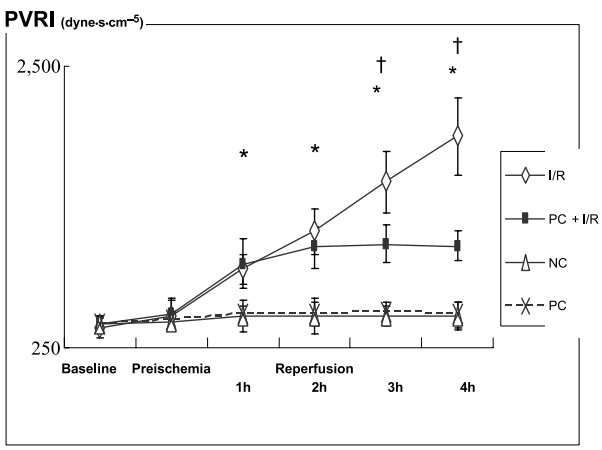

FIG. 4. **The PVRI response.** *$P < 0.05$ between the I/R group and the NC or PC group. †$P < 0.05$ between the I/R group and the PC plus I/R group.

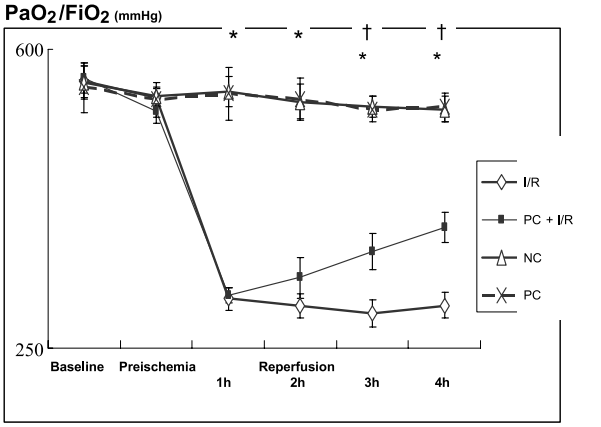

FIG. 5. **Arterial blood gas Pao_2/Fio_2 values among groups.** *$P < 0.05$ between the I/R group and the NC or PC group. †$P < 0.05$ between the I/R group and the PC plus I/R group.

Arterial blood gas analysis

These four groups of animals exhibited no significant differences in the values of the baseline and preischemia Pao_2/fraction of inspired oxygen (Fio_2) (Fio_2 = 1.0). The postreperfusion Pao_2/Fio_2 values in the NC group and the PC group were insignificantly changed compared with its own baseline data (Fig. 5). However, the postreperfusion Pao_2/Fio_2 values in the I/R and the PC plus I/R groups were significantly lower than those of the other two groups (NC and PC) or their own baseline values.

Lung histology and neutrophil infiltration

There was no significant difference in the baseline values of the lung neutrophil infiltration or injury score (Figs. 6 and 7) among these four groups. The differences between the I/R group and the NC group or in preischemia values of these two parameters were insignificant. However, the PC plus I/R group and the PC group have significantly higher preischemia values of lung neutrophil infiltration and injury score than those of the other two groups. These might reflect their

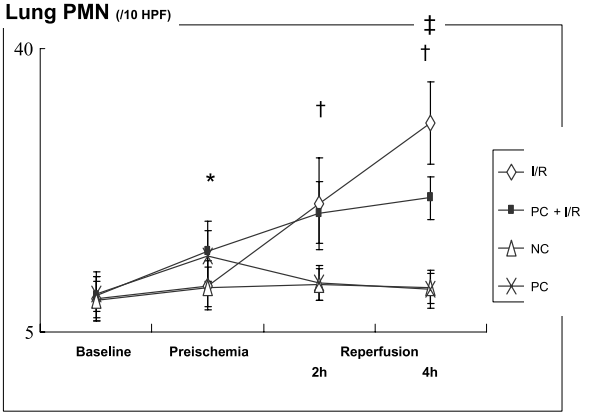

FIG. 6. **Lung neutrophil infiltration values among groups.** *$P < 0.05$ between the PC (PC + IR or PC) and non-PC (I/R or NC) groups. †$P < 0.05$ between the I/R group and the NC or PC group). ‡$P < 0.05$ between the I/R group and the PC + I/R group.

