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ORIGINAL ARTICLE

Protection against acute porcine lung ischemia/reperfusion injury by systemic preconditioning via hind limb ischemia

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Summary

Previous work on various organs and tissues has shown that ischemic preconditioning protects against reperfusion injury in these organs and also against secondary effects in the lung. In contrast, the purpose of this study was to investigate the effects of preconditioning in a remote organ (hind limb ischemia) on an ischemia/reperfusion (I/R) treatment of the lung itself. A porcine model of *in situ* left lung ischemia (90 min) and reperfusion (5 h) was used. Systemic preconditioning was induced by clamping the left common femoral artery (3×5 min). Lung injury was assessed in terms of pulmonary vascular resistance, pulmonary artery pressure, pulmonary venous and arterial pO_2, and tissue macrophage counts. The zymosan-stimulated release of reactive oxygen species (ROS) in whole blood was determined by a chemiluminometric procedure. Inflammatory cytokines (interleukin-1β and interleukin-6) were measured in arterial plasma as indicators of a systemic inflammatory reaction. Preconditioning by hind limb ischemia completely prevented the I/R-induced functional impairment of the lung, the pulmonary hypertension and the reduced oxygenation capacity. The plasma levels of interleukin-1β and the macrophage counts in preconditioned animals were reduced to control values, whereas the levels of interleukin-6 and the release of ROS were not affected by preconditioning. In conclusion, systemic preconditioning by repeated hind limb ischemia protects against acute I/R injury of the lung but not against all indices of reperfusion-associated systemic inflammation.

Introduction

Lung transplantation has become an accepted treatment for patients with end-stage lung diseases. Ischemia/reperfusion (I/R) injury is inevitably associated with organ transplantation, and I/R-induced lung functional impairment remains a significant cause of early graft failure [1].

One of the strategies to prevent the consequences of I/R is ischemic preconditioning. This term refers to a mechanism whereby a brief exposure to I/R or repeated, short episodes of I/R render the pretreated organ resistant to an extended ischemic phase later on. Since the first description by Murry, Jennings, and Reimer in 1986 [2], using a canine model of myocardial ischemia, this phenomenon was also investigated in various lung models. Two different experimental approaches were used: preconditioning by a short ischemic episode of the lung itself (organ preconditioning), or ischemia in another organ to protect this organ against subsequent, extended I/R and, at the same time, to attenuate systemic effects on the lung which are secondary to the I/R injury in the remote organ (systemic preconditioning).

Organ preconditioning of the lung was evaluated in several animal models and experimental settings [3–10]. The treatment has been shown to attenuate the detrimental effects of I/R, pulmonary hypertension, impaired gas

exchange, and the inflammatory response. Furthermore, organ preconditioning reduced the damage to the donor lung during prolonged ischemic storage for organ transplantation experiments in rats [4], rabbits [9], and dogs [6]. Repeated, short episodes of ischemia proved to be superior to a single treatment, with a narrow time window for optimal effects [5,9,10]. Systemic preconditioning, meaning attenuation of remote organ dysfunction in the lung secondary to brief I/R and subsequent, extended ischemia in a remote organ, was investigated in several experimental animal systems [11–19]. The effect was frequently observed in the liver [11,14–16,18], but similar results were found in myocardium [12], intestine [13], and skeletal muscles of the hind limbs [17]. This form of ischemic preconditioning has been shown to be clinically effective in patients having valve replacement [12].

The purpose of the present study is to investigate the effects of a remote preconditioning event on a subsequent, extended I/R treatment in the lung itself and not on the effects secondary to the I/R injury in other, distal organs. To our knowledge, the potential benefits of this form of systemic preconditioning have never been evaluated in the lung. Since the same *in situ* porcine model of single lung warm ischemia (90 min) and reperfusion (5 h) was used before to evaluate chemical preconditioning by nitric oxide (NO) inhalation [20], the advantages of both procedures can be compared.

Materials and methods

Surgical protocol

The protocol was approved by the Animal Care Section of the Saxonian Government (Dresden, Germany) and the Animal Care Committee of the Technical University of Dresden. Animal experiments met the 'Principles of laboratory animal care' (NIH publication No. 88-23, revised 1985). In total 20 domestic pigs weighing 26–33 kg were used in this investigation.

