Anoxia-reoxygenation versus ischemia in isolated rat lungs

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Zhao, Guochang, Abu B. Al-Mehdi, and Aron B. Fisher. Anoxia-reoxygenation versus ischemia in isolated rat lungs. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L1112–L1117, 1997.—Oxidant generation in anoxia-reoxygenation and ischemia-reperfusion was compared in isolated rat lungs. Anoxia-reoxygenation was produced by N2 ventilation followed by O2 ventilation. After anoxia, lung ATP content was decreased by 59%. Oxygenated ischemia was produced by discontinuing perfusion while ventilation with O2 was maintained. With anoxia-reoxygenation, oxidant generation, evaluated by oxidation of dichlorodihydrofluorescein (H2DCF) to fluorescent dichlorofluorescein, increased 3.6-fold, lung thiobarbituric acid reactive substances (TBARS) increased 342%, conjugated dienes increased 285%, and protein carbonyl content increased 46%. Pretreatment of lungs with 100 µM allopurinol inhibited the reoxygenation-mediated increase in lung fluorescence by 75% and TBARS by 69%. Oxygenated ischemia resulted in an approximately eightfold increase in lung H2DCF oxidation and a fourfold increase in TBARS, but allopurinol had no effect. On the other hand, 100 µM diphenylidionium (DPI) inhibited the ischemia-mediated increase in lung fluorescence by 69% and lung TBARS by 70%, but it had no effect on the increase with anoxia-reoxygenation. Therefore, both ischemia-reperfusion and anoxia-reoxygenation result in oxidant generation by the lung, but a comparison of results with a xanthine oxidase inhibitor (allopurinol) and a flavoprotein inhibitor (DPI) indicate that the pathways for oxidant generation are distinctly different.

THE BIOCHEMICAL MECHANISMS for cell damage during ischemia and posts ischemic reperfusion have been described for heart, brain, intestine, kidney, and other organs (13, 26). In these organs, ischemia is invariably accompanied by tissue anoxia, whereas reperfusion reintroduces O2. Therefore, ischemia-reperfusion in essence is equated with anoxia-reoxygenation. A significant biochemical change in tissues during the ischemic phase is the sharp decrease in levels of ATP and phosphocreatine and the corresponding increase in the ATP degradation products hypoxanthine and xanthine. The latter are substrates for the enzyme xanthine oxidase that can generate superoxide when O2 reenters the tissue with reperfusion (26).

It is important to recognize that ischemia-reperfusion in lungs is physiologically different from that in organs with systemic circulation (18). Lung ischemia, for example that seen with pulmonary artery occlusion, does not necessarily result in tissue anoxia because lung ventilation can continue. Supporting evidence is the observation that the ATP content of lung tissue does not change significantly during ischemia in continuously ventilated isolated lungs (18). Therefore, ischemia-reperfusion in lungs does not equate to anoxia-reoxygenation. However, as with anoxia-reoxygenation in systemic organs, lung ischemia-reperfusion does result in oxidant generation and oxidative damage to lung tissue (2–4, 7, 16, 18). Oxidant generation actually occurs during the ischemic phase, provided that tissue oxygenation is maintained through ventilation.

A basic question is whether anoxia with reoxygenation, as distinct from ischemia-reperfusion, results in lung oxidant generation and, if so, whether the mechanisms may differ. Oxidant generation with reoxygenation has been demonstrated in atelectatic lungs after reexpansion (22), although additional factors associated with lung collapse could have contributed to the observed oxidant injury. In the present study, we utilized the isolated rat lung ventilated with gases of varying O2 content to separate anoxia-reoxygenation effects from possible changes due to ischemia or the mechanical effect of atelectasis. We used the isolated lung to evaluate mechanisms and biochemical pathways rather than to simulate pathophysiological conditions. We found generation of oxidants with anoxia-reoxygenation similar to that observed with lung ischemia with or without reperfusion. However, based on response to inhibitors, we found that the pathways for oxidant generation differ in the two models.

