Role of neutrophils in xanthine/xanthine oxidase-induced oxidant injury in isolated rabbit lungs

MASASHI KISHI,1 LOIS F. RICHARD,3 ROBERT O. WEBSTER,2,3 AND THOMAS E. DAHMS1–3
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Kishi, Masashi, Lois F. Richard, Robert O. Webster, and Thomas E. Dahms. Role of neutrophils in xanthine/xanthine oxidase-induced oxidant injury in isolated rabbit lungs. J. Appl. Physiol. 87(6): 2319–2325, 1999.—Reactive oxygen species have been shown to play an important role in the pathogenesis of lung injury. This study was designed to clarify the role of intrapulmonary neutrophils in the development of xanthine/xanthine oxidase (X/XO)-induced lung injury in isolated buffer-perfused rabbit lungs. We measured microvascular fluid filtration coefficient (Kf) and wet-to-dry weight ratio to assess lung injury. X/XO induced a significant increase in Kf and wet-to-dry weight ratio in neutrophil-replete lungs, whereas the lung injury was attenuated in neutrophil-depleted lungs. A neutrophil elastase inhibitor, ONO-5046, also attenuated the lung injury. In addition, X/XO induced a transient pulmonary arterial pressure (Ppa) increase. The thromboxane inhibitor OKY-046 attenuated the Ppa increase but did not alter the increase in permeability. Neutrophil depletion reduced the Kf increase but had no effect on the Ppa increase. These results suggest that intrapulmonary neutrophils activated by X/XO play a major role in development of the lung injury, that neutrophil elastase is involved in the injury, and that the X/XO-induced vasoconstriction is independent of intrapulmonary neutrophils.

permeability; neutrophil elastase; perfused lungs

REACTIVE OXYGEN SPECIES (ROS) have been shown to play an important role in the pathogenesis of acute respiratory distress syndrome (13, 25). Xanthine (X)/xanthine oxidase (XO) is known as one of the major sources of ROS (6, 20, 25). Weinbroum et al. (33) demonstrated that circulating XO after ischemia-reperfusion of the liver could cause an increase in pulmonary permeability in isolated rat liver and lungs. Steinberg et al. (28) demonstrated that superoxide radicals, generated by hypoxanthine and XO, resulted in alteration of endothelial cell function, including a progressive fall in the cellular transport of 14C-labeled 5-hydroxytryptamine, and lung edema in isolated perfused rat lungs. However, the involvement of neutrophils in the oxidant injury is unclear in these studies. Adkins and Taylor (1) demonstrated that neutrophils and intrinsic XO were involved in the increased capillary permeability associated with ischemia and reperfusion in isolated rabbit lungs, because prevention of neutrophil adhesion with the monoclonal antibody IB4 and inhibition of XO by allopurinol attenuated the change in fluid filtration coefficient (Kf). On the other hand, Deeb et al. (11) demonstrated in isolated rat lungs that toxic oxygen metabolites were involved in ischemia-reperfusion injury but neutrophils were not, because added neutrophils did not enhance the injury. It is reported that accumulation of activated neutrophils in the lung can cause lung injury induced by ischemia-reperfusion in vivo (27) or by the chemotactic peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine in isolated perfused lungs (29). However, the involvement of intrapulmonary neutrophils in X/XO-induced oxidant lung injury has not been well characterized.

This study was designed to evaluate whether intrapulmonary neutrophils were involved in the X/XO-induced lung injury and, if so, to examine the role of neutrophil elastase in the lung injury. To achieve these objectives, neutrophil-depleted lungs and the selective neutrophil elastase inhibitor ONO-5046 (18) were used. It has been shown that oxygen metabolites generated by purine plus XO stimulated thromboxane (Tx) production and vasoconstriction in isolated saline-perfused rabbit lungs (30). To evaluate whether suppression of a transient increase in pulmonary arterial pressure (Ppa) attenuates the injury in neutrophil-replete lungs, the Tx synthesis inhibitor OKY-046 was used.