The surgical techniques of thoracotomy and I/R were outlined in a previous communication [21]. Briefly, anesthesia was induced by an injection (i.m.) of azaperon (200 mg), diazepam (10 mg) and atropine (0.5 mg), and sustained by infusion of thiopental sodium (6 mg/kg/h) and fentanyl (5 mg/kg/h). Animals were kept in supine position for tracheostomy and pressure-regulated, volume-controlled ventilation (8–10 ml/kg body weight, fraction of inspired oxygen 0.5, positive end-expiratory pressure 3 mmHg). This setting was temporarily changed for single lung ventilation during left lung ischemia. The animals were heparinized (initially 500 IU/kg, later 160 IU/kg every 2 h). Catheters were advanced through the right jugular vein into the right pulmonary artery, through the right carotid artery, and after 90 min of ischemia into a pulmonary vein of the left lung. The animals were positioned on the right side for left lateral thoracotomy in the fifth intercostal space and dissection of the pulmonary hilum. The animals were placed on a heating pad throughout the experiment to achieve normothermic conditions.

Study groups

In pigs of the ischemia group ($n = 7$), the left pulmonary artery and the pulmonary veins were clamped, and the left main bronchus was closed with a Fogarty catheter. The ventilation frequency was twofold increased at 50% tidal volume. These conditions of normothermic ischemia were maintained for 90 min, followed by 5-h reperfusion. A catheter was placed into the left inferior pulmonary vein at the end of the ischemic period to collect blood directly from the ischemic organ. Animals of the preconditioned group ($n = 8$) were treated in the same way, except that the left hind limb was made ischemic by clamping the A. femoralis communis for 5 min. After reperfusion for 10 min, this cycle was repeated two more times. This treatment was applied 30–40 min before thoracotomy. Animals in the sham controls group ($n = 7$) received sham surgery without ischemic preconditioning and without I/R. The other surgical procedures were the same as in the other study groups.

Hemodynamic measurements

Numerous hemodynamic and respiratory parameters as well as blood gases and pH were measured and recorded as outlined in a previous communication [21]. The

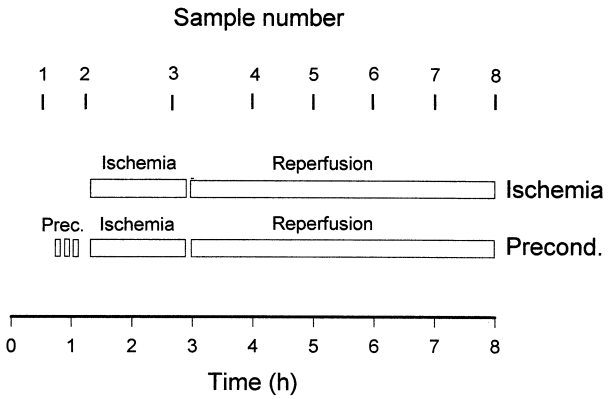

Figure 1 Protocols of lung I/R experiments. The bars on top indicate points in time of blood and tissue sampling as well as hemodynamic measurements during preconditioning, ischemia, and reperfusion. Sample 1, after initial surgery; sample 2, after preconditioning and dissection of the hilum; sample 3, after 80 min of ischemia; samples 4–8, after 1, 2, 3, 4, and 5-h reperfusion.

timing of data assessment is illustrated in a schematic diagram of the study protocol (Fig. 1).

Macrophage counts

Representative tissue samples from the left lung were excised after dissection of the hilum (sample 2 in Fig. 1) and at the end of the observation period (sample 8). The incisions were sealed with a 3/0 polypropylene suture. In order to detect and identify macrophages, lung tissue samples were fixed in 4% formaldehyde and embedded in paraffin for histochemical staining with biotin-conjugated lectin from Dolichos biflorus by an established procedure [22]. The lectin (Vector Laboratories, Burlingame, CA, USA) was applied in a dilution of 1:800.

Cytokine determinations

The concentrations of interleukin (IL)-1β and IL-6 in plasma were quantified using commercial assay kits (Quantikine colorimetric ELISA; R&D Systems GmbH, Wiesbaden, Germany) according to the instructions of the manufacturer.

Measurement of ROS

The zymosan-induced release of reactive oxygen species (ROS) from phagocytic leukocytes was determined in EDTA-whole blood samples by a chemiluminometric procedure using luminol and lucigenin [21]. With the experimental conditions used, luminol detected primarily OH^- and H_2O_2 whereas lucigenin detected O_2^- [21].

