Oxidative Stress in Early Stage of Acute Lung Injury Induced with Oleic Acid in Guinea Pigs

Changqing Yang,1) Hiroshi Moriuchi,* Junko Takase, Yoichi Ishitsuka, Mitsuru Irikura, and Tetsumi Irie

Department of Clinical Chemistry and Informatics, Graduate School of Pharmaceutical Sciences, Kumamoto University; 5–1 Oe-honmachi, Kumamoto 862-0973, Japan. Received September 17, 2002; accepted December 26, 2002

Changes in several biomarkers in bronchoalveolar lavage fluid (BALF) during an early stage of lung injury induced with oleic acid were examined in guinea pigs. In addition, a possible contribution of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase to the oxidative changes in the lung injury was investigated. An intravenous injection of oleic acid increased the levels of lipid peroxidation products, lactate dehydrogenase, and total proteins, decreased the ratio of glutathione to glutathione disulfide in the BALF, and also affected the levels of other oxidative biomarkers such as superoxide dismutase and catalase in the BALF in a dose-dependent manner. Diphenyleneiodonium chloride, a NADPH oxidase inhibitor, inhibited the oxidative changes in the BALF and the decrease in partial pressure of oxygen in artery induced with oleic acid, while allopurinol, a xanthine oxidase inhibitor, had no inhibitory effects. The results demonstrate that oxidative stress would be an important mechanism of oleic acid-induced lung injury, and indicate that the NADPH oxidase-dependent pathway contributes significantly to the generation of reactive oxygen species in oleic acid-induced lung injury.

Key words: oleic acid; acute lung injury; oxidative stress; nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; diphenyleneiodonium chloride; bronchoalveolar lavage fluid

Reactive oxygen species (ROS) play an important role in several models of acute lung injury including ischemia/reperfusion and lung injuries induced with endotoxin, paraquat, oleic acid and phosgene.2–6) ROS production systems from activated polymorphonuclear leukocytes (PMNs) and endothelial cells are considered to be two major pathways in acute lung injuries.7) Extensive studies have demonstrated that sources for ROS in the lungs are reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (including the phagocytic cell oxidase and nonphagocyte oxidase in the vascular endothelial cells), xanthine dehydrogenase/xanthine oxidase (XDH/XO), and mitochondrial respiration.7) Recently, several papers reported that NADPH oxidase-like enzymes and XO in lung microvascular endothelial cells are important sources of ROS generation,8–13) and the ROS-generating pathways are distinct in different models of lung injuries.11,12) For example, endothelial NADPH oxidase is known to participate in the ROS-generating pathway with ischemia/reperfusion lung injury,14) while xanthine oxidase is involved in the ROS-generating pathway with anoxia-reoxygenation.11)

ROS generated from cells are scavenged by the antioxidant defense system, which includes enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase, as well as antioxidants such as glutathione (GSH) and vitamins A, C, and E. When production of ROS in tissues exceeds the ability of the antioxidant system to eliminate them, oxidative stress occurs, which leads to an accumulation of lipid peroxidation products (e.g., malondialdehyde (MDA)), an increase in oxidized glutathione (GSSG), and a decrease in the ratio of GSH/GSSG.12,13)

Oleic acid-induced lung injury is well known as a model of acute respiratory distress syndrome (ARDS).14) Intravenous administration of oleic acid can produce neutrophil activation15,16) through aggregation, attachment to endothelial cells,17) and activated neutrophils can injure the capillary endothelium by releasing ROS such as superoxides.16,18) A possible mechanism of oleic acid-induced lung injury is that superoxide anion is converted to an active oxidant-hydroxyl radical through the Fenton reaction, and the radical may cause direct tissue injuries by lipid peroxidation.19–21) Lipid peroxidation may lead to loss of the functional integrity of the cell membranes, culminating in an acute increase in alveolar-capillary permeability. There is some evidence demonstrating that lipid peroxidation may contribute to oleic acid-induced lung injury.21,22) The contributions of NADPH oxidase, XDH/XO, and effects of the antioxidant defense system have been reported in animal models of acute lung injuries.2,6,9,11,23,24) but only a few papers demonstrated an involvement of the system in oleic acid-induced lung injury.24) In addition, the characteristics of the ROS-generating pathway with oleic acid-induced lung injury are unclear.