MATERIALS AND METHODS

Reagents. Allopurinol, aprotinin, ATP, butylated hydroxytoluene, 2,4-dinitrophenylhydrazine (DNPH), glucose-6-phosphate dehydrogenase, hexokinase, leupeptin, β-NADP, pepstatin, phenylmethylsulfonyl fluoride (PMSF), and thiobarbituric acid (TBA) were purchased from Sigma Chemical (St. Louis, MO). 2',7'-Dichlorodihydrofluorescein (H2DCF) diacetate was obtained from Molecular Probes (Eugene, OR). Diphenylidionium chloride (DPI) was obtained from ICN Biochemicals (Cleveland, OH). Protein assay reagent concentrate and γ-globulin were from Bio-Rad Laboratories (Richmond, CA). All other chemicals used were of analytic grade.

Lung perfusion. The isolated perfused rat lung model used for this study has been described previously (17). Briefly, lungs were isolated from Sprague-Dawley male rats (Charles River Breeding Laboratories, Kingston, NY) weighing 180–200 g and anesthetized with 30 mg/kg intraperitoneal pentobarbital sodium while ventilation was maintained at 60 cycles/min at 2 ml tidal volume and 2 cmH2O end-expiratory pressure. The pulmonary circulation was cleared of blood by gravity flow of perfusate with 25 cmH2O pressure through a cannula inserted in the main pulmonary artery. The perfusate medium, Krebs-Ringer bicarbonate buffer (pH 7.4) containing 10 mM glucose and 3% (wt/vol) fatty acid-free bovine serum albumin, was preincubated with the same gas mixture subsequently used for lung ventilation. The cleared lungs were freed of cardiac and other nonpulmonary tissues were suspended in a water-jacketed perfusion chamber maintained at 37°C. Perfusion was maintained using a peristaltic pump at a constant flow rate of 10 ml/min with a recirculating volume of...
40 ml. Global ischemia was produced by discontinuing perfusion for 1 h while ventilation was continued. In some experiments, ischemic lungs were reperfused for 1 h after the 1 h of ischemia. To produce anoxia, lungs were ventilated with either 95% N₂-5% CO₂ or 95% CO-5% CO₂ while perfusion was maintained; for all other experimental conditions, lungs were ventilated with 95% O₂-5% CO₂. After 5 min of ventilation with H₂DCF diacetate (0.05 mM), lungs were perfused with the fluorophore for 30 min before initiation of anoxia or ischemia. The frozen lungs were homogenized under N₂ in 10 volumes of ice-cold 0.9% sodium chloride containing 0.2% butylated hydroxytoluene. An aliquot of the homogenate was extracted with trichloroacetic acid (TCA) and was reacted with TBA at 95°C for 15 min. TBARS were determined by reaction with DNPH as described previously (8). Briefly, a portion of frozen lung tissue was extracted with cold ethanolic perchloric acid and was assayed enzymatically using reactions coupled to hexokinase and glucose-6-phosphate dehydrogenase. Xanthine oxidase activity was measured in lung homogenates (30). Frozen lungs were homogenized in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol, and 1 mM PMSF. The homogenate was centrifuged at 25,000 g for 30 min at 4°C. The resultant supernatants were chromatographed to remove endogenous substrates on Sephadex G-25 columns (Pharmacia Biotech) equilibrated with extraction buffer. The column eluate was incubated at 25°C for 5 min with 100 µM xanthine as the substrate. Uric acid formation was measured spectrophotometrically at 295 nm using the maximum linear rate and a millimolar extinction coefficient of 12.2. One unit of xanthine oxidase activity represents the conversion of 1 µmol of xanthine to uric acid/min. The protein content of the lung homogenate was measured by the Coomassie blue method using bovine γ-globulin as the standard (10).

Statistical analysis. Data were analyzed by way of analysis of variance for multiple comparisons followed by Bonferroni’s test using SigmaStat software (Jandel Scientific, San Rafael, CA). The level of statistical significance was taken as P < 0.05.

RESULTS

ATP content. Lung ATP content was significantly decreased during anoxia. At the end of 1 and 2 h of N₂ ventilation, the mean level of ATP had decreased by 59 and 70%, respectively (P < 0.05, n = 4; Table 1). After 1 h of reoxygenation following 1 h of anoxia, ATP content was restored by 31% but had not returned to the control level. The change in ATP content when lungs were ventilated for 1 h with 95% CO was similar to that for ventilation with N₂ (Table 1). CO and N₂ were compared because the former inhibits cytochrome oxidase in addition to replacing O₂ in the ventilating gas and precludes an effect of undetected gas leaks (8). The similar ATP change with the two different gases showed that N₂ ventilation indeed resulted in tissue anoxia.