Statistical analysis

All values are expressed as mean ± SD. Differences between groups over time were analyzed with an ANOVA for repeated measurements. After global testing, *post hoc* Bonferroni procedures were applied. Values of $P < 0.05$ were considered statistically significant.

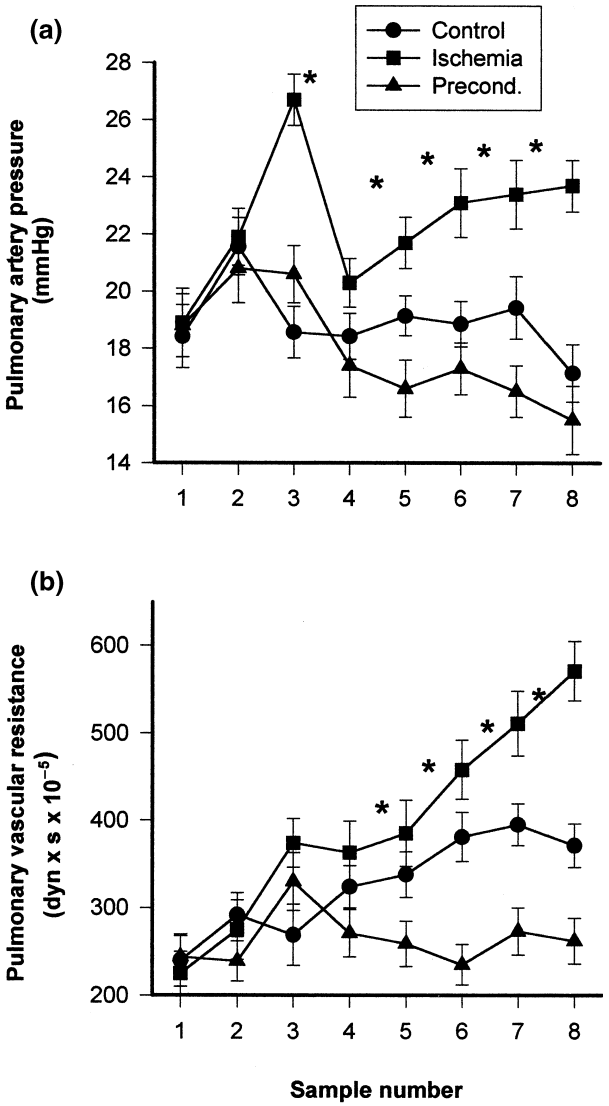

Figure 2 Pulmonary vascular resistance and pulmonary artery pressure as affected by I/R and preconditioning. The numbering at the X-axis indicate the time points of measurements according to Fig. 1. Pulmonary artery pressure (a) and pulmonary vascular resistance (b). *$P < 0.05$, ischemia versus preconditioned group.

Results

Hemodynamic effects of I/R and preconditioning

Preconditioning by repeated hind limb ischemia had a pronounced effect on lung hemodynamic parameters (Fig. 2). The 1.4-fold increase of pulmonary artery pressure (PAP) over baseline during initial surgery and lung ischemia (sample 3) in the ischemia group was abrogated in the preconditioned group. Values in the latter group showed a tendency to decline during reperfusion, even below sham controls, while unprotected lung I/R resulted in a significant increase of PAP during reperfusion. A 1.5-fold higher value (23.7 mmHg) in the ischemia group when compared with the preconditioned group was measured at the end of the reperfusion phase (Fig. 2a).

Determination of pulmonary vascular resistance (PVR) also revealed pronounced differences between groups (Fig. 2b). Unprotected lung I/R resulted in an almost continuous increase to a 2.5-fold higher value when compared with baseline after 5-h reperfusion, and a 1.5-fold increase of PVR was observed in the sham control group. In contrast, the value of PVR in the preconditioned group did not differ from baseline during reperfusion, and the

values were significantly lower compared with the control group. At the end of the reperfusion phase (sample 8), PVR in the ischemia group was 2.2-fold higher than in the preconditioned group.