The increase in concentrations of bronchoalveolar lavage fluid (BALF) biomarkers has been proposed to be one of the sensitive indicators of lung injury.25,26) In the present study, we examined oxidative changes in acute lung injury induced by oleic acid using BALF biomarkers such as thiobarbituric acid reactive substances (TBARS), lactate dehydrogenase (LDH) activity, total proteins, GSH, SOD, and catalase. We also assessed whether or not NADPH oxidase and/or xanthine oxidase contribute to ROS generation in oleic acid-induced lung injury using diphenyleneiodonium chloride (DPI), a NADPH oxidase inhibitor, or allopurinol, a xanthine oxidase inhibitor.

MATERIALS AND METHODS

This study was approved by the Animal Care and Use Committee of Kumamoto University. The care and handling of the animals were performed in accordance with National Institutes of Health guidelines for the care and handling of animals.

* To whom correspondence should be addressed. e-mail: moriuchi@gpo.kumamoto-u.ac.jp © 2003 Pharmaceutical Society of Japan
Drugs and Chemicals  Oleic acid was purchased from ICN Biomedicals Inc. (Aurora, Ohio, U.S.A.). DPI and allopurinol were purchased from SIGMA Chemical Co. (St. Louis, MO, U.S.A.).

Experimental Procedure  Hartley strain guinea pigs of either sex were obtained from KBT-Oriental (Tosu, Saga, Japan), and fasted for 24 h before starting the experiments. Fifteen µl/kg of oleic acid was used in all studies, except for the dose-dependent study of oleic acid in which doses of 15, 30, 60 µl/kg oleic acid were used. Six hundred µg/kg DPI or 10 mg/kg allopurinol was injected 30 min before the oleic acid injection in the test groups. Intravenous injections of oleic acid were conducted without any vehicle using a micro-syringe. A small amount of saline was used to flush out the oleic acid from the catheter into the vein.

Oleic Acid-Induced Changes in Biomarkers in BALF  Animals were randomly assigned to the study groups. Twenty (642±29 g, one saline group and four oleic acid groups) and 40 guinea pigs (491±33 g, one saline group and three oleic acid groups) were used in a time- and dose-dependent study of oleic acid on oxidative changes in the BALF, respectively. Forty guinea pigs (498±38 g, saline, oleic acid, DPI+oleic acid, and allopurinol+oleic acid groups) were used to examine the effects of DPI and allopurinol on oxidative stress induced with oleic acid. The animals were anesthetized with pentobarbital sodium (25 mg/kg i.p.), and an operation was performed under local anesthesia with 2% procaine to kill the pain further and diminish reflective movement to pain. A catheter (1.1 mm outer diameter) was inserted into a unilateral subclavian artery for blood sampling, and the other catheter was inserted into the other unilateral subclavian vein for the injection of saline, DPI, allopurinol, and oleic acid. To perform the bronchoalveolar lavage, further administration of pentobarbital sodium (50 mg/kg i.p.) was done 15 min before the bronchoalveolar lavage. The whole lungs were then lavaged twice with 10 ml of ice cold saline (the recovery was routinely 90% or greater), and the two BALFs were combined and centrifuged at 400 g for 10 min at 4°C. The aliquots of the supernatant were frozen at −85°C for subsequent assayng. In the time-dependent study, the animals underwent bronchoaveolar lavage 0.5, 1, 1.5, or 2 h after injection of oleic acid. In the other studies, bronchoalveolar lavage was done 1.5 h after oleic acid injection.

Effects of DPI and Allopurinol on Partial Pressure of Oxygen in Artery (PaO₂)  Thirty guinea pigs (502±44 g) were randomly divided into three groups: oleic acid alone, DPI+oleic acid, or allopurinol+oleic acid. The animals were pretreated with saline, DPI, or allopurinol, respectively, 30 min before the oleic acid injection. Oleic acid was then injected and the blood gas was measured. The partial pressure of arterial oxygen (PaO₂), partial pressure of carbon dioxide in arterial blood (PaCO₂), and pH were measured 5 min before the oleic acid injection (defined as the values at 0 min), and 6, 10, 15, 35, 55, and 75 min after the injection. For the measurements of blood gas parameters, 150 µl of blood was collected and analyzed with a blood gas analyzer (ABL300-Acid-Base Laboratory, Radiometer Co., Japan).

