Preventive Effect of Phosphoenolpyruvate on Hypoxemia Induced by Oleic Acid in Guinea Pigs

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Oleic acid-induced hypoxemia is an animal model of acute respiratory distress syndrome (ARDS). Increased capillary permeability is a cause of hypoxemia in lung injury. Endothelial cells form a major capillary barrier, and disruption of the barrier appears to involve a decreased level of ATP in the cells. Phosphoenolpyruvate (PEP) is an endogenous substance that is one of the ATP precursors and can cross some cell membranes via anion exchanger. We examined the effect of PEP on oleic acid-induced lung injury in guinea pigs. An intravenous injection of oleic acid (15 μL/kg) caused severe hypoxemia. Pretreatment with PEP at a dose of 2, 20, or 200 μmol/kg attenuated the oleic acid-induced decrease in the arterial partial pressure of oxygen in a dose-dependent manner. Furthermore, PEP attenuated the oleic acid-induced increase in vascular permeability in the proximal and distal bronchi, as indicated by the extravascular leakage of Evans Blue dye. The combination of PEP with ATP (4 μmol/kg) showed no additional inhibitory effect on oleic acid-induced lung injury, compared with PEP alone. We suggest that PEP is a promising candidate to prevent hypoxemia in acute lung injuries associated with increased vascular permeability, such as ARDS.

Key words oleic acid; phosphoenolpyruvate; ATP; lung injury; hypoxemia

Hypoxemia is very dangerous state that can lead to brain damage and/or death. Acute respiratory distress syndrome (ARDS) is a diffuse lung injury that results in severe hypoxemia (impaired gas exchange), loss of lung compliance, and noncardiogenic edema. This condition is the end result of common pathways initiated by a variety of local or systemic insults leading to diffuse damage to the pulmonary parenchyma. Despite the accumulation of abundant information regarding the physiological and cellular basis of lung injury and increasingly sophisticated intensive care, improved prognosis has lagged behind. It has become clear that there is not one mediator responsible for ARDS but rather a complex interplay between diverse proinflammatory agents (e.g., lipopolysaccharide, complement products, cytokines, chemokines, reactive oxygen species, and eicosanoids) and antiinflammatory (interleukin-10, interleukin-1-RA, PGI2) mediators. The proinflammatory mediators may finally disturb the energy generation that is necessary for maintaining the normal functions and structure of the lung.

Oleic acid-induced lung injury, a well-described laboratory model of acute lung injury, has morphologic and cellular changes similar to those in ARDS. It is characterized by diffuse interstitial and alveolar edema with focal hemorrhage and vascular congestion and by interstitial and alveolar infiltration of leukocytes. Lung compliance and arterial oxygenation are decreased, whereas pulmonary arterial pressure and extravascular lung fluid are increased. There is a decrease in vascular perfusion of the edematous lung regions and increased heterogeneity of perfusion.

Decreased ATP levels in the lung tissues can be induced by impaired gas exchange. Sanders and Baylin showed that in lung injuries caused by ischemic and 100% O2 exposure, there was a marked decrease in ATP levels in pulmonary tissues. There appears to be a relationship between lung injury and ATP level in lung cells in terms of the permeability barrier. Increased pulmonary capillary permeability is one cause of severe hypoxemia in lung injuries. Endothelial cells form a major part of the capillary permeability barrier, and changes in the cells are associated with increased capillary permeability. Microfilaments are a major cytoskeletal structure involved in maintaining endothelial cell shape. Disruption of microfilaments is an ATP-dependent phenomenon.

Phosphoenolpyruvate (PEP) is an intermediate substance of glycolysis. It can cross the cell membrane and is converted to enolpyruvate by pyruvate kinase. With the transfer of the high-energy phosphate group to adenosine diphosphate (ADP), intracellular ATP is replenished. One molecule of ATP is generated from one molecule of PEP. It has been reported that PEP improves the energy status after ischemia of the heart and skeletal muscle. PEP has the potential to improve energy metabolism immediately after hepatic ischemia and reperfusion in rats.