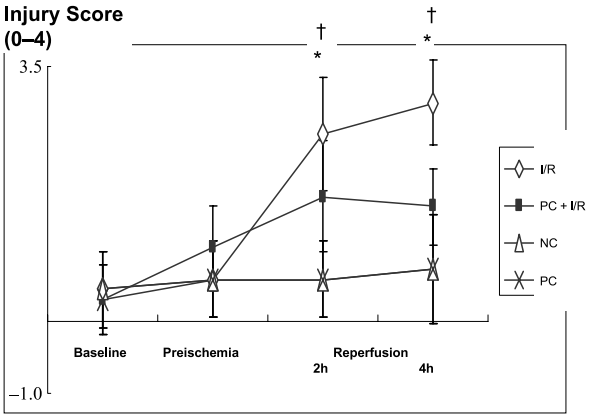

FIG. 7. **Lung injury scores among groups.** *$P < 0.05$ between the I/R group and the NC or PC group. †$P < 0.05$ between the I/R group and the PC plus I/R group.

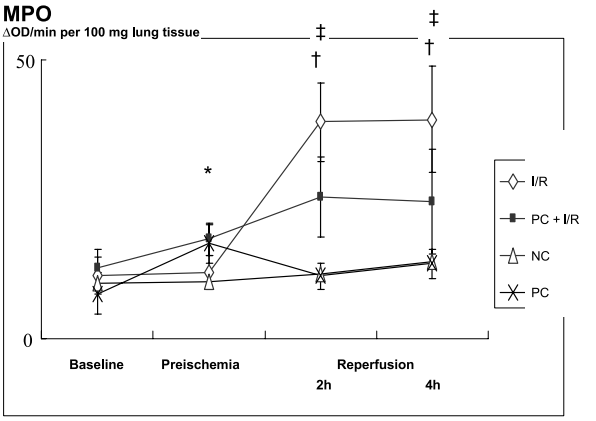

FIG. 9. **Lung MPO assays among groups.** *$P < 0.05$ between the PC (PC + IR or PC) and non-PC (I/R or NC) groups. †$P < 0.05$ between the I/R group and the NC or PC group). ‡$P < 0.05$ between the I/R group and the PC plus I/R group.

responses to the hyperthermic PC. After reperfusion, the values of these two parameters in the I/R or PC plus I/R group were significantly higher than the NC or PC group. Pretreatment with hyperthermic PC (PC + I/R group) can significantly decrease the degree of lung neutrophil infiltration and injury score relative to that of the I/R (I/R group) after 4 and 2 h of reperfusion, respectively.

Lung W/D weight ratio

There was no significant difference in the baseline and preischemia W/D ratio among these four groups (Fig. 8). The preischemia value of the PC plus I/R group and the PC group was higher than the other two groups, which reflected the effect of PC. However, the differences were not statistically significant. This ratio of the I/R group became significantly higher than its baseline value after I/R. In the PC plus I/R group, the W/D ratio also increased after reperfusion. However, this value was significantly lower than that of the I/R group.

Lung tissue MPO assay

There was no significant difference in the baseline lung tissue MPO assay among these four groups (Fig. 9). In the PC plus I/R group, the preischemia MPO value was significantly higher than the other three groups, which was compatible with the phenomenon of higher white blood cell infiltration in histological examination and reflected the effect of PC. After reperfusion in the I/R and PC plus I/R groups, the lung tissue MPO assay became higher than the NC or PC group and their own baseline values. However, the PC plus I/R group has significantly lower values of MPO assay than those in the I/R group.

Serum IL-1β, IL-6, and TNF-α assay

The concentration of IL-1β, IL-6, and TNF-α in serum was determined at various time points throughout the study (Figs. 10–12). There was no significant difference in the baseline and preischemia (post-PC in the PC plus I/R group) values of these proinflammatory cytokines among these four groups. After reperfusion, serum levels of these cytokines in the I/R group started to increase until the end of the observation period. In the PC plus I/R group, these values only moderately increased, which were significantly lower than values of the I/R group after 2 h of reperfusion. In the NC and PC groups, only modest increases of these values were noted.