Oxygenation as affected by I/R and preconditioning

In order to further characterize the lung function during I/R, the pO_2 in pulmonary venous blood ($pvpO_2$) was determined during the reperfusion phase, and the arterial pO_2 (apO_2) was measured throughout the observation period (Fig. 3). The values of $pvpO_2$ in the preconditioned animals increased during the early phase of reperfusion. After 3 h, the $pvpO_2$ was not significantly different in controls and the preconditioning group (Fig. 3a). In the ischemia group, this functional recovery of the organ was not observed, and lower values of $pvpO_2$ than in the preconditioning group were found (49% at 3 h).

The apO_2 in preconditioned animals showed a tendency to drop below the level in sham controls, but the difference was never significant (Fig. 3b). In contrast, the apO_2 in unprotected animals was significantly lower than in controls (69%) and in the preconditioned group (76%) at the end of the 5-h reperfusion phase.

Cytokine measurements

In order to characterize the systemic inflammatory response, cytokine levels in arterial plasma were determined (Fig. 4). In the ischemia group, a pronounced increase (2.5-fold over baseline) of the early phase cytokine IL-1β after 2 h reperfusion was observed. After an

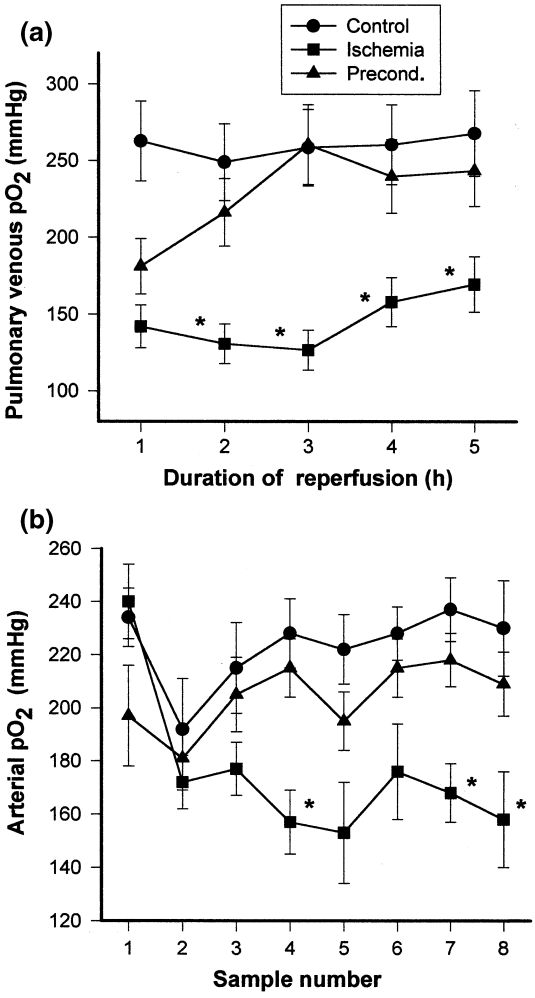

Figure 3 Effects of preconditioning and I/R on pulmonary venous pO_2 (a) and arterial pO_2 (b). The sample numbering in (b) refers to Fig. 1. *$P < 0.05$, ischemia versus preconditioned group.