Oxidant generation with anoxia-reoxygenation. Anoxia-reoxygenation of rat lungs resulted in increased

Table 1. ATP content of rat lungs after anoxia with or without reoxygenation

<table>
<thead>
<tr>
<th>Ventilation Gas (95%)</th>
<th>1-h Perfusion</th>
<th>2-h Perfusion</th>
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<tr>
<td>O₂</td>
<td>9.4 ± 0.5*</td>
<td>9.4 ± 0.5*</td>
</tr>
<tr>
<td>N₂</td>
<td>2.8 ± 0.5</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>N₂ → O₂</td>
<td>5.6 ± 0.5*</td>
<td>5.6 ± 0.5*</td>
</tr>
<tr>
<td>CO</td>
<td>3.8 ± 0.5</td>
<td>3.8 ± 0.5</td>
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</table>

Data are means ± SE for 4 lungs under each condition and are expressed as µmol/g dry wt. Ventilation gas also contained 5% CO₂. N₂ → O₂, 1 h of anoxia and 1 h of reoxygenation; O₂, control. *P < 0.05 compared with each of the other groups.
generation of oxidants as evidenced by increased fluorescence of DCF, the oxidized product of H$_2$DCF (Fig. 1). Fluorescence intensity of DCF in homogenate of lungs subjected to 1 h of N$_2$ ventilation followed by 1 h of O$_2$ ventilation increased 360% compared with control (continuously oxygenated lungs; P < 0.05, n = 4; Fig. 1). Anoxia-reoxygenated lungs perfused in the absence of fluorophore showed no change in fluorescence (data not shown).

Tissue oxidation with anoxia-reoxygenation. Lipid peroxidation was stimulated by anoxia-reoxygenation in isolated perfused rat lungs. TBARS at the end of the reoxygenation period were increased 240% (P < 0.05, n = 4) above the value for control lungs that were continuously perfused for 2 h (Fig. 2). No change in TBARS was observed after ventilation with N$_2$ for 2 h (Fig. 2). Conjugated dienes with anoxia-reoxygenation essentially paralleled the observations for TBARS and increased by 156% above control (P < 0.05, n = 4; Table 2).

Protein oxidation as measured by protein carbonyl formation also was detected with anoxia-reoxygenation in the isolated perfused rat lungs. Lungs subjected to 1 h of anoxia induced by N$_2$ ventilation did not display significant changes in protein oxidation; however, after 1 h of reoxygenation, protein oxidation indicated by DNP-reactive protein carbonyls increased by 46% from the control level (P < 0.05, n = 4; Table 2). Ventilation of lungs with N$_2$ during the second hour prevented the oxidant injury (Table 2). Lung protein carbonyl content increased from 4.0 ± 0.3 (control) to 6.0 ± 0.3 nmol/mg protein (P < 0.05, n = 4) after 1 h of reoxygenation following 1 h of CO-induced anoxia.

Oxidant generation and tissue oxidation with ischemia-reperfusion. Compatible with our previous results (3, 4, 18), ischemia resulted in increased oxidant generation and lipid peroxidation as detected by lung DCF fluorescence and TBARS. DCF fluorescence (Fig. 1) and TBARS (Fig. 2) of lung tissue after 1 h of oxygenated ischemia were significantly increased by 8.1- and 3.9-fold, respectively, compared with control. DCF fluorescence and TBARS after reperfusion (1 h after 1 h of ischemia) were essentially unchanged from the end-ischemic values (Figs. 1 and 2).
Effect of allopurinol and DPI. Addition of a xanthine oxidase inhibitor, 100 µM allopurinol, to the perfusate before anoxia-reoxygenation inhibited the subsequent increase in lung DCF fluorescence by 75% and the increase in TBARS by 69% (Fig. 3). By contrast, allopurinol added to the perfusate before ischemia had no effect on the subsequent increase in lung fluorescence or TBARS (Fig. 3). DCF fluorescence at the end of 1 h of reperfusion after 1 h of ischemia also was unchanged by pretreatment with allopurinol (data not shown). Xanthine oxidase activity of lung homogenate after ischemia (1.36 ± 0.14 µIU/mg protein; n = 4) and after anoxia-reoxygenation (1.26 ± 0.06 µIU/mg protein; n = 4) were not significantly different and essentially were not different from control (1.10 and 1.14 µIU/mg protein; n = 2). Xanthine oxidase activity of lung homogenate was inhibited completely by the addition of 50 µM allopurinol. Activity in lungs was not evaluated after perfusion with allopurinol because this low-molecular-weight inhibitor is removed by the column chromatography.

