

## Neutrophil Activation in Ischemia-reperfusion Injury of the Lung

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Activated neutrophils and oxygen free radicals have been implicated in the pathogenesis of lung injury associated with ischemia reperfusion. This study tests whether postischemic reperfusion in the lung activates neutrophils and whether superoxide dismutase (SOD) could block this event. Ten anesthetized mongrel dogs underwent three hours of warm ischemia to their left lung followed by 120 minutes of reperfusion. Pulmonary venous blood was assayed for neutrophil hydrogen peroxide production and plasma thiobarbituric acid reactive materials (TBARM) immediately before (baseline) and during reperfusion. Neutrophil hydrogen peroxide production, as assayed flow cytometrically by dichlorofluorescein oxidation, was elevated significantly to  $123.9 \pm 21.1\%$  of baseline at 30 minutes of reperfusion. Plasma TBARM was elevated significantly to  $124.2 \pm 23.6\%$  of baseline at 60 minutes after reperfusion. However, in six animals which received human recombinant SOD (intravenously, 35000U/kg) at the onset of reflow, neither neutrophil hydrogen peroxide production nor plasma TBARM changed during reperfusion. Treatment with SOD was also associated with significant improvement in the recovery of lung function. In conclusion, these data demonstrate that post-ischemic lung reperfusion enhances neutrophil hydrogen peroxide and free radical production, and can be blocked by intravenous SOD.

Lung transplantation is performed successfully at a number of centers around the world and the survival rate improved yearly. Primary graft failure, however, is the main cause of perioperative death.<sup>1)</sup> Ischemia reperfusion injury in the lung increases capillary permeability, pulmonary edema, and appears to be due to primary graft failure.

Experimental studies have demonstrated that oxygen free radicals play a role in ischemia-reperfusion injury. In addition, the role of neutrophils in lung injury at the time of reperfusion after a period of ischemia has been demonstrated in vitro.<sup>2)</sup> The purpose of this study is to investigate neutrophil activation and free radical generation after 3hr of normothermic ischemia and to evaluate the ability of superoxide dismutase (SOD) to prevent these events.

### Methods

Sixteen mongrel dogs (8-13kg) were anesthetized intravenously, with 0.5ml/kg of sodium pentobarbital solution. Each animal received humane care in compliance with the Guidelines for Animal Experimentation of Nagasaki University. After tracheal intubation, ventilation was carried out at a tidal volume of 30ml/kg and a rate of 13 cycles/min. with a volume-cycled ventilator. The fraction of inspired oxygen was maintained at 100%. A 5F Swan-Ganz catheter introduced into the external jugular vein was positioned in the main pulmonary artery. A left thoracotomy was performed at the fifth intercostal space. Both main pulmonary arteries (PA) and both main bronchi were isolated with slings. The left pulmonary veins were exposed at their junction with the left atrium.

The right pulmonary artery and main bronchus were occluded with clamps for ten minutes to measure the baseline function of the left lung. After baseline measurements were taken, the clamps were released. Function parameters measured included arterial oxygen tension (PaO<sub>2</sub>), pulmonary arterial pressure (PAP), left atrial pressure (LAP), airway pressure and cardiac output (CO). LAP was measured through a 22G needle placed in the left atrium. Pulmonary vascular resistance (PVR) was calculated from the formula:  $PVR = 79.92 (PAP-LAP)/CO$  (dynes•sec•cm<sup>-5</sup>). Static lung compliance (Cst) was calculated as tidal volume/pressure at 1.4 seconds in the end-inspiratory plateau (ml/cmH<sub>2</sub>O).<sup>3)</sup> Following intravenous administration of 200U/kg heparin, the left lung was isolated by clamping the left PA and the left atrial cuff from which the pulmonary veins of the left lung originated. The left bronchus was clamped to maintain inflation of the lung with 100% oxygen.

After three hours of normothermic ischemia, the left lung reperused and ventilation resumed. Animals were divided into two groups: no treatment, n = 10 (control group) and recombinant-human SOD infusion (35000U/kg intravenously for 40 minutes starting ten minutes before the end of ischemia), n = 6 (SOD group).