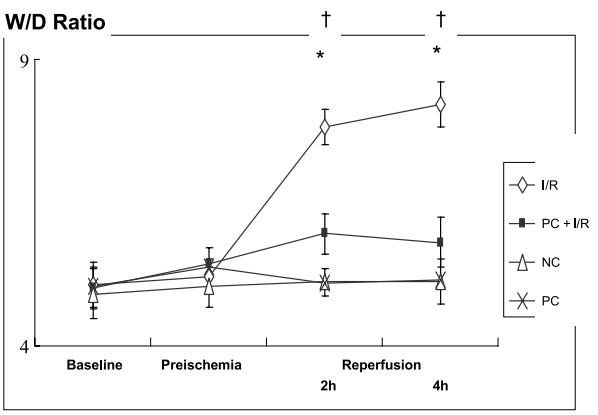

FIG. 8. **Lung W/D ratio among groups.** #$P < 0.05$ between the PC (PC + IR or PC) and non-PC (I/R or NC) groups. *$P < 0.05$ between the I/R group and the NC or PC group. **$P < 0.05$ between the I/R group and the PC plus I/R group.

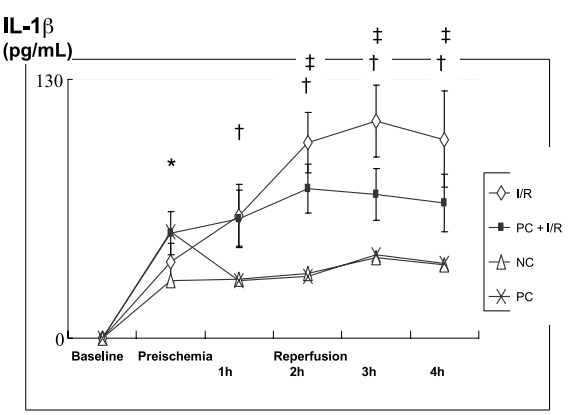

FIG. 10. **Serum IL-1β assay among groups.** *$P < 0.05$ between the PC (PC + IR or PC) and non-PC (I/R or NC) groups. †$P < 0.05$ between the I/R group and the NC or PC group. ‡$P < 0.05$ between the I/R group and the PC plus I/R.

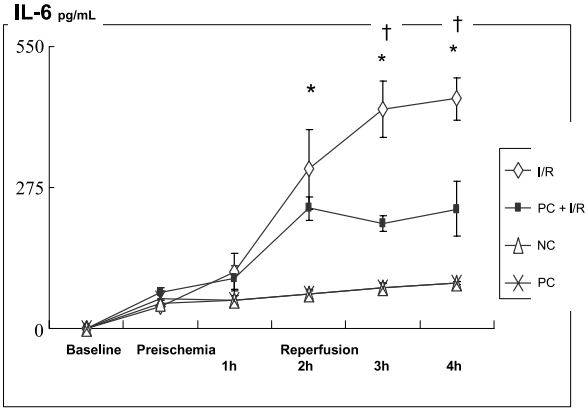

FIG. 11. **Serum IL-6 assay among groups.** *$P < 0.05$ between the I/R group and the NC or PC group. †$P < 0.05$ between the I/R group and the PC plus I/R group.

Tissue HSP-70 Western immunoblotting

In this experiment, the PC plus I/R group revealed increased and persistent expression of HSP-70 than that in the I/R group (Fig. 13). The PC group revealed transient and much less degrees of HSP-70 expression than the PC plus IR group. These results proved that the expression of HSP-70 for lungs undergoing I/R can be induced by antecedent hyperthermal PC. Moreover, this type of PC would only induce mild and transient increases in the expression of HSP-70 if it were not followed by I/R.

DISCUSSION

We hypothesize that hyperthermic PC can attenuate the subsequent pulmonary I/R injury. In this study, a new and clinically applicable model of hyperthermic gas inhalation, instead of saline immersion of the lung through the thoracotomy wound being published before (21, 35, 36), was established. It was shown that this type of PC can significantly ameliorate the subsequent pulmonary I/R injury, as demonstrated by decreased pulmonary vascular resistance, increased