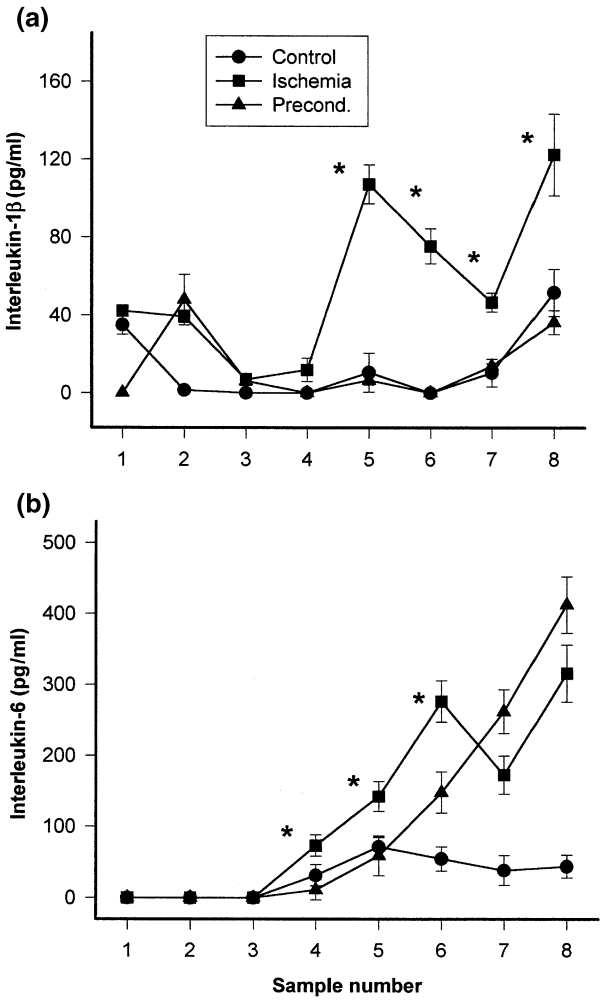

Figure 4 ELISA for cytokines IL-1β and IL-6. The cytokines IL-1β (a) and IL-6 (b) were tested in plasma samples which were taken at the time points of Fig. 1 (*x*-axis). *$P < 0.05$, ischemia versus preconditioned group.

intermediary drop to near baseline values after 4-h reperfusion (sample 7), IL-1β increased again at the end of the observation period. This response was completely blocked in preconditioned animals. The IL-1β levels in this group were not different from sham controls throughout the reperfusion phase (Fig. 4a).

In contrast, the level of IL-6 in the preconditioned group started to increase at 2 h of reperfusion (sample 5). At the end of the reperfusion phase, the highest value (413 pg/ml) of all groups was measured (Fig. 4b). In sham controls, only a minor increase over the detection limit was observed at the early reperfusion phase. In the ischemia group, the IL-6 concentration increased at the beginning of the reperfusion to higher values when compared with the preconditioned group, but IL-6 showed an intermediary decrease, similar to IL-1β, at the end of reperfusion.

Tissue macrophage count

Macrophages in representative lung tissue sections were quantified by a histochemical procedure using Dolichos biflorus lectin for staining. The results in Fig. 5 show that ischemia induced a pronounced increase (4.8-fold over sham controls) of the lectin-binding macrophages at the end of the reperfusion period (sample 8). This effect was abrogated (1.4-fold increase) by preconditioning.

Whole blood chemiluminescence

The ROS from polymorphonuclear granulocytes contribute to the deleterious effects of I/R-associated inflammation. The granulocyte activity in arterial whole blood was therefore quantified by detection of the zymosan-stimulated release of ROS by means of enhanced chemiluminescence with two chemiluminogenic probes. In our experimental setting, luminol detected primarily OH^- and H_2O_2 whereas lucigenin reacted with O_2^- [21].

In both ischemia groups, a pronounced increase of the luminol chemiluminescence was observed (Fig. 6a). During the last 4 h of reperfusion, the values in these groups were significantly higher compared with controls (3.1-fold at 4 h reperfusion, ischemia versus control). Significant differences between the ischemia and the preconditioned groups were not detected. A similar situation was found when the release of O_2^- was measured with lucigenin

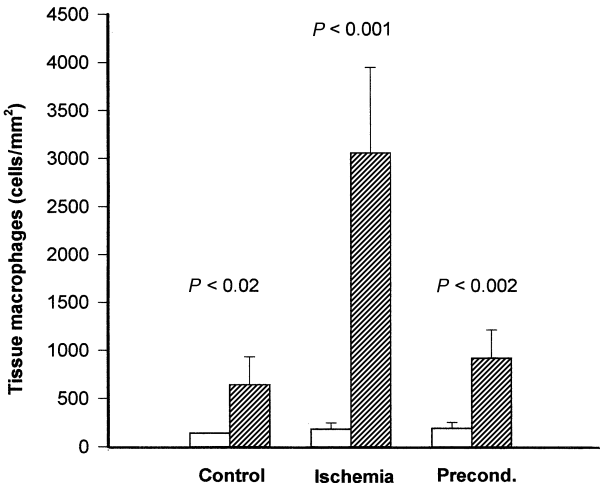

Figure 5 Macrophage counts in lung tissue samples. Lung tissue was sampled before ischemia (sample 2, open column) and at the end of the reperfusion phase (sample 8, closed column). The difference between the ischemia and the preconditioned group (sample 8) is significant ($P < 0.05$). The *P*-values of the difference between samples 2 and 8 are indicated in the figure.