Functional data were obtained in the same fashion as the baseline measurements at 10, 60, 90, 120 minutes of reper-

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fusion. Pulmonary arterial and venous blood was sampled for determinations of plasma thiobarbituric acid-reactive materials and neutrophil hydrogen peroxide production. Samples were obtained immediately before reperfusion, and at 10, 30, 60, and 120 minutes after reperfusion, while clamping the right main pulmonary artery.

#### *Hydrogen peroxide production by neutrophils*

A flow cytometric assay (previously described by Bass and colleagues<sup>4)</sup>) was used to quantitate the oxidation of intracellular 2', 7'-dichlorofluorescein (DCFH) during an oxidative burst of neutrophils.

Heparinized whole blood (0.1ml)<sup>5)</sup> was preincubated for 15 minutes with a 5  $\mu$ mol/L solution of DCFH diacetate in phosphate-buffered saline (PBS) at a final volume of 2ml with horizontal agitation in a shaking water bath at 37 °C. To each sample we added 0.5ml of 24mmol/L ethylenediaminetetraacetic acid dissolved in PBS and incubated each sample with or without 10  $\mu$ l of phorbol myristate acetate (15  $\mu$ g/ml) in the horizontal shaking water bath at 37 °C for 25 minutes. After incubation, the cells were washed with PBS by centrifugation at 300G for 10 minutes. Erythrocytes were lysed with cold 0.87% NH<sub>4</sub>Cl. Following addition of PBS, the remaining cells were then centrifuged at 300G for 10 minutes, the supernatant was discarded, and the pellet was resuspended in PBS to be introduced into the flow cytometer. Cellular fluorescence was determined using the flow cytometer, Spectrum III (Ortho Diagnostic Systems, Inc.). Fluorescence emission (510 to 550nm) was recorded and polymorphonuclear leukocytes were distinguished analytically from the other cell types by their characteristic forward and right-angle scattering properties. The intensity of intracellular DCF fluorescence in individual PMN and the corresponding cell counts were estimated. Hydrogen peroxide production was expressed as the mean fluorescence intensity of cells. The result for every sample was expressed as percent baseline in mixed venous blood which was obtained just before reperfusion.

#### *Plasma thiobarbituric acid reactive materials (TBARM) assay*

Lipid peroxidation was estimated by the assay of plasma thiobarbituric acid reactive materials (TBARM). Plasma TBARM were measured by the method of Yagi.<sup>6)</sup> Briefly, a heparinized blood sample was centrifuged at 300G for 10min. to separate plasma and then 0.02ml of the plasma was transferred to a centrifuge tube and 4.0ml of 0.083N H<sub>2</sub>SO<sub>4</sub> was added and mixed. Then, 0.5ml of 10% phosphotungstic acid was added and mixed. After standing at room temperature for 5min., the mixture was centrifuged at 3000G for 10min. The supernatant was discarded, and the sediment was mixed with 2.0ml of 0.083N H<sub>2</sub>SO<sub>4</sub> and 0.3ml of 10% phosphotungstic acid, and the mixture was

centrifuged at 3000G for 10min. The sediment was suspended in 4.0ml of distilled water and 1.0ml of TBA reagent was added. The reaction mixture was boiled for 60min. After, cooling with tap water, 5.0ml of n-butanol was added and the mixture was shaken vigorously. After centrifugation at 3000G for 15min., the n-butanol layer was taken for fluorometric measurement at 515nm excitation and 553nm emission. The concentration of TBARM was determined by reference to a standard curve created with 0.5nmol of tetraethoxypropane. The plasma protein content was measured by the method of Lowry *et al.*<sup>7)</sup> using bovine serum albumin as the standard. The concentration of TBARM was corrected for the plasma protein content. The result for every sample was expressed as percent baseline in mixed venous blood which was obtained just before reperfusion.

#### *Measurement of lung water content*

Upon completion of the study, the upper lobe was removed from the left lung, weighed (wet weight) and stored in a heating chamber at 70 °C until it reached a constant weight. After this drying process, the lung was reweighed (dry weight) for calculation of the wet/dry weight (W/D) ratio. W/D ratios were used as a measure of pulmonary edema.

