Continuous Observation of Superoxide Generation in an In-Situ Ischemia–Reperfusion Rat Lung Model

Jun Midorikawa, MD; Kazuhira Machara, MD; Hiroyuki Yaoita, MD; Tatsuya Watanabe, MD; Hiroshi Ohtani, MD; Shinichi Ushiroda, MD; Yukio Maruyama, MD

To investigate the time course of superoxide generation in ischemia-reperfusion in the in-vivo rat lung, the present study used an enhanced chemiluminescence method with 2-methyl-6-[p-methoxyphenyl]-3,7-dihydromidazo[1,2-][pyrazin-3-one (MCLA) as a specific probe. The right pulmonary artery was occluded for 120 min, followed by 90-min reperfusion. Chemiluminescence induced by MCLA was continuously monitored by a photomultiplier exposed to the right lung. Chemiluminescence increased gradually in 30 min of reperfusion and remained elevated throughout reperfusion. The ratio of the luminescence count during reperfusion to the preischemic value increased to 2.0±0.31 (mean±SEM) (p<0.02 vs preischemic level), 2.50±0.39 (p<0.005), and 2.69±0.44 (p<0.005), at 30, 60, and 90 min of reperfusion, respectively. Bolus administration of superoxide dismutase during the reperfusion period significantly attenuated the chemiluminescence by 45.0±6.7% (p<0.01). The present results suggest that increasing oxygen radical formation leading to ischemia–reperfusion lung injury may occur even after a short period of occlusion of the pulmonary artery alone in vivo. (Jpn Circ J 2001; 65: 207–212)

Key Words: Chemiluminescence; Reperfusion lung injury; Superoxide

Pulmonary ischemia–reperfusion injury is an important contributor to a number of pulmonary diseases, including pulmonary embolism and the acute respiratory distress syndrome, and to post-transplant complications. Studies in the heart, intestines and other tissues have suggested that reperfusion-induced injury is mediated by the production of oxygen free radicals. The same mechanism appears to be involved in the effect of ischemia–reperfusion on the pulmonary circulation, but it remains unclear how oxygen free radicals are generated after a short period of occlusion of the pulmonary artery alone. Most previous studies investigating oxygen free radicals in reperfusion injury of the lungs have been performed in isolated lungs or in animals with obstruction of both the airway and the pulmonary artery: that is, hilar occlusion. In order to simulate clinical pulmonary artery occlusion and reperfusion, the generation of oxygen free radicals should be evaluated without obstructing either the airway or the bronchial circulation in in-vivo blood-perfused lungs. Although the lung is relatively resistant to reperfusion injury, Murata et al reported that 24-h reperfusion after only 2 h of occlusion of the pulmonary artery alone induced numerous foci of hemorrhagic necrosis with disrupted alveoli and accumulation of leukocytes, and these pathological changes were significantly attenuated by superoxide dismutase (SOD). Okubo et al recently reported that the continuous production of oxygen free radicals, using a chemiluminescence (CL) method in a 110-min hilar occlusion reperfusion, was significantly reduced by SOD. However, alveolar hypoxia may have affected the generation of oxygen free radicals in their study. Accordingly, it still remains unclear how superoxide is generated in reperfusion after a short period of pulmonary artery occlusion. Because of this lack of data, the best time of onset and the duration of administration of radical scavengers, if needed, is still unclear. Thus, we investigated the time course of the generation of superoxide in reperfusion after a 2-h pulmonary artery occlusion using 2-methyl-6-[p-methoxyphenyl]-3,7-dihydromidazo[1,2-][pyrazin-3-one (MCLA), which is a sensitive and specific CL probe for the detection of superoxide, as well as the effect of administration of SOD on CL, in the in-situ rat lung.

Methods

Photon Counting System

The CL monitoring system (Fig 1) has been described previously. Briefly, experiments were carried out in a special light-proof box with an R375 photomultiplier tube (Hamamatsu Photonics Inc, Hamamatsu, Japan), sensitive in the range of 160–850 nm, and a dry air jacket located in front of the shutter and window of the photomultiplier tube to avoid moisture condensation. We measured CL at 10 s intervals and the data were displayed as the count per 10 s.