Determination of Biomarkers in the BALF  The extent of lipid peroxidation was estimated as the concentration of TBARS using a lipid peroxidation test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). LDH and total protein in the BALF were analyzed using a bio-analyzer (Hitachi 7600; Japan). The levels of GSH and GSSG were determined using a GSH/GSSG assay kit (Bioxytech GSH/GSSG-412, Oxis International, Inc., Portland, U.S.A.). The SOD and catalase activities were determined using an SOD assay kit (Bioxytech SOD-525, Oxis International, Inc., Portland, U.S.A.) and a catalase assay kit (Amplex Red Catalase Assay Kit, Molecular Probes, Inc., Eugene, U.S.A.), respectively.

Statistical Analysis  Results are expressed as the mean±S.E.M. In the assessment of oxidant changes among the values of groups, Bartlett’s test was employed to examine the uniform variance of data. Significant differences were then identified after the data were further analyzed by Dunnett’s or Tukey’s multiple comparison test. In the comparison of blood gas measurements, the F test was employed to examine the uniform variance of data. Significant differences were then identified after the data were further analyzed by Student’s t-test. A p<0.05 was considered significant.

RESULTS

Oleic Acid-Induced Changes in Biomarkers in BALF  An intravenous injection of oleic acid at a dose of 15 µl/kg to guinea pigs time-dependently increased the level of TBARS in the BALF (Fig. 1). The TBARS levels in the BALF 1.5 h and 2 h after the oleic acid injection significantly increased to 2.46 (p<0.05) and 3.50 (p<0.01) times the value in the control group, respectively, but the TBARS levels in the plasma were not significantly different between the saline and oleic acid groups at 1.5 h (2.56±0.17 and 3.53±0.62 nmo/l/ml, p=0.1241). Table 1 shows the dose-dependent effects of oleic acid on biomarkers in the BALF 1.5 h after the oleic acid injection. The TBARS levels increased to 2.49 (p<0.01), 2.96 (p<0.01), and 6.22 (p<0.01) times the value in the control group after the oleic acid injection at doses of 15, 30, and 60 µl/kg, respectively.

The LDH activity in the BALF served as a marker of alveolar cell injury, and the total protein concentration in the BALF was determined as a marker of alveolar-capillary membrane compromise. In the present study, LDH activity increased to 30.5 (p<0.01), 22.7, and 133.1 (p<0.01) times the level in the control group after 15, 30, and 60 µl/kg oleic acid injection, respectively, and the total protein level increased to 4.33 (p<0.05), 4.89 (p<0.01), and 15.25 (p<0.01) times the value in the control group after 15, 30, and 60 µl/kg oleic acid injection, respectively.
Changes Induced by Oleic Acid

The TBARS level in the BALF significantly (p<0.01) times the level in the control group after 15, 30, and 60 μl/kg oleic acid injection, respectively.

The intravenous injection of oleic acid decreased the GSH level and its ratio to GSSG in the BALF in a dose-dependent manner. The GSH level decreased to 86.7% (p<0.05), 93.8% (p<0.05) and 84.4% (p<0.05) of the value in the control group after oleic acid injection at doses of 15, 30, and 60 μl/kg, respectively. The GSH/GSSG ratio also decreased to 68.2% (p<0.01), 70.4% (p<0.01), and 57.1% (p<0.01) of the value in the control group after oleic acid injection at doses of 15, 30, and 60 μl/kg, respectively.

SOD activity also increased to 3.74 (p<0.01), 4.12 (p<0.01), and 6.92 (p<0.01) times the value in the control group after the injection of 15, 30, and 60 μl/kg oleic acid, respectively. Catalase concentration in the BALF was decreased to 76.5% (p<0.01), 86.9% (p<0.01), and 93.1% (p<0.05) of the value in the control group after the injection of 15, 30, and 60 μl/kg oleic acid, respectively.