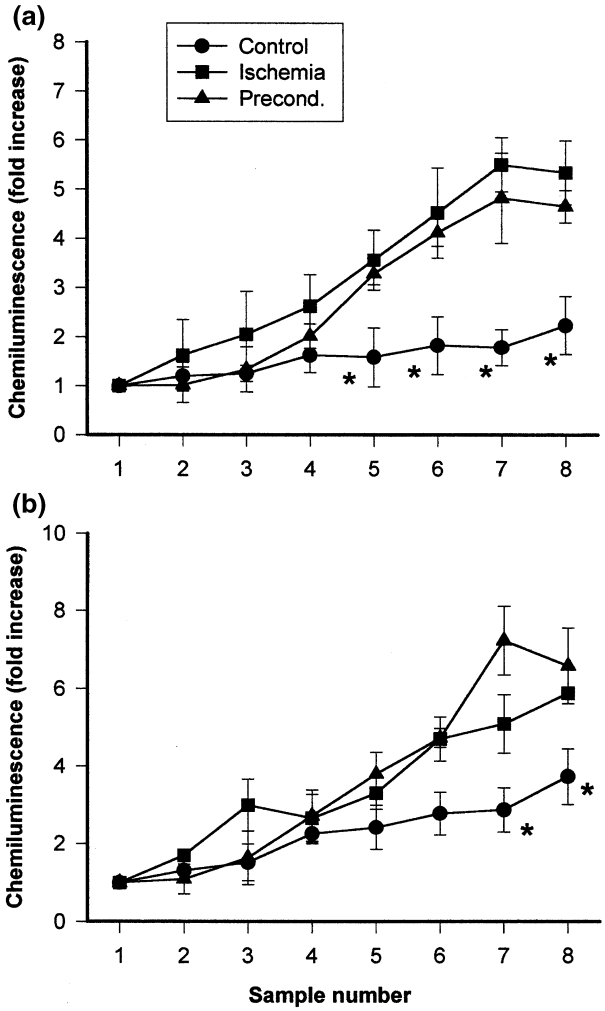

Figure 6 Effects of preconditioning and I/R on the zymosan-stimulated release of ROS from whole blood. ROS were determined by a chemiluminometric procedure using luminol (a) and lucigenin (b). The sample numbering (*x*-axis) refers to Fig. 1. $*P < 0.05$, controls versus ischemia and preconditioning.

(Fig. 6b). There was no indication of an attenuating effect by preconditioning. At the end of the reperfusion phase, the values in the preconditioned animals showed rather a tendency to increase over the ischemia group.

Discussion

The main objective of the present investigation was to demonstrate that attenuation of acute I/R damage to the lung can be achieved with brief, repeated phases of sublethal ischemia in a remote tissue, the hind limb skeletal muscle. The results show that the detrimental effects of I/R on the lung function, pulmonary hypertension and impaired gas exchange, were completely reversed. The values of PAP and PVR were even better compared with controls receiving sham surgery. To our best knowledge, this is the first report that ischemic preconditioning in a remote organ is sufficient to protect the lung against later, extended ischemia of the pulmonary tissue itself.

Extended ischemic episodes followed by reperfusion are inevitably associated with a systemic and local inflammatory response [1]. The systemic preconditioning by hind limb artery clamping had contradictory effects on different indicators of inflammation. The I/R-induced increase of the early phase cytokine, IL-1β, was completely abrogated, whereas the plasma level of IL-6 was largely the same as in the ischemia group. As tissue macrophages are a major source of IL-1β [23], the blunting of the IL-1β release may be directly related to the pronounced reduction of lectin-stained macrophages in the lung tissue (Fig. 5). In contrast, the zymosan-induced release of ROS from whole blood was not affected by preconditioning. The luminol/lucigenin chemiluminescence test used for detection of ROS mainly measures the oxidative burst capacity of leukocytes, preferentially of neutrophils [17,24]. Obviously, the priming of phagocytic leukocytes was elevated to the same level as in unprotected animals, whereas the lung tissue was not functionally impaired. Taken together, these results show that the systemic preconditioning by hind limb arteria clamping was not sufficient to completely ameliorate the systemic inflammation, but this treatment preserved the organ in an excellent functional state during I/R challenge.