#### *Histological studies*

Following reperfusion, a biopsy specimen was always taken from the lateral aspect of the left lower lobe in every animal, fixed in formalin and stained with hematoxylin and eosin for histologic examination.

#### *Statistics*

Values are expressed as the mean  $\pm$  standard deviation. Statistical analysis was performed by the Mann-Whitney nonparametric U test for comparison of data between groups and the Wilcoxon nonparametric U test for comparison of data at different time points. A p-value of less than 0.05 was considered significant.

## **Results**

Pulmonary function data are shown in Table I. There was a significant fall in arterial oxygen tension on 100% oxygen in both groups, however, the SOD group maintained significantly better gas exchange than did the control group at 90 and 120 minutes after reperfusion. Pulmonary vascular resistance (PVR) rose significantly in control animals, but not in the SOD group following reperfusion. At 10 minutes, PVR was significantly lower in the SOD group than in the control group. During reperfusion, both groups experienced a fall in static lung compliance. There was no significant

**Table 1.** Function parameters in reperfused lungs

reperfusion time	PaO <sub>2</sub>		PVR		Cst	
	control	SOD	control	SOD	control	SOD
baseline	414.1 ± 84.8	435.5 ± 61.5	1352 ± 228	1254 ± 311	32.2 ± 5.2	32.7 ± 3.6
10 min	317.5 ± 107.6*	360.7 ± 22.8*	2423 ± 554*	1749 ± 620	28.9 ± 3.9*	29.9 ± 2.9
60 min	275.6 ± 94.7*	352.6 ± 39.2*	2408 ± 313*	1984 ± 475	25.9 ± 3.8*	27.2 ± 4.2
90 min	219.1 ± 79.5**	302.3 ± 63.4**	2586 ± 592*	2132 ± 492	24.9 ± 3.1**	25.9 ± 4.9
120 min	168.3 ± 72.2**	286.2 ± 73.4**	2500 ± 262*	2129 ± 755	22.6 ± 3.5**	23.3 ± 4.9

Note PaO<sub>2</sub>: arterial oxygen tension (mmHg)

PVR: pulmonary vascular resistance (dynes·sec·cm<sup>-5</sup>), Cst: static lung compliance (ml/cmH<sub>2</sub>O)

#: P < 0.05 vs. control group

\*: P < 0.05 vs. baseline

\*\* : P < 0.01 vs. baseline

difference between the two groups.

The wet/dry lung weight ratio at the conclusion of experimentation was  $7.99 \pm 0.72$  and  $7.11 \pm 0.47$  in the control and SOD groups, respectively. This difference was significant ( $p < 0.05$ ).

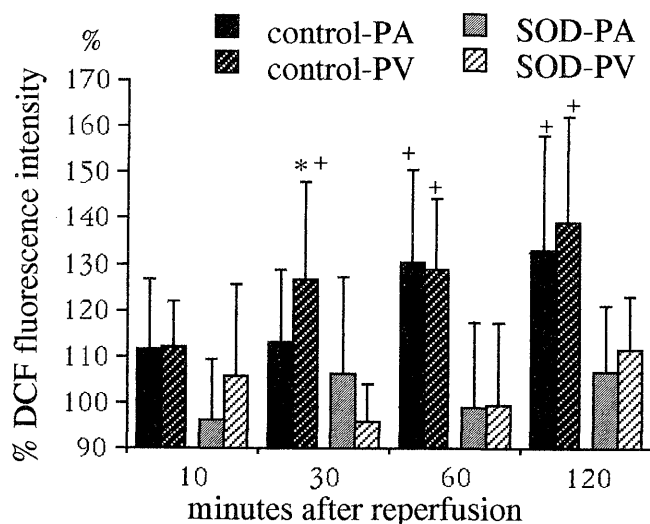
#### Neutrophil hydrogen peroxide production (Fig. 1)