**Effects of DPI and Allopurinol on Lung Oxidative Changes Induced by Oleic Acid**

The TBARS level in the BALF significantly (p<0.01) increased in the 15 μl/kg oleic acid groups compared with the control group. The increase in the TBARS level was significantly inhibited by DPI (p<0.01), but was still higher (p<0.01) than in the control group (Table 2). The LDH activity increased to 30.2 (p<0.01) times that of the control group after 15 μl/kg oleic acid and the increase was significantly inhibited by DPI (p<0.01). Total proteins also increased in parallel with the increase in LDH, which was 5.08 (p<0.01) times the level of the value in the control group after 15 μl/kg oleic acid, and the increase was also significantly inhibited by DPI (p<0.05). However, allopurinol had no inhibitory effects on the increase in LDH and total proteins in the BALF.

The decrease in the GSH/GSSG ratio in the BALF induced with oleic acid was significantly inhibited by DPI pretreatment, but the level of GSH was not affected (Table 2). SOD activity increased significantly (p<0.01) in the 15 μl/kg oleic acid group compared with that in the control group, and the rise in SOD activity was significantly inhibited by DPI (p<0.01), but was still higher (p<0.05) than that in the control group. However, allopurinol had no inhibitory effects on these parameters associated with the oxidant changes induced by oleic acid.

**Effects of DPI and Allopurinol on PaO₂ in Oleic Acid-Induced Lung Injury**

The pretreatment of DPI (i.v.) significantly inhibited the decreases in PaO₂ 6, 10, and 15 min after oleic acid injection, while allopurinol had no inhibitory effects (Table 3). On the other hand, in all groups, no significant changes in PaCO₂ and pH were observed after the oleic acid injection, compared with the value at 0 min and between the control and test group (the data not shown).

### Table 1. Dose-Dependent Effect of Oleic Acid on Changes in Bronchoalveolar Lavage Fluid Biomarkers

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>15 μl/kg</th>
<th>30 μl/kg</th>
<th>60 μl/kg</th>
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<tbody>
<tr>
<td>TBARS (nmol/ml)</td>
<td>0.19±0.02</td>
<td>0.47±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.56±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactate dehydrogenase (U/ml)</td>
<td>1.2±0.8</td>
<td>30.2±2.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.2±7.2</td>
<td>159.7±28.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total protein (mg/ml)</td>
<td>16.3±1.3</td>
<td>70.5±5.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>79.7±4.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>248.6±23.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSH (μmol/ml)</td>
<td>2.42±0.18</td>
<td>1.73±0.10</td>
<td>2.11±0.15</td>
<td>1.90±0.16</td>
</tr>
<tr>
<td>GSH/GSSG ratio</td>
<td>7.90±0.72</td>
<td>5.39±0.98&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.56±1.09&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.51±0.59&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOD (units/ml)</td>
<td>2.47±0.54</td>
<td>9.23±0.51&lt;sup&gt;e&lt;/sup&gt;</td>
<td>17.39±1.06&lt;sup&gt;e&lt;/sup&gt;</td>
<td>17.11±2.32&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Catalase (IU/ml)</td>
<td>3.49±0.06</td>
<td>2.67±0.104&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3.03±0.048&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3.25±0.066&lt;sup&gt;f&lt;/sup&gt;</td>
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</tbody>
</table>

*<sup>a</sup> p<0.05, compared with saline groups. All values are shown as the mean±S.E.M. (n=10).*

### Table 2. Effects of DPI and Allopurinol on Changes in Bronchoalveolar Lavage Fluid Biomarkers

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Oleic acid (15 μl/kg)</th>
<th>DPI+oleic acid (15 μl/kg)</th>
<th>Allopurinol+oleic acid (15 μl/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS (nmol/ml)</td>
<td>0.20±0.02</td>
<td>0.48±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.37±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.33±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactate dehydrogenase (U/ml)</td>
<td>1.0±0.7</td>
<td>36.6±6.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.8±5.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.0±11.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total protein (mg/ml)</td>
<td>13.5±1.4</td>
<td>68.8±4.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.4±5.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>64.1±9.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSH (μmol/ml)</td>
<td>2.42±0.18</td>
<td>1.95±0.17</td>
<td>2.48±0.24</td>
<td>1.89±0.16</td>
</tr>
<tr>
<td>GSH/GSSG ratio</td>
<td>9.53±1.69</td>
<td>4.28±0.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.79±1.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.30±3.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOD (units/ml)</td>
<td>2.27±0.60</td>
<td>5.30±0.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.03±0.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.79±1.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Catalase (IU/ml)</td>
<td>3.48±0.02</td>
<td>2.81±0.085&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.523±0.011&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.394±0.050&lt;sup&gt;b&lt;/sup&gt;</td>
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*<sup>a</sup> p<0.05, compared with saline groups.  <sup>b</sup> p<0.05, compared with 15 μl/kg oleic acid groups. All values are shown as the mean±S.E.M. (n=10).*