Animals

Twenty-two male Wistar rats, weighing 350–450 g, were housed in a constant-temperature facility, exposed to a 12-h light–dark cycle, and given standard laboratory chow and water ad libitum. The experimental protocol was approved by Animal Research Committee in accordance with the Guidelines on Animal Experiments of Fukushima Medical University and the Japanese Government Animal Protection and Management Law (No. 115).
Surgical Preparation

The rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and maintained by further injections of pentobarbital, as needed. Animals were intubated via tracheostomy and mechanically ventilated with room air at 60 breaths/min at a tidal volume of 1.0 ml/100 g of body weight. Catheters were inserted into both jugular veins for MCLA and drug infusions. The left carotid artery was also cannulated for monitoring of arterial blood pressure and heart rate. After a midline thoracotomy, the right main pulmonary artery was dissected free from the surrounding tissue. A thread was passed around it and brought through a long rigid polyethylene tube, which served as a reversible snare occluder operated from outside the light-proof box. The surface of the right lung was then exposed in situ with a right lateral thoracotomy. The rats were placed in the box as close as possible to the window of the photomultiplier tube for measurement of the light emission from the right lung surface, the area of which was approximately 3 cm². Areas other than the target area, which could interfere with the photon count, were masked with a black cloth. The rectal temperature was monitored and maintained at approximately 37°C using circulating water-jacket (Fig 1).

Chemicals

MCLA (Tokyo Kasei Co, Tokyo, Japan) was dissolved in 0.9% saline and the solution was then divided into 10-ml aliquots for storage at –80°C and diluted to a concentration of 200 μmol/L with saline just before use. The concentration was calculated using the value of ε_{430nm}=9,600 (mol/L)⁻¹·cm⁻¹. Bovine erythrocyte SOD, which has an enzyme activity of 4,800 U/mg, was obtained from Sigma Chemicals (St Louis, MO, USA).

Experimental Protocol

After 10 min observation of natural light emission in the ischemia–reperfusion group (n=16), MCLA (200 μmol/L) was rapidly injected (1.5 ml/kg), followed by a continuous infusion of MCLA (1.5 ml·kg⁻¹·h⁻¹) until the end of the experiment. CL was observed for 45 min after injection of...
MCLA, and then the right pulmonary artery was occluded for 120 min followed by a 90-min reperfusion. Light emission was continuously observed during the experimental period. Bolus injections of SOD were administered into the jugular vein of 8 ischemia–reperfusion rats (20 mg/kg, n=3 or 40 mg/kg, n=5) when the CL value exceeded the preischemic value during the 90-min reperfusion. We also examined a sham-operated group (n=6) without right pulmonary artery occlusion and reperfusion.

Quantification of Enhanced CL During Reperfusion

The increase in enhanced CL during reperfusion was evaluated in terms of the relative intensity ratio (RIR), which was defined as the ratio of the luminescence count above natural light emission during reperfusion (C2) to the steady-state level following injection of MCLA before ischemia (C1) as shown in Fig 2. This ratio was assessed in order to correct for the nonspecific luminescence induced by MCLA.

We measured changes in enhanced CL from the steady state before occlusion of the right pulmonary artery following MCLA administration to just before administration of SOD (L1), and to the nadir following SOD (L2). SOD-induced attenuation of CL during reperfusion was estimated by the equation: \((L_1 - L_2)/L_1 \times 100\) (%) (Fig 2). This effect of SOD was evaluated for the time course of the increase in CL during reperfusion in terms of the ratio of the CL just before administration of SOD (L1, Fig 2) to the peak value after the onset of reperfusion (Lpeak, Fig 2): L1/Lpeak \times 100\%.

Statistical Analysis

Data are expressed as the mean±SEM. Factorial analysis of variance (ANOVA) was used to examine differences among the various stages in the ischemia–reperfusion and sham-operated groups. If the results were significant, data were analyzed using Fisher’s protected least significant difference (PLSD) test. Differences in the CL count before and after administration of SOD were examined by the paired t test. Unpaired t test was used to examine the difference between the effects of 20mg/kg and 40mg/kg of SOD. A p value <0.05 was considered statistically significant.

Results

Hemodynamics

The mean blood pressure and heart rate remained almost constant during the experimental period in the sham-operated group. The arterial pressure was 90.2±9.2 (n=16) mmHg during the preischemic period in the ischemia–reperfusion group and fell to 76.9±7.4 (n=16) mmHg at 60 min after the onset of ischemia and to 68.3±7.6 (n=15) mmHg at 60 min after the onset of reperfusion. These values did not differ significantly. The heart rate remained almost constant throughout the experiment in the ischemia–reperfusion group. There were no significant differences in mean blood pressure and heart rate at any stage between the ischemia–reperfusion and sham-operated groups. Data obtained 60 min after the onset of reperfusion in one rat were omitted because of an interruption in the observation by a sudden drop in arterial pressure.