Upon PMA stimulation in the control group, there was no significant increase in the hydrogen peroxide production by neutrophils collected from pulmonary artery (PA) at 10 or 30 minutes and from the pulmonary vein (PV) at 10 minutes when compared with baseline levels. However, neutrophil hydrogen peroxide production rose significantly to  $123.9 \pm 21.1\%$  at 30 minutes of reperfusion in PV neutrophils. Thereafter, this increased level was main-

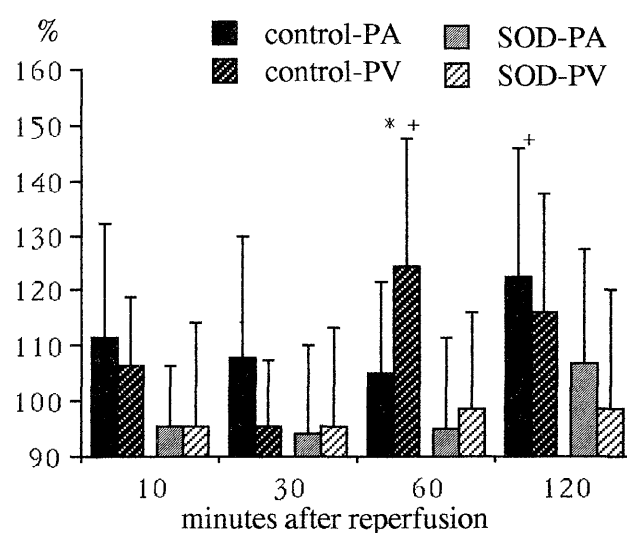
tained. Neutrophils collected from PV produced significantly more hydrogen peroxide than did those from PA only at 30 minutes of reperfusion. In the SOD group, upon PMA stimulation, there was no significant increase in neutrophil hydrogen peroxide production at any time point and there was no difference between neutrophil hydrogen peroxide production in PA and in PV. When no PMA stimulation was applied, there were few fluorescent cells, and the fluorescence intensity could not be measured.

#### Plasma TBARM assay (Fig. 2)

In the control group, levels of PV plasma TBARM increased to  $105.0 \pm 12.6\%$  of baseline at 10 minutes of reperfusion (not significant compared with baseline), fell to  $94.0 \pm 12.0\%$  at 30 minutes of reperfusion, and then increased again to  $124.2 \pm 23.6\%$  (significant compared



**Fig. 1.** Hydrogen peroxide production by neutrophils in the pulmonary artery and vein. Values are expressed as percent baseline value (before reperfusion). In control animals, hydrogen peroxide production by the PV neutrophils increased significantly (\*:  $p < 0.03$ ) when compared to PA neutrophils only at 30 minutes of reperfusion. In SOD-treated animals, there was no difference in hydrogen peroxide production between PA and PV neutrophils. +:  $P < 0.03$  vs baseline of the same group.