### Table 3. Effects of DPI and Allopurinol on Changes in Partial Pressure of Oxygen in the Artery by Oleic Acid

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Partial pressure of oxygen in the artery (mmHg)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Oleic acid (15 μl/kg)</td>
</tr>
<tr>
<td>0</td>
<td>103.2±3.2</td>
</tr>
<tr>
<td>6</td>
<td>53.3±3.5</td>
</tr>
<tr>
<td>10</td>
<td>48.2±3.6</td>
</tr>
<tr>
<td>15</td>
<td>52.1±4.0</td>
</tr>
<tr>
<td>35</td>
<td>69.8±3.3</td>
</tr>
<tr>
<td>55</td>
<td>80.2±2.7</td>
</tr>
<tr>
<td>75</td>
<td>88.6±3.0</td>
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</tbody>
</table>

*<sup>a</sup> p<0.05, compared with the oleic acid group at each time; mean±S.E.M. (n=10).*
DISCUSSION

We measured the changes in the levels of TBARS, LDH, total proteins, GSH, SOD, and catalase in the BALF during the early stage of the oleic acid-induced lung injury to gain further insight into the mechanism(s).

Since TBARS, including MDA and other lipid peroxides, are made from oxidized membrane lipids, the level of TBARS in the BALF can be used to assess the oxidative damage to the cell membranes in the lungs. Simultaneously, we measured LDH activity as a marker of alveolar cell injury, and total proteins as a marker of alveolar-capillary membrane compromise. Our data showed that an injection of oleic acid increased TBARS levels in the BALF in a dose- and time-dependent manner. In addition, LDH activity and total proteins in the BALF increased with increasing doses of oleic acid, and these increases were parallel to the increase in the TBARS level. These results indicate that the increased TBARS level in the BALF reflects enhanced oxidative damage to the cell membranes of alveolar cells and pulmonary endothelial cells, and suggest that the cell injury may at least partially contribute to oleic acid-induced pulmonary hyperpermeability. Zhao and coworkers reported that increased TBARS levels in lung tissue after oleic acid injection would be an important mechanism of the oleic acid-induced lung injury. In contrast, Ward et al. reported that the plasma levels of conjugated dienes, another index of lipid peroxidation in the oleic acid-injected rats, were not significantly different from those in saline-injected animals. Their results seem to suggest that oleic acid-induced lung injury is an oxygen radical-independent lung injury. However, in our study, although TBARS levels in BALF were significantly different between the saline and oleic acid groups, there was no significant difference in the plasma levels. Taken together, we believe that oxidative indexes of lipids in BALF can reflect the changes in lung tissue in oleic acid-induced acute lung injury, while those in the plasma can not. Therefore, the suggestion by Ward et al. that oleic acid-induced lung injury is an oxygen radical-independent lung injury should be reconsidered.

GSH is an intracellular thiol that is ubiquitously present in all tissues and in various body fluids, with especially high levels in lung tissue and BALF. When mammalian cells are exposed to increased oxidative stress, GSH is able to react with and scavenge $H_2O_2$ to form GSSG, following which the GSH/GSSG ratio decreases. Therefore, glutathione is potentially an important antioxidant, and measurement of the GSH level or determination of the GSH/GSSG ratio is a useful indicator of oxidative stress. The present data indicate that the GSH/GSSG ratio in the BALF decreased significantly in response to oleic acid injection in a dose-dependent manner, demonstrating that oleic acid injection may produce oxidative stress.