Pretreatment of lungs with 100 µM DPI gave results that were the inverse of those with allopurinol. This agent had no significant effect on increased DCF fluorescence or TBARS induced by lung anoxia-reoxygenation. However, DPI inhibited the ischemia-mediated increases in lung DCF fluorescence by 69% and TBARS by 70% (Fig. 3). Therefore, allopurinol was an inhibitor for anoxia-reoxygenation-induced oxidant generation by the lung, whereas DPI inhibited oxidant generation with lung ischemia.

DISCUSSION

Previous data from this laboratory have demonstrated that isolated lungs produce oxidants during ischemia, provided that tissue oxygenation is maintained through continued ventilation. Oxidant generation and tissue oxidative injury during ischemia were indicated in isolated lungs by oxidation of fluorophores, by depletion of tissue glutathione, and by tissue lipid and protein oxidation (3, 4, 6, 7, 16, 18). In the oxygenated ischemic model, oxidative injury depended on ventilation gas PO2 and was prevented by ventilation with N2 (3, 4, 7, 18). The present results confirm the oxidation of H2DCF and lipid during oxygenated ischemia.

The present study shows that anoxia-reoxygenation also leads to oxidant generation in isolated rat lungs. As expected, oxidant generation was not detected with anoxia alone but required the reintroduction of O2. DCF fluorescence increased 3.6-fold in lungs subjected to anoxia-reoxygenation, and oxidation of lipid (TBARS and conjugated dienes) and protein also indicated increased oxidant generation. Oxidation of H2DCF can occur via H2O2 (plus intracellular peroxidases), lipid hydroperoxides, peroxynitrite, ·OH, and perhaps other oxidants so that this fluorophore is not a specific indicator but can be considered a general index for generation of reactive O2 species (ROS; see Refs. 3 and 14). Compared with oxygenated ischemia, the changes in lung fluorescence and protein oxidation observed with anoxia-reoxygenation were somewhat less, although lipid peroxidation was similar. The increase in TBARS may reflect, in part, flux through cyclooxygenase/lipoxygenase pathways in addition to nonspecific lipid peroxidation (7). Therefore, both ischemia and anoxia-reoxygenation result in oxidant generation and tissue oxidant injury. Our previous results with combined ischemia and anoxia-reoxygenation are compatible with an additive effect on tissue TBARS (16).

Table 2. Lipid and protein oxidation in isolated rat lungs during anoxia-reoxygenation

<table>
<thead>
<tr>
<th>Condition</th>
<th>Conjugated Dienes, mOD/mg protein</th>
<th>Protein Carbonyls, nmol/mg protein</th>
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<tr>
<td>Control</td>
<td>4.8 ± 0.3</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>N2 (1 h)</td>
<td>4.0 ± 0.2</td>
<td></td>
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<tr>
<td>N2 → O2</td>
<td>12.3 ± 0.9*</td>
<td>5.4 ± 0.1*</td>
</tr>
<tr>
<td>N2 (2 h)</td>
<td>3.9 ± 0.2</td>
<td></td>
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Data are means ± SE for 4 lungs under each condition. Control lungs were continuously ventilated with O2 and were perfused for 2 h. mOD, milli-optical density units. *P < 0.05 compared with control.