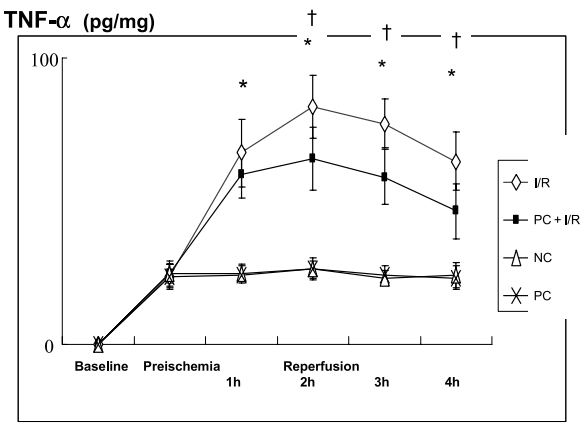

FIG. 12. **Serum TNF-α assay among groups.** *$P < 0.05$ between the I/R group and the NC or PC group). †$P < 0.05$ between the I/R group and the PC plus I/R group.

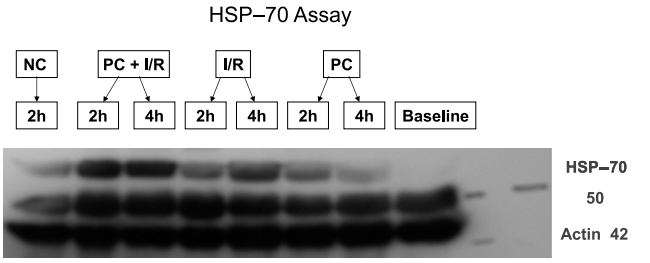

FIG. 13. **Tissue HSP-70 expression in the four groups (NC, PC + I/R, I/R, and PC groups) 2 and 4 h after the I/R in the I/R and PC plus I/R groups, and after the equivalent time point (without I/R) in the PC or NC groups.** Please see Figure 2 for the experimental design.

arterial oxygen tension, decreased W/D lung weight ratio, lung injury, or neutrophil infiltration, and decreased tissue MPO assay and proinflammatory cytokine secretion. This study also demonstrates that this unique type of hyperthermal PC, although only slightly and transiently increases the expression of HSP-70, can result in significant and persistent increases in its expression while the PC was followed by I/R, which can also be observed in other models of hyperthermic PC (36). Simple I/R in the present study can also increase the expression of HSP-70. However, the duration and level of its expression is much less than that in animals receiving PC before I/R. Heat shock protein expression has been found to prevent the tissue from subsequent I/R injury (37), and our present study proved that increased HSP expression might contribute in attenuating subsequent lung injuries in animals undergoing hyperthermal PC before I/R.

Previous work by our group has demonstrated that a variety of the I/R model, such as *in situ* 1-lung I/R (4, 38), warm ischemia after donor lung retrieval (5), or isolated lung I/R (39), can result in significant deterioration of pulmonary functional and histopathological parameters. Lung I/R injury plays an important role in many clinical issues, including thromboembolism, trauma, thermal injury, hypovolemic and endotoxin shock, organ transplantation, and many respiratory diseases in critical care (1–6).

As observed by animal models of reported series, not only the I/R but also other types of lung injuries, such as hemorrhagic shock (40) or air emboli (21), can be attenuated by antecedent sublethal stimuli. Organ PC was first recognized by Murry et al. (41) in 1986, who originally tried to create a larger area of myocardial infarction by performing several brief episodes of myocardial ischemia before a protracted ischemia, but got paradoxical results. This concept and detailed mechanisms on a variety of organs, such as liver, brain, lung, or skeletal muscles, in studies of human or animals have been uncovered by subsequent studies (14–18, 42–46). However, there are relatively limited data about the similar effects on lungs (22–33, 45).

How does the PC protect the tissue from injury? The cellular and molecular mechanisms of tissue I/R injury are related to the cellular activation (neutrophils, T lymphocyte, and antigen-presenting cells), formation of proinflammatory mediators (cytokines and chemokines), expression of adhesion molecules, activation of nitric oxide synthase system, and the