SOD catalyzes the dismutation of superoxide radicals into hydrogen peroxide and oxygen. The hydrogen peroxide can lead to the production of oxidant-hydroxyl radical through the Fenton reaction, and then the oxidant-hydroxyl radical can cause direct tissue injury by lipid peroxidation. In the present study, SOD activity in the BALF was significantly increased by oleic acid dose-dependently. The result indicates that superoxide production is increased as the dose of oleic acid increases, and also indicates that the contribution of SOD to oleic acid-induced lung injury may be harmful because an increase in SOD activity can facilitate production of hydrogen peroxide and further increases the level of the toxic oxidant-hydroxyl radical, which seems to cause increases in the TBARS level (Table 1). Therefore, the result further confirms that oxidative stress contributes to oleic acid-induced lung injury.

In general, catalase activity increases in parallel to SOD activity in lung injuries such as bleomycin- and ethanol-induced lung injuries. In the present study, we did not examine the activities of enzymes in lung tissue, but did show that catalase activity decreased and SOD activity increased in the BALF due to the oleic acid injection. At the present time, the precise mechanism cannot be determined. However, one possible explanation is that there might have been an overuse of catalase which led to the decrease in catalase activity, if there had been a difference in the relative amount between SOD and catalase in terms of scavenging activity. In addition to their activity in BALF, whether SOD and catalase in lung tissue are affected by oleic acid or not is intriguing. Therefore, we are planning to examine this further in the near future.

There are two major sources of the production of superoxide anions. One is NADPH-dependent oxidase, which is not only present in the plasma membrane of phagocytes and macrophages, but also present in pulmonary endothelial cells. The other is xanthine oxidase.

The doses of DPI, a NADPH oxidase inhibitor, and allopurinol, a xanthine oxidase inhibitor, to inhibit superoxide generation were chosen as follows. Some in vitro studies have reported that DPI at concentrations ranging from 10 to 100 $\mu$m exerted an inhibitory effect on superoxide generation from pulmonary microvascular endothelial cells stimulated with paraquat or phorbol ester (PMA), and the inhibition of NADPH oxidase activity in neutrophils was complete at DPI concentrations above 10 $\mu$m. A dose of 600 $\mu$g/kg DPI (i.v.) was chosen here because intravenous administration of this dose would produce a maximum blood concentration of about 30 $\mu$m based on the assumption that the blood volume of the guinea pig is about 5.8% of body weight and DPI is only distributed to the blood initially. On the other hand, Hultkvist-Bengtsson and Martensson reported that pretreatment with 10 mg/kg allopurinol completely inhibited the elevation in uric acid concentration in the plasma after oleic acid infusion. Their results suggest that the oxygen radical producing hypoxanthine/xanthine oxidase (XDH/XO) reaction contributes to oleic acid-induced lung injury because XDH catalyzes the reactions of purine metabolism to produce uric acid, and XDH can be converted to XO, following which NO uses molecular oxygen as its electron carrier, producing superoxide. However, in their study they did not assess any oxidant changes. A recent study using isolated perfused rat lung has shown that ROS production is inhibited by allopurinol (100 $\mu$m) in a model of anoxia/reoxygenation lung injury. Based on the above observations, an intravenous dose of 10 mg/kg allopurinol was assumed to produce a maximum blood concentration of 1.25 mm in guinea pigs, and the level could be enough to inhibit ROS production by hypoxanthine/xanthine oxidase. Our results clearly demonstrate that DPI inhibited the oxidative changes in the BALF and the decrease in $P_{O_2}$ induced by oleic acid, while allopurinol had
no inhibitory effects.

Our previous study has shown that neutrophils activated by oleic acid release oxygen free radicals.\textsuperscript{16} In such a situation, NADPH oxidase from neutrophils is thought to be one of the most promising ROS generating enzymes in oleic acid-induced lung injury. A recent study has demonstrated that NADPH oxidase is responsible for the generation of superoxide in mouse lung microvascular endothelial cells stimulated with PMA, and that the superoxide release is inhibited by DPI.\textsuperscript{16} These results indicate that NADPH oxidases, originating from lung microvascular endothelial cells as well as from neutrophils, participate in superoxide generation.

Oxidative stress may be an important pathogenetic mechanism of oleic acid-induced lung injury and the NADPH oxidase-dependent pathway may contribute significantly to the generation of ROS in oxidative stress.

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1) Present address: Department of Pharmacy, Affiliated Hospital of Yanbian University Medical College; Department of Pharmaceutical Sciences, College of Pharmacy, Yanbian University, Yanji City, Jilin Prov. 133000 China.


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