Fig. 3. Effect of allopurinol and diphenyliodonium (DPI) on DCF fluorescence (A) and TBARS (B) produced by anoxia-reoxygenation or oxygenated ischemia. Data are means ± SE for 4–6 lungs for each condition. Actual values for control lungs (without inhibitors) are shown in Figs. 1 and 2. Percentage of control was calculated after subtraction of baseline values for each parameter. *P < 0.05 vs. control.
In the present study, the expected decrease in lung ATP content with anoxia was observed. Degradation of ATP can provide the substrate to xanthine oxidase for generation of oxidants. Evidence that the generation of oxidants with anoxia-reoxygenation occurs, at least in part, through the xanthine oxidase pathway was obtained by addition of allopurinol to the perfusate before anoxia. This agent markedly attenuated the increase in DCF fluorescence and TBARS with reoxygenation. At the concentration used in this study, allopurinol is a xanthine oxidase inhibitor that has been studied widely for protection against oxidant-mediated ischemia-reperfusion injury in various organs and tissues such as heart (19), kidney (31), liver (24), skeletal muscle (5), and intestine (32). Allopurinol also has been studied in several models of lung ischemia (anoxia)-reoxygenation (reoxygenation). Kennedy et al. (25) and Adkins and Taylor (1) observed in isolated rabbit lungs that ischemia (2–3 h) in the absence of ventilation followed by reperfusion (with reventilation) resulted in an oxidant-mediated microvascular injury that was significantly attenuated by 100 µM allopurinol. These published reports indicate that xanthine oxidase is involved in lung damage associated with a nonventilated lung model of ischemia-reperfusion. The present findings suggest that it is the tissue anoxia component with nonventilated ischemia that is responsible for increased flux through the xanthine oxidase pathway with reoxygenation and the protective effect of allopurinol. A recent immunohistochemical study has demonstrated a widespread distribution of xanthine oxidase in bronchial and alveolar tissues of the rat lung (27). Therefore, several cellular sites may be involved in the allopurinol-sensitive generation of oxidants with anoxia- reoxygenation, although the precise cells involved remain to be determined.

Several findings indicate that the pathways for oxidant generation in anoxia-reoxygenation and oxygenated ischemia differ. First, the effect of ischemia is not due to ATP depletion because previous data from this laboratory have shown that lung tissue ATP content is not significantly changed with ischemia when ventilation is maintained (2, 18). Second, in contrast to anoxia-reoxygenation, oxidant generation during oxygenated ischemia was not inhibited by allopurinol. Finally, addition of DPI to the perfusate before oxygenated ischemia significantly attenuated the increase in DCF fluorescence and TBARS but had no significant effect on oxidant generation with anoxia- reoxygenation.

DPI is a probe that binds and inhibits flavoproteins and has been widely used for assessing flavin-linked NADPH oxidase activity in both phagocytic and nonphagocytic cells (15, 20, 23, 28). The observed inhibitory effect of DPI on oxidant production in the isolated lung suggests a role for NADPH oxidase in oxidant generation by rat lungs subjected to oxygenated ischemia. The presence of this enzyme complex in endothelium has been suggested, and the presence of several key components has been demonstrated in human umbilical vein endothelial cells (23). Al-Mehdi et al. (2–4) previously have demonstrated that pulmonary capillary endothelium is a major site for ischemia-mediated oxidant generation. Therefore, we postulate that endothelial generation of ROS occurs through the NADPH oxidase pathway. Alternatively, DPI could limit H$_2$DCF and tissue oxidation, at least in part, through inhibition of NO synthase (29), thereby inhibiting the formation of ONOO$^-$. The latter has been shown to contribute to oxidative stress in lung ischemia-reperfusion (21). Other flavin-linked oxidases also represent potential DPI-sensitive pathways for generation of oxidants with lung ischemia and cannot as yet be excluded.

Although endothelium appears to be the predominate cellular source of oxidants with lung ischemia, other cell types, such as the macrophage that is known to have NADPH oxidase activity (20), may contribute to the lung oxidant burden. As discussed previously (18), polymorphonuclear leukocytes (PMN) are not present in significant numbers and therefore do not appear to play a significant role in oxidant generation in this isolated perfused lung preparation. However, PMN could contribute to O$_2^·$-derived radical production via the NADPH oxidase or other pathways in vivo or in isolated blood-perfused lungs.

In summary, our studies demonstrate that lung ATP content decreases during anoxia but not during oxygenated ischemia. In isolated blood-free lungs, both ischemia with or without reperfusion and reoxygenation after anoxia lead to oxidant generation and tissue oxidation. Inhibition of xanthine oxidase with allopurinol attenuates this effect in reoxygenated lungs but not in ischemic lungs. On the other hand, DPI, an inhibitor of flavoproteins such as NADPH oxidase, attenuates oxidant generation with ischemia but has no significant effect with anoxia-reoxygenation. We conclude that the generation of oxidants occurs through distinctly different pathways with ischemia and with reoxygenation after anoxia.

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