production of reactive oxygen species (1–6). The detailed mechanisms of PC varied from different animal species or organ systems. However, in general, these could be classified as the immediate acquisition, which is related to the posttranslational modification, or delayed induction, which is related to the new protein synthesis (16). These "biphasic" phenomena of PC have been observed in the brain, heart, and liver tissues in human or other animals (13, 17, 18, 26, 47–49). The molecular mechanisms of PC included inhibition of inflammatory cytokines, such as TNF-α, IL-1β, and IL-8 (13, 22), with subsequent reducing activation and infiltration of neutrophils (24), decrease of lipid peroxidation (20), reduction of apoptosis of lung cells *in vivo* by upregulating bcl-2 protein expression (28), increase in the production of endogenous calcitonin gene related peptide (45, 50), reducing production of oxygen free radicals (45). In addition, PC can induce the production of HSPs, especially HSP-70 or HSP-32 (HO-1), which can reduce the nuclear binding of proinflammatory transcription factors and increase the oxidant capacity of the cells (36, 51, 52). In our present study, improved hemodynamics and gas exchange function, decreased tissue edema and neutrophils infiltration, and inhibition of TNFα, IL-1β, and IL-6 release with increased HSP expression postischemia reperfusion were noted after antecedent PC by hyperthermic gas administered intratracheally. Although transient and mildly increased in serum IL-1β and IL-6 expression as well as neutrophil colonization in lung tissues immediately after PC are found in these animals in the present study, these rapidly return to normal value within 2 h. Therefore, we think that these phenomena caused by PC are only "inflammatory cell aggregation," without resulting in subsequent physiological or histopathological changes. The elevation of IL-6 serum level has been reported to be a better predictor of the severity of the organ I/R injury than TNF-α, which is difficult to keep at high serum level because of the subsequent tissue binding after release (12, 13). In our study, similar results were also noted after this model of pulmonary I/R. However, hyperthermic PC can still result in statistically, but not really clinically, significant decrease in the TNF-α serum levels.

The protective effects about the ischemic PC from I/R injuries of the lung have been shown in previous literature, in experimental guinea pig (20), canine (44, 45), and rabbit (43) models. There were several types of PC other than ischemia for lung being published in previous literature. Hyperthermia has been applied as one of the common types of PC. A traditional model of hyperthermic PC in rat is 41°C for 15 min (21, 48). However, this model would result in more cellular damage. A less severe thermal PC, using water bath to elevate 1°C core temperature for 15 min for 5 consecutive days, can also achieve PC protective effects (36). In our present study, sublethal damage by PC can be only influence locally, such as pulmonary neutrophil infiltration, instead of systemic effects, such as some of the serum cytokines (IL-6 and TNF-α), hemodynamics, and gas exchange function. The reason why this PC resulted in such effects needs further studies to verify it and uncover detailed mechanisms. However, it can be explained that the "sublethal" effects of PC are only short-term and self-limited, and the subsequent protective effects after I/R can also prove its effectiveness.

The hyperthermal PC can attenuate pulmonary dysfunction after various animal models of injuries mimicking clinical conditions, such as organ transplantation, pulmonary air emboli, hemorrhagic shock, or abdominal surgery. In our present study, we have established a new pulmonary hyperthermic PC model, which has the advantages of less adverse effects to the whole body, more specific to the target organ, clinically feasible and easily manipulated, and it has been proven effective in tissue protection from subsequent I/R injury through this study.

Pharmacological agents, after the discovery of related molecular mechanisms of PC, gradually replace ischemia or hyperthermia, as the specific "targeted" pretreatment methods to protect organs from various types of injuries. However, related studies about pulmonary PC were relatively rare and were still under developing. Some agents, such as diethylmaleate (an intracellular pro-oxidant agent), geldanamycin, 3-nitropropionate (an inhibitor of the mitochondrial complex II), or *N*-acetyl-L-cysteine (an oxidant scavenger which promotes glutathione formation), can be used as PC agents to prevent subsequent pulmonary I/R injury (40, 53–55). The pharmacological PC can also gradually replace the traditionally ischemic or thermal PC if their molecular mechanisms become clearer in the future.

In conclusion, we have shown herein that a brief episode of hyperthermic gas inhalation before I/R of the lung is sufficient to attenuate its induced early pulmonary dysfunction and injury. Further studies, such as how to change the episodes, duration, and the temperature at this type of PC to achieve maximal protective effect, and its protective effects as well as detailed mechanism at latent phase, would be valuable. Release of some proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, can also be influenced by this type of PC. In the future, more molecular markers can be applied in this model to monitor the extent of pulmonary injury at different stages. Increased heat shock protein expression after this type of hyperthermic PC can be observed in this study, and this might contribute to the pulmonary protection after I/R. Its detailed mechanism and cellular signal transduction pathway will be an interesting issue in further studies.

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