CL Response

A strong burst of CL from the surface of the lung was observed after injection of MCLA (Fig 3), reflecting the increase in nonspecific CL by auto-oxidation of MCLA.10,18 During continuous infusion of MCLA, CL decreased, reaching a steady-state level in approximately 30 min and remaining fairly constant thereafter until the end of the observation period in the sham-operated group without ischemia (data not shown). In contrast, during reperfusion after the pulmonary artery occlusion, CL increased gradually and remained elevated. The overall RIRs of cases without SOD in the ischemia–reperfusion group were 2.20±0.31 at
30 min after the onset of reperfusion (n=8), 2.50±0.39 at 60 min (n=7), and 2.69±0.44 at 90 min (n=7) (Fig 4). These CL values were significantly higher during reperfusion compared with the preischemic levels (p<0.02, p<0.005 and p<0.005 vs preischemic level, respectively). The corresponding ratios in the sham-operated group were maintained at nearly 1.0.

**Effects of SOD**

Bolus injection of SOD did not affect CL at any time during the observation period in the sham-operated group. Fig 5 is a representative case in which a bolus injection of SOD administered 35 min after the onset of reperfusion caused a transient decrease in CL. The mean increase in CL was 104±25 counts/10 s just before administration of SOD in the ischemia–reperfusion group (Fig 6); the nadir of CL after injection of SOD was 65±18 counts/10 s. Thus, SOD administered at 44.8±5.3 min after reperfusion significantly inhibited the increase in CL by 45.0±6.7% (p<0.01) (Fig 6A), indicating that superoxide contributed to the increase in CL after reperfusion. There was no significant difference between the effects of 20 mg/kg of SOD (n=3, 43.2±18.9%) and 40 mg/kg of SOD (n=5, 46.1±4.2%). SOD was administered at 73.9±9.0% of the peak CL after reperfusion (Fig 6B). The peak CL in 3 rats was observed just before administration of SOD because superoxide-induced inhibition of CL persisted 90 min after the onset of reperfusion.

**Discussion**

The main findings of this study were as follows. First, a burst-like increase in CL was not observed; instead, CL was gradually and continuously produced during reperfusion after a 2-h occlusion of the pulmonary artery alone in the in-situ rat lung. Second, SOD was always effective, irrespective of the timing of its administration. These results suggest that superoxide was increasingly generated until at least the 90-min reperfusion period after the 2-h occlusion of pulmonary artery.

Previous studies have examined the generation of oxygen radicals in ischemia–reperfused lungs at either specific
times or intermittently. In addition, oxygen free radical formation has been often indirectly estimated from the preventive effects of oxygen radical scavengers, such as SOD, catalase, and dimethylthiourea or the effects of inhibitors of radical production, such as verapamil, tungsten, allopurinol, loxodamide, ATP-MgCl₂, and nitric oxide on reperfusion lung injury in isolated or in vivo lungs. These methods do not permit the continuous observation of oxygen free radical generation in ischemia–reperfusion lungs and have not provided direct evidence of oxygen free radical generation in an in vivo ischemia–reperfusion lung model. The present study is the first to continuously and directly show superoxide generation in an in situ pulmonary artery occlusion model followed by reperfusion.

**Methodological Considerations**

Analysis of CL permits continuous monitoring of the generation of oxygen free radicals without damage to biological systems. MCLA, a cypridina luciferin analog, has been found to be a highly sensitive and specific CL probe for the identification of superoxide generated by activated leukocytes and monocytes. Takahashi et al used MCLA to observe superoxide generation in an in-situ rat lung model treated with drugs. However, MCLA has a weak nonspecific luminescence that is derived from auto-oxidation at a light emission at 465nm which is not affected by SOD. In the present study, this nonspecific luminescence was represented by the steady-state level of CL following a strong burst. We corrected for this nonspecific luminescence using the ratio of the increased CL after the onset of reperfusion to the nonspecific luminescence at the preischemic stage. Moreover, CL from the surface of the lung is likely to be the predominant source of light detected, but it remains to be clarified which part(s) of the lung actually generates CL.

In the present study, CL was significantly reduced during the pulmonary artery occlusion in which MCLA was supplied only via bronchial circulation, indicating decreased amounts of MCLA because of reduced blood volume in the ischemic lung. Therefore, it is quite probable that the change in pulmonary blood flow after reperfusion might have affected CL via a change of the amount of MCLA in the lung tissue, even though hemodynamic variables did not significantly change during the reperfusion period.