The protection of the lung against injury after remote organ I/R by ischemic preconditioning in these organs was observed in several animal models and experimental systems [11–19]. The mechanism of interorgan transfer is not known, but evidence has been presented showing that reduced levels of inflammatory mediators, especially of tumor necrosis factor α (TNF-α) [11,16], and a diminished number of circulating neutrophils [17] may be causally related to this effect. In addition, the levels of circulating xanthine oxidase and xanthine were lowered by ischemic preconditioning of the liver, and evidence for a role of this effect in the protection of the lung after liver I/R was presented [14].

Recently, the effects of ischemic preconditioning in the hind limbs on pulmonary dysfunction because of systemic I/R injury were investigated using the same porcine model as in the present study, except that preconditioning and the subsequent extended ischemia phase were both carried out by occlusion of the external iliac artery [17]. Although the I/R injury of the lung was caused in different ways, by direct ischemia of the lung in the present study and by a systemic effect in the study of Harkin *et al.* [17], preconditioning ameliorated the I/R-induced functional impairments, reduced oxygenation capacity and pulmonary hypertension, largely to the same extent. In addition, Harkin *et al.* [17] showed that other signs of lung damage, edema and tissue leukosequestration, were attenuated. In marked contrast to the present investigation, the indicators of systemic inflammation, the increased plasma level of IL-6 and the activation of circulating leukocytes, were clearly reduced. Furthermore, the plasma level of the early phase cytokine, TNF-α, showed no difference among groups in the experiment of Harkin *et al.* [17], whereas a pronounced increase during lung reperfusion of another early phase mediator of inflammation, IL-1β, was observed in this study. Preconditioning against lung dysfunction secondary to I/R in a remote organ is obviously based on mechanisms which are different from the mechanisms of lung protection in our experimental setting. The blocking of interorgan mediators which confer I/R injury to the lung is basically sufficient to protect the remote organ.

In this study, we show that the functional integrity of the lung can be preserved during I/R in spite of the incomplete blocking of the systemic inflammatory response, especially without reduced phagocytic leukocyte priming. In consideration of the detrimental role of these cells in lung tissue, it has to be assumed that the prevention of lung invasion by circulating phagocytic leukocytes, possibly by lowered expression of adhesion molecules [25], played a major role in systemic preconditioning. The vehicles by which the protection was conferred from the hind limb skeletal muscle to the lung are not known, but the suppression of early phase mediators of inflammation like IL-1β (Fig. 4) may have been involved.

In addition to brief episodes of ischemia, preconditioning can also be induced by exposure to increased temperature or by administration of pharmacological agents. Recently, the porcine experimental model of lung I/R of this study was used to investigate the effects of a chemical preconditioning variant, the brief inhalation of NO (15 p.p.m., 10 min) before the onset of lung ischemia

[20]. As otherwise strictly the same experimental system was used, the benefits of both procedures can be directly compared. Topical application of NO resulted in the complete recovery of the I/R-impaired gas exchange, to the same extent as under systemic preconditioning, whereas systemic preconditioning was the superior technique for the attenuation of the pulmonary hypertension. In contrast, NO inhalation resulted in a more pronounced blunting of the systemic inflammation. The plasma levels of both cytokines, IL-1β and IL-6, were clearly reduced, and the onset of polymorphonuclear priming was delayed. However, the lung tissue counts of macrophages was not reduced, in marked contrast to systemic preconditioning. Taken together, both methods for preconditioning exert distinct and largely complementary sets of effects. Lung function parameters were better after ischemic preconditioning, and NO inhalation preferentially abrogated inflammation. Therefore, the combination of both methods could be a new strategy for the protection against I/R injury of the lung.

In conclusion, we have shown that preconditioning by brief episodes of hind limb ischemia can ameliorate major I/R injury of the lung. As the surgical strain is minimal, this procedure may be a feasible alternative to direct lung I/R preconditioning and pharmacological preconditioning in clinical situations of lung ischemia. Obviously, further studies are warranted to elucidate the – so far unknown – underlying mechanisms of this form of systemic preconditioning. The extent of I/R injury is still a crucial factor for the outcome of lung transplantations. As preconditioning by hind limb clamping is inevitably dependent on a systemic response which mediates the effects to the remote organ, the potential application of this procedure – not only for the treatment of the donor but also to achieve a preconditioned situation in the recipient – should be evaluated in future studies.

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