METHODS

Isolated Lung Preparation

New Zealand White rabbits (2.5–3.0 kg) were obtained from Charles River Rabbitry (Wilmington, MA). All animal experiments were conducted in accordance with the rules and regulations of St. Louis University regarding the care and use of experimental animals. Rabbits were anesthetized with pentobarbital sodium (30 mg/kg iv). After a tracheostomy was performed, the animals were ventilated with room air during the lung isolation procedure. The rabbits were then heparinized (1,000 U/kg) via a cannula placed in the internal carotid artery. The heparin was allowed to circulate for 2 min, and the animals were exsanguinated. The chest was opened by median sternotomy, and the pulmonary artery was cannulated through an incision in the right ventricle. Perfusion with a Krebs-Henseleit bicarbonate buffer (pH 7.40 ± 0.05) containing 6% BSA (Intergr, Purchase, NY) was begun slowly. A cannula was placed in the left atrial appendage via an incision in the apex. The heart and lungs were removed on bloc and suspended from a force transducer (model FT 03, Grass Instruments, Quincy, MA) for continuous weight measurement in a well-humidified 37°C chamber. Ppa and left atrial pressure (Pla) were measured by pressure transducers (model P23i, Statham) with zero pressure reference at the level of the lung apex. The lung was perfused in a nonrecirculating manner until the effluent from the cannula in the pulmonary vein appeared clear. Thereafter, the lung was perfused in a recirculating system with a volume of 200 ml. The buffer contained <10³/mm³ erythrocytes, 10³/mm³ leuko-

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cytes, and $5 \times 10^9$/mm$^3$ platelets in our preliminary study. The pH of the perfusate was maintained between 7.30 and 7.50 by balancing the CO$_2$ tension in the buffer exposed to a gas mixture of 90% O$_2$-10% CO$_2$ and the alveolar CO$_2$ tension by ventilating the lungs with 15% O$_2$-6% CO$_2$-79% N$_2$ (Harvard small animal ventilator; tidal volume 15 ml, rate 15 breaths/min). The flow rate was gradually increased to a constant flow of 100 ml/min. The P$_{pa}$ was adjusted at 3–5 cmH$_2$O, which resulted in a P$_w$ of $-12$ cmH$_2$O, and also was greater than mean airway pressure (P$_{mwp}$), so that lungs were maintained in West's zone 3 condition (P$_{pa}$ > P$_{la}$ > P$_{aw}$). P$_{mwp}$, P$_{la}$, P$_{aw}$, and lung weight changes were continuously recorded on a polygraph (Grass Instruments). Pulmonary vascular resistance (PVR) was calculated as follows

$$PVR = \frac{(P_{pa} - P_{la})}{\text{flow rate}}$$

All the procedures of lung isolation were performed within 20 min after the start of invasive procedures. A stabilization period of $>10$ min was allowed for pressures to become constant and the preparations to become isogravimetric. The few lungs that were not isogravimetric within 30 min of isolation were replaced.

Measurement of Microvascular Hydrostatic Pressure

After the stabilization period, pulmonary microvascular pressure was measured by the double-occlusion method of Hakim et al. (15). Double-occlusion pressure (P$_{do}$) was measured by simultaneously occluding the perfusion inflow and outflow lines with solenoid valves (Anger Scientific, Cedar Knolls, NJ). The estimated P$_{ib}$ was measured by using the stable portion of the P$_{pa}$ and P$_{ib}$ traces during occlusion.

Measurement of $K_f$

Measurement of $K_f$ as an index of vascular permeability is based on the forces described in the Starling equation as follows

$$Q_f = K_f[(P_{mv} - P_i) - \sigma(\Pi_{mv} - \Pi_i)]$$

where $Q_f$ is net flux of fluid across the microvascular wall, P$_{mv}$ and P$_i$ are the microvascular and interstitial pressures, respectively, $\Pi_{mv}$ and $\Pi_i$ are the colloid oncotic pressures of plasma and interstitium, respectively, and $\sigma$ is the protein reflection coefficient. After the preparation reached an isogravimetric state, where $Q_f$ was $\sim 0$, P$_{do}$ was measured. P$_{la}$ was increased by 8–9 cmH$_2$O, resulting in increased P$_{mv}$, the other forces in the equation are not