**Effects of SOD**

SOD was administered from 23 to 62 min after the onset of the reperfusion. The CL was from 30% to 100%, regarding the \( L_{i}/L_{\text{peak}} \times 100\% \), throughout this period, during which SOD was always effective irrespective of the timing of its administration. However, SOD did not reduce CL to the preischemic baseline level in the present study (data not shown), which may be related to the brief half-life of SOD (6–10 min). After the generation of superoxide in the intravascular space, SOD would have been able to immediately catalyze the dismutation of superoxide. However, it is possible that SOD, a high molecular weight protein, failed to reach the interstitial space in which superoxide can be generated via the vascular endothelium. Superoxide anions reportedly increase the permeability of the lung thus the superoxide generated in the reperfused lung may enhance the vascular leakage of plasma containing MCLA. Therefore, part of the increase in CL during reperfusion may reflect not only superoxide generation, but also a nonspecific luminescence from the increased interstitial fluid. The dose of SOD did not appear to be insufficient to abolish the generation of superoxide during reperfusion because there was no significant difference between the effects of low-dose (20 mg/kg) and high-dose (40 mg/kg) SOD on CL.

**Occlusion and Reperfusion of the Pulmonary Artery Alone**

The occlusion of the pulmonary artery, bronchus and pulmonary vein showed a biphasic pattern of lung injury, as measured by changes in vascular permeability, even after a 90-min ischemia followed by reperfusion, at 30 min and 4 h of reperfusion. This hilm occlusion model has sometimes been used to investigate lung injury during ischemia–reperfusion, but we must be careful to extend those findings to the lung injury following pulmonary arterial circulatory disturbance. Namely, the lung is relatively resistant to pulmonary arterial occlusion alone, because oxygen is supplied via 2 other sources: the bronchial arterial system and the airways. Obermiller et al demonstrated that the tidal reverse pulmonary venous flow reached the alveolar tissues even when both pulmonary and bronchial inflows were obstructed in goats. Further, as for the pulmonary reperfusion injury, Modry and Chiu demonstrated that in-vivo canine lungs showed minimal morphological damage after a 1-h reperfusion preceded by a 5-h occlusion of the pulmonary artery. Bishop et al reported that a 48-h occlusion of the pulmonary artery followed by a 4-h reperfusion caused marked edema and inflammatory infiltrates in the in-vivo dog lung. Horgan et al reported that vascular permeability was increased in isolated rabbit lungs subjected in vivo to a 24-h occlusion of the pulmonary artery followed by a 2-h reperfusion; this increase was mediated by the generation of oxidants during reperfusion. Murata et al reported that pulmonary necrosis occurred after a 24-h reperfusion following a 2-h occlusion of the right pulmonary artery branch in an in-vivo rat model; necrosis was prevented by SOD, indicating that superoxide generation was involved in the reperfusion injury of the lung. Occlusion was performed for relatively short periods in the present study compared with the previous in vivo studies. It is of interest that even a 2-h ischemia of the pulmonary artery alone caused superoxide generation during subsequent reperfusion and the radicals were generated in an earlier phase than in the previous in vivo studies.

**Time Course and Possible Sources of Oxygen Free Radical Generation**

The sources of the oxygen free radicals generated during ischemia–reperfusion in the lung are not clear. Although pulmonary endothelial cells, smooth muscle cells, lung macrophages and neutrophils have been implicated, this is still controversial. Moreover, although there are many oxidase enzymes capable of forming superoxide in the lung (eg, xanthine oxidase, arachidonic acid peroxidases, nitric oxide synthase (all of which are present in endothelial cells), NADPH oxidase and NADH oxidase), it remains to be determined which is predominant. Bursts of oxidant formation within several minutes of reperfusion have been observed in other organs, and xanthine oxidase-derived oxidants have been proposed as the source. However, we did not observe a typical burst of superoxide generation soon after the onset of reperfusion, which suggests that xanthine oxidase-derived oxidants may not be critically important in ischemia–reperfusion injury of the lung. Also, the present finding was in contrast to that of Eppinger et al.
who showed the early (stimulated macrophage-mediated) and late (neutrophil-mediated) phase of reperfusion injury obtained from the left hilar occlusion in rats. Our data were obtained in a condition similar to a clinically observed disturbance of the pulmonary circulation. It is of interest that, even with a 2-h occlusion of the pulmonary artery alone followed by reperfusion with normoxic ventilation, superoxide production increased more than twice at 30 min following the reperfusion, and tended to rise further.

Conclusions

We continuously monitored superoxide generation in the in-vivo rat lung during reperfusion after a 2-h occlusion of the right pulmonary artery alone using an MCLA-enhanced CL method. Superoxide generation increased gradually, beginning less than 30 min after the onset of reperfusion and continued to increase for up to 90 min, although no burst was detected. The pathophysiology of pulmonary thromboembolism may be more complex than that represented by the present model, and therefore caution should be used when extrapolating our experimental data to the clinical setting. However, the present results suggest that even a relatively short occlusion of the pulmonary artery alone results in superoxide generation for a long time during reperfusion, and that ischemia–reperfusion lung impairment related to oxygen radical formation may occur earlier than previously suspected if continuous treatment for superoxide generation is not administered.

References


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