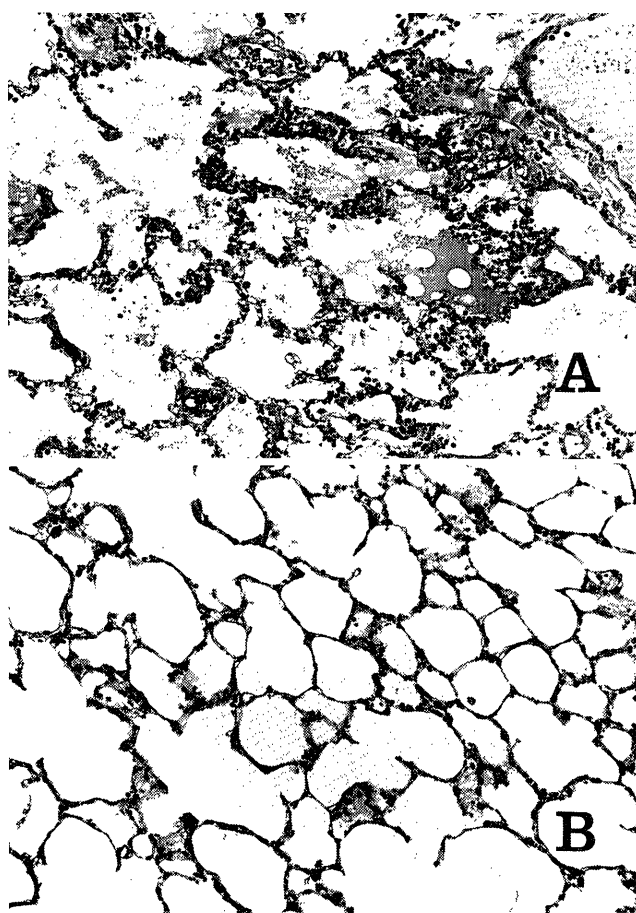


**Fig. 2.** Plasma TBARM in pulmonary artery and vein. Values are expressed as percent baseline value (before reperfusion). In control animals, TBARM in PV plasma increased significantly (\*:  $p < 0.02$ ) compared to those in PA plasma only at 60 minutes of reperfusion. In SOD-treated animals, TBAR did not change. +:  $p < 0.03$  vs baseline of the same group.

with baseline) at 60 minutes of reperfusion. The plasma TBARM in PV was significantly higher than that in PA only at 60 minutes of reperfusion. In the SOD group, there was no significant increase in plasma TBARM in any sample.

### Histology (Fig. 3)

Postmortem histological examination of lungs showed focal alveolar injury accompanied by intravascular leukocyte aggregation, interstitial edema, alveolar congestion and alveolar bleeding to a much greater extent and intensity in the control group than in the SOD group.



**Fig. 3.** Histological appearance of the lung subjected to three hours of warm ischemia followed by two hours of reperfusion. Interstitial edema, alveolar congestion and alveolar bleeding is more extensive and intense in the (A) control group than in the (B) SOD group. (Hematoxylin and Eosin,  $\times 200$ )

### Discussion

Ischemia reperfusion injury has been studied extensively in a wide variety of organs, and much of the results suggest that toxic oxygen radicals play an important role in reper-

fusion injury.<sup>8</sup> Previous findings indicate that oxygen free radicals also play a role in ischemia reperfusion injury in the lung.<sup>9-11</sup> Pulmonary ischemia reperfusion injury is characterized by increased pulmonary capillary permeability, pulmonary edema, and respiratory failure.<sup>12</sup>

After reperfusion of ischemic tissue begins, oxygen free radicals are generated by at least two sources: xanthine oxidase and neutrophils. Xanthine dehydrogenase is converted to xanthine oxidase by an activated cytosolic protease during ischemia. Xanthine oxidase then produces oxygen free radicals when the necessary substrates are available, namely, hypoxanthine which accumulates during ischemia and oxygen introduced at reperfusion.<sup>8,13</sup> Allopurinol, a xanthine oxidase inhibitor that decreases the formation of superoxide anions, can preserve some function in dog lungs subjected to two hours of warm ischemia followed by one hour of reperfusion *in vivo*.<sup>14</sup> Pulmonary endothelial cells, in particular, seem to be affected, and the damage to their cell membrane leads to cell edema.<sup>15</sup>

Oxygen free radicals may also be generated by neutrophils which marginate in the reperfused lung. Neutrophils produce superoxide anions by their membrane-bound NADPH oxidase system.<sup>16</sup> Using an isolated rabbit lung perfusion model, Breda and colleagues<sup>2</sup> reported that reperfusion with leukocyte-depleted blood protected lungs subjected to cold preservation for 24 hours against reperfusion injury. *In vivo* studies using animal models have shown that lung injury is attenuated markedly in the absence of circulating neutrophils.<sup>17</sup> Consequently, it is assumed that neutrophils play a very important role in lung ischemia-reperfusion injury.

Our results indicate that neutrophils are activated *in vivo* following ischemia and subsequent reperfusion of the lung. Without PNA stimulation, neutrophils isolated after reperfusion did not produce hydrogen peroxide. With PMA stimulation, production of hydrogen peroxide by neutrophils increased to 124% of baseline at 30min. after reperfusion. This indicates that the neutrophils may be primed *in vivo* to react more vigorously to external stimuli. The exact mechanism of this priming remains unknown and endothelial cells seem to play some role. Neutrophil priming is likely during reperfusion in the lung because both alveolar hypoxia and toxic oxygen metabolites lead to the production of leukotrienes.<sup>18</sup> Leukotrienes and platelet activating factor are potent priming agents for neutrophils.<sup>19</sup> The production of oxygen free radicals by the xanthine oxidase pathway results in the production of chemotactic factors during the metabolism of arachidonic acid.<sup>20</sup> This process, itself, leads to the sequestration and activation of neutrophils, further production of oxygen radicals and the formation of potent cytotoxic halide radicals via the myeloperoxidase pathway.