In light of its pharmacological properties, PEP may be effective in preventing lung injury caused by oleic acid. In this study the possible preventive effect of PEP and PEP plus ATP on oleic acid-induced hypoxemia was evaluated. Furthermore, we examined the effect of PEP on increased vascular permeability following oleic acid injection.

MATERIALS AND METHODS

This study protocol 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.

Materials Male Hartley strain guinea pigs (500–800 g; Ark Resource Co., Ltd., Kumamoto, Japan) with free access

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to food and water were used. Sodium phosphoenolpyruvate monohydrate was kindly donated by Ube Kousan (Ube, Yamaguchi, Japan). Oleic acid was purchased from ICN Biomedicals Inc. (Aurora, OH, U.S.A.). ATP and Evans Blue were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Saline was commercially obtained from Otsuka Pharmaceutical Co. (Naruto, Tokushima, Japan). Other materials were of reagent grade, and deionized double-distilled water was used.

**Blood Gas Parameters**  Animals were randomly assigned to the treatment or control groups. An operation was performed under sodium pentobarbital (25 mg/kg i.p.) anesthesia, during which both subclavian arteries and one of the subclavian veins were catheterized. Arterial catheters were used for blood sampling, and the venous catheter was used for the injection of reagents. Lung injury was induced by a single injection of oleic acid (15 μl/kg). Five test groups with the respective control groups were studied. The animals in groups I to III received 2, 20, and 200 mol/kg of PEP, respectively. The animals in group IV were injected with 2 μmol/kg of PEP plus 4 μmol/kg of ATP. These injections were performed 20 min before oleic acid injection. Animals with PaO2 less than 80 mm/Hg at time 0 were excluded from the experiment. In the case of normalization of PaO2, the value at time 0 was considered to be 100% and measurements at the other times were calculated as follows:

\[ \text{PaO}_2 (\%) = \frac{\text{PaO}_2 (\text{mm/Hg}) \text{ at a time}}{\text{PaO}_2 (\text{mm/Hg}) \text{ at time 0}} \times 100 \]

For measurements of blood gas parameters, 200 μl of blood was collected and analyzed with a blood gas analyzer (IL 1304, Instrumentation Laboratories, Lexington, MA, U.S.A.).

**Pulmonary Vascular Permeability**  Evans Blue dye was used as a marker of airway vascular permeability. Male Hartley strain guinea pigs (500—800 g) were anesthetized with sodium pentobarbital (25 mg/kg i.p.). An operation was performed with local anesthesia as explained in the section above describing the measurement of blood parameters. A catheter was inserted in one of the subclavian veins. The animals in the treatment and control groups received an injection of PEP (200 μmol/kg) and saline (1 ml/kg) 20 min before oleic acid injection, respectively. Evans Blue (30 mg/kg) was administered 1 min before oleic acid injection (15 μl/kg).

In the control (saline+saline) group, saline 15 μl/kg was administered instead of oleic acid. Further sodium pentobarbital (8.3 mg/kg i.p.) was administered 30 and 60 min after oleic acid injection. Ninety minutes after oleic acid injection, the chest cavity was opened. Evans Blue dye was washed out from vascular space by perfusion with 100 ml of saline. For this purpose, a 13-gauge blunt cannula was inserted and clamped through a right ventriculotomy into the pulmonary artery. The left atrium was opened for perfusate outflow. The lungs were perfused in situ using an infusion pump (Eyela Micro Tube Pump MP-3, Kikakikai, Co., Ltd., Tokyo, Japan) at the rate of 3 ml/min. The airways with the parenchyma were then removed and the parenchyma was carefully scraped off. The airways were separated into the trachea, main bronchus, and intrapulmonary branches. The intrapulmonary branches were further subdivided into the proximal and distal parts. Each wet tissue was then weighed. Evans Blue dye was extracted in 2 ml of 100% formamide after 18 h incubation at 37 °C. The dye concentration was determined by light absorbance at 620 nm with a spectrophotometer (U 3200, Hitachi, Tokyo, Japan). Interpolation of data was performed using a standard curve for absorbance of various concentrations of Evans Blue ranging from 0 to 5 μg/ml. The amount of extracted dye from the tissue was expressed as ng/mg of tissue wet weight.

**Statistical Analysis**  Comparisons between paired values were performed using the paired Student's t-test. Comparisons between unpaired values were performed using the unpaired Student's t-test or Mann-Whitney U-test after an examination of uniformity of variance with the F-test. Multiple comparisons were made to examine the statistical significance of the data on pulmonary vascular permeability. After an examination of uniform variance using Bartlett analysis (p<0.05), significant differences (p<0.05) were identified using Scheffe's test.

## RESULTS

### Effects on Blood Gas Parameters

In the control groups, an injection of oleic acid 15 μl/kg caused a significant decrease in PaO2. The maximal decrease of 45—55% of the value at time 0 was observed 6 or 10 min after oleic acid injection, after which PaO2 was normalized over a period of 50 min. Pretreatment with PEP (2 μmol/kg) attenuated the decrease in PaO2 compared with the control groups 6, 10, 15, 35, 55, and 75 min after oleic acid injection (Table 1). When each PaO2 value was compared with the value at time 0,
there were less decrease in PaO₂ in the PEP groups than in the control groups. Similarly, PEP 20 and 200 μmol/kg significantly prevented arterial oxygen reduction at nearly all the examined times (Tables 2 and 3). When PaO₂ was normalized based on the values at time 0 being 100%, there was a significant difference between some PaO₂ values in the PEP groups and corresponding values in the control groups (data for PEP 200 μmol/kg are shown as a representative example in Fig. 1).

In Fig. 2, an attenuation of the decrease in PaO₂ is estimated using a comparison of the cumulative percentage of change in PaO₂ up to 75 min after oleic acid injection (hypoxemic effect), calculated by summing the area above (negative values) and below (positive values) the control level (PaO₂=100%). Pretreatment with PEP at a dose of 2, 20, or 200 μmol/kg attenuated the oleic acid-induced decrease in the arterial partial pressure of oxygen in a dose-dependent manner. As shown in Tables 1 to 3, the fluctuation in PaCO₂ and pH was less in the PEP groups compared with the control groups. The addition of ATP did not significantly affect the influence of PEP on PaO₂ (Table 4).

**Effects on Pulmonary Vascular Permeability** Oleic acid caused an increase in vascular permeability, which was reflected in higher concentrations of Evans Blue dye in the tissue samples. This increase in permeability was especially conspicuous in the intrapulmonary airways. PEP (200 μmol/kg) showed a significant effect in reducing vascular permeability (Fig. 3).

**DISCUSSION**

The most severe functional consequence of lung injury as-

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**Table 2. Blood Gas Parameters in the Control and PEP (20 μmol/kg) Groups**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>6</th>
<th>10</th>
<th>15</th>
<th>35</th>
<th>55</th>
<th>75</th>
</tr>
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<tbody>
<tr>
<td>Control (n=8)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>PaO₂</td>
<td>93.9±2.2</td>
<td>50.9±4.3†</td>
<td>55.8±4.7††</td>
<td>59.6±5.5†††</td>
<td>74.7±5.6†</td>
<td>82.7±3.9†</td>
<td>91.7±3.3</td>
</tr>
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<td>PaCO₂</td>
<td>36.7±0.6</td>
<td>35.3±0.8</td>
<td>34.5±1.4</td>
<td>34.0±1.4</td>
<td>34.2±1.6</td>
<td>33.7±2.0</td>
<td>33.6±6.2</td>
</tr>
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<td>pH</td>
<td>7.40±0.01</td>
<td>7.37±0.01†</td>
<td>7.36±0.01†</td>
<td>7.36±0.01†</td>
<td>7.38±0.01</td>
<td>7.38±0.01</td>
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<tr>
<td>PEP (20 μmol/kg) (n=8)</td>
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<tr>
<td>PaO₂</td>
<td>98.1±4.5</td>
<td>77.0±8.0*</td>
<td>78.3±7.5*</td>
<td>82.7±8.1†</td>
<td>92.0±7.5</td>
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<td>PaCO₂</td>
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<td>36.3±1.3</td>
<td>35.6±1.9</td>
<td>34.5±1.5</td>
<td>34.6±1.4</td>
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<tr>
<td>pH</td>
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<td>7.37±0.01</td>
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<td>7.39±0.01</td>
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</table>

Each value represents mean±S.E. *p<0.05 versus control group. †p<0.05, ††p<0.01 versus the value at time 0.

**Table 3. Blood Gas Parameters in the Control and PEP (200 μmol/kg) Groups**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>6</th>
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<th>75</th>
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<tbody>
<tr>
<td>Control (n=8)</td>
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</tr>
<tr>
<td>PaO₂</td>
<td>93±2.0</td>
<td>42.6±2.5†††</td>
<td>44.0±2.2†††</td>
<td>47.3±2.2†††</td>
<td>57.3±3.9†††</td>
<td>67.6±5.4†††</td>
<td>75.1±2.1†††</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>39.7±1.4</td>
<td>37.2±1.2</td>
<td>37.4±1.7</td>
<td>39.0±1.3</td>
<td>37.5±1.2</td>
<td>37.9±1.4</td>
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</tr>
<tr>
<td>pH</td>
<td>7.38±0.01</td>
<td>7.37±0.01</td>
<td>7.37±0.01</td>
<td>7.36±0.01</td>
<td>7.36±0.01</td>
<td>7.37±0.01</td>
<td>7.38±0.01</td>
</tr>
<tr>
<td>PEP (200 μmol/kg) (n=8)</td>
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<tr>
<td>PaO₂</td>
<td>88.4±2.4</td>
<td>69.6±5.8***</td>
<td>73.5±6.9**</td>
<td>75.3±7.1**</td>
<td>77.1±7.0*</td>
<td>83.8±5.6</td>
<td>89.5±4.5*</td>
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<tr>
<td>PaCO₂</td>
<td>37.1±1.8</td>
<td>38.3±2.3</td>
<td>38.7±2.4</td>
<td>38.6±2.0</td>
<td>37.3±2.5</td>
<td>36.5±2.3</td>
<td>35.8±2.1</td>
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<tr>
<td>pH</td>
<td>7.37±0.01</td>
<td>7.36±0.01</td>
<td>7.36±0.01</td>
<td>7.37±0.01</td>
<td>7.37±0.01</td>
<td>7.39±0.01</td>
<td>7.40±0.01</td>
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</table>

Each value represents mean±S.E. *p<0.05, **p<0.01, ***p<0.001 versus control group. †p<0.05, ††p<0.01, †††p<0.001 versus the value at time 0.
associated with vascular hyperpermeability is the inability to maintain effective gas exchange, leading to progressive hypoxemia and respiratory failure. The primary disturbance of ARDS is pulmonary edema characterized by an increase in the permeability of the alveolo-capillary membrane to fluid, solutes, and formed elements of the blood.

In the present study, the effects of PEP on oleic acid-induced hypoxemia and pulmonary vascular hyperpermeability were examined. We have demonstrated for the first time that PEP can prevent oleic acid-induced hypoxemia and reduce vascular hyperpermeability. The decrease in hyperpermeability by oleic acid can be lead to the attenuation of the decrease in PaO\textsubscript{2}, since we demonstrated the dose dependency of both the decrease in PaO\textsubscript{2} and the increase in pulmonary vascular permeability induced by oleic acid.

Eiermann and Dickey\textsuperscript{9} showed that a significant influx of polymorphonuclear leukocytes into bronchoalveolar lavage fluid from rats occurred after oleic acid injection. We previously demonstrated the participation of elastase and superoxides in the pathogenesis of oleic acid-induced lung injury in \textit{vivo}.\textsuperscript{10} In addition, we showed that superoxides were released from polymorphonuclear leukocytes after an application of oleic acid \textit{in vitro}.\textsuperscript{10} It was reported that oxidant-induced endothelial leak correlated with the decrease in intracellular ATP level.\textsuperscript{11} Rojanasakul \textit{et al}.\textsuperscript{12} also investigated the oxidative cell injury occurring in alveolar cells after exposure to various pneumotoxic agents. They showed the similarity of the time profiles of ATP depletion and cellular injury, suggesting that ATP depletion may also be involved in the loss of cell viability. Therefore the beneficial effects of PEP on lung injury can be attributed to an attenuation of endothelial injury that is caused by active oxygen released from polymorphonuclear leukocytes.

An increase in ATP in polymorphonuclear leukocytes may be another mechanism of the effect of PEP on oleic acid lung injury. It has been postulated that during lung inflammation, membrane stimulation by complement fragments can activate polymorphonuclear leukocytes and promote the release of intracellular enzymes (myeloperoxidase, lysozymes), acid hydrolases, and neutral proteases into the vascular space that can, in turn, produce endothelial injury. On the other hand, Wilkinson and Robinson\textsuperscript{13} showed that the discharge of intracellular enzymes into medium during prolonged incubation of human leukocytes and rat lymphocytes is inversely related to their ATP content. The prevention of the release of destructive enzymes by the intracellular increase in ATP in polymorphonuclear leukocytes may be another mechanism of attenuation of lung injury.

Superoxides may be involved in the nitric oxide (NO) system. Under physiological conditions, the powerful vasodilator NO is continuously released by endothelial cells to regulate organ blood flow and perfusion pressure.\textsuperscript{14} Endothelial-derived NO formation is catalyzed by endothelial NO synthase (eNOS).\textsuperscript{15} Under inflammatory conditions, a calcium-independent isofrom of NOS (iNOS) that produces copious amounts of NO is induced.\textsuperscript{16} The coupled production of excess NO and superoxide leads to the formation of an unstable intermediate termed peroxynitrite (ONOO\textsuperscript{-}).\textsuperscript{17} ONOO\textsuperscript{-} formation is kept to a minimum under normal conditions by endogenous superoxide dismutase (SOD), which removes superoxides, and by the limited capacity of eNOS to form NO. However, when iNOS is expressed in conditions where superoxide formation is increased, or SOD activity is decreased, ONOO\textsuperscript{-} is formed in excess. ONOO\textsuperscript{-} is commonly described as a toxic oxidant that inhibits cellular respiration\textsuperscript{18—20} and may contribute to endothelial dysfunction.\textsuperscript{21—23} ONOO\textsuperscript{-} inhibits cellular respiration directly by inhibiting mitochondrial electron transport and indirectly by activating the nuclear enzyme polyadenosine 5’-diphosphoribose synthase (PARS).\textsuperscript{18} ONOO\textsuperscript{-} causes DNA breaks that consequently trigger a futile cycle by the PARS pathway, resulting in depletion of nicotinamide adenine dinucleotide (NAD\textsuperscript{+}) and ATP.\textsuperscript{24} This mechanism could be another reason for the preventive effect of PEP on oleic

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<th>Time (min)</th>
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<th>55</th>
<th>75</th>
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<tbody>
<tr>
<td>PaO\textsubscript{2}</td>
<td>97.4±2.1</td>
<td>51.5±4.2 \textsuperscript{†††}</td>
<td>48.4±4.2 \textsuperscript{†††}</td>
<td>53.4±4.7 \textsuperscript{†††}</td>
<td>63.9±4.8 \textsuperscript{††}</td>
<td>73.4±4.7 \textsuperscript{††}</td>
<td>81.1±3.9 \textsuperscript{†}</td>
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<td>PaCO\textsubscript{2}</td>
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<tr>
<td>pH</td>
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Each value represents mean±S.E. *p<0.05, †††p<0.001 versus the value at time 0.

![Graph](image_url)

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Table 4. Blood Gas Parameters in the Control and Treatment Groups That Received PEP (2 μmol/kg) and ATP (4 μmol/kg) 20 min before Oleic Acid Injection.
acid-induced hypoxemia.

Another consequence of oxidant injury is changes in endothelial cell morphology. Endothelial cells form a major part of the normal capillary permeability barrier. Changes in endothelial cell shape which occur during inflammation (e.g., ARDS) have been associated with increased capillary permeability. Two major cytoskeletal structures involved in maintaining cell shape are microfilaments and microtubules. Microtubules made up of polymers of the protein tubulin lend support to overall shape and are quite sensitive to elevation of intracellular calcium levels. An increase in the intracellular concentration of calcium induced by oxidant injury has been correlated closely with the break up and ultimate depolymerization of microtubules. Microfilaments, primarily composed of the protein actin, play an important role in cellular adherence to other cells or the extracellular matrix. Microfilament disruption into bundles was found to be an ATP-dependent phenomenon. The approximate threshold level of ATP in endothelial cells for microfilament disruption and bundle formation is 15—20% of normal levels. This process is reversible if ATP synthesis can be restored. The loss of both cytoskeletal structures is followed by dramatic cell contraction and rounding.5) These mechanisms can partially explain endothelial cell dysfunction and capillary leak in ARDS and also might be a reason for the protective effect of PEP. It seems reasonable to assume that these mechanism may be involved in the effect of PEP, a membrane-permeable agent, on oleic acid-induced lung injury, while ATP, a membrane-impermeable agent, could not enhance the effect.

As a source supplying superoxide, polymorphonuclear leukocytes seem to play important roles in oleic acid-induced lung injury, leading to a decrease in ATP in the cells. To clarify the effect of PEP on polymorphonuclear leukocytes, we are planning to examine infiltration of polymorphonuclear leukocytes into pulmonary cells and how PEP influences their activation. In addition, we will examine the effect of PEP on another animal model of ARDS, such as lung injury induced by lipopolysaccharide, so that we can further confirm the usefulness of PEP.

In our experiments, PaCO₂ and pH were not affected by oleic acid. It is known that CO₂ is about 10000 times more permeable to the alveolar-capillary barrier than O₂, and that the blood pH is mainly controlled by the kidneys as well as the lungs. This may be why the two parameters were not affected by a “modest” dose (15 μl/kg) of oleic acid. However, we showed that high-dose of oleic acid significantly changed blood pH.25) We used 15 μl/kg of oleic acid so that we could easily detect the potential effect of PEP, because severe lung injury induced by high dose oleic acid may mask the effects of PEP on PaO₂.

Based on our results, two important areas of focus for therapy may be enhancement of intracellular antioxidant defenses as shown by Pacht and Abernathy.26) They showed the effects of exogenous glutathione and N-acetylcysteine in the prevention of intracellular ATP depletion after oxidant injury to rat type II alveolar epithelial cells. The maintenance or restoration of cellular ATP levels as we showed in the present experiments is the second area of focus.

It is suggested that PEP is candidate for the treatment of lung injuries associated with pulmonary vascular hyperpermeability such as ARDS. We hope that the results of this study will provide better insights into the pathogenesis of the lung injury and lead to novel therapeutic strategies to prevent or ameliorate ARDS.

REFERENCES

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