Another important finding was that there was no significant elevation in hydrogen peroxide production by PA neutrophils within 30 minutes of reperfusion, whereas

hydrogen peroxide production in PV neutrophils increased significantly during 30 minutes of reperfusion. Thus, neutrophils were activated by their passing through the ischemic lung only at 30 minutes after reperfusion and were not activated by passing through the previously ischemic lung after 60 minutes. This pattern differs from the neutrophil activation seen in skeletal muscles<sup>21)</sup> and heart<sup>22)</sup> in ischemia-reperfusion. Neutrophil hydrogen peroxide production in skeletal or cardiac muscles peaks within 15 minutes of reperfusion and then subsides gradually. Although unclear, it is likely that this time delay is related to physiological and anatomical differences, as the vascular bed in the lung has a lower pressure and is broader than in skeletal or cardiac muscles.

An increase of plasma TBARM suggests greater oxygen free radical generation.<sup>23)</sup> In our study, levels of PV plasma TBARM in control animals increased slightly at 10 minutes after reperfusion, fell to the baseline at 30 minutes, and then increased again to peak at 60 minutes after reperfusion. Levels of PV plasma TBARM were significantly higher than that of PA plasma TBARM only at 60 minutes after reperfusion. This indicates that lipid peroxidation in the ischemic lung occurs only at 60 minutes of reperfusion. It is possible that the increase in plasma TBARM seen at 10 minutes is caused by lipid peroxidation by xanthine oxidase in endothelial cells and that the increase seen at 60 minutes is caused by activated leukocytes. Hope and colleagues<sup>24)</sup> used a spin-trapping technique to demonstrate two peaks of oxygen free radical production in myocardial tissues within the first hour of myocardial reperfusion in a pig model. They speculated that the first peak is due to endogenous production, whereas the second may be due to neutrophil activation.

SOD, when given during reperfusion, is known to prevent repeatedly and successfully, reperfusion injury in a wide variety of organs.<sup>9)</sup> Isolated rabbit lungs subjected to four hours of warm ischemia rapidly develop edema on reperfusion accompanied by an increase in oxygen free radical formation—all of this can be prevented by superoxide dismutase.<sup>10)</sup> In the current study, the infusion of 35,000U/kg of SOD during reflow reduced edema formation and lipid peroxidation in the lung after three-hours of warm ischemia. This also inhibited the increase in hydrogen peroxide production by neutrophils. Some authors have expressed reservations about using large molecules, such as SOD, as an active oxygen scavenger because it would be expected to penetrate poorly the endothelial cell. However, Ratych and colleagues,<sup>25)</sup> who subjected cultured rat pulmonary artery endothelial cells to anoxia-reoxygenation reported that when SOD and catalase were added immediately prior to reoxygenation, cell damage was reduced. They discovered that anoxia-reoxygenation injury by superoxide produced by xanthine oxidase in endothelial cells occurred within or near the intravascular space. Thus, SOD may prevent endothelial cell damage by oxygen free

radicals<sup>26)</sup> and inhibit the release of neutrophil-stimulating materials (e.g., leukotriene B4 and platelet activating factor) from endothelial cells. In addition, SOD also diminishes neutrophil adherence to the endothelium.<sup>27)</sup> Therefore, SOD acts on both endothelial cells and neutrophils and thereby prevents the deterioration of lung function when ischemia is followed by reperfusion.

In conclusion, we have demonstrated that ischemia-reperfusion injury in the lung activates neutrophils, as evidenced by increased hydrogen peroxide production and free radical generation. SOD prevents these changes and protects lung function after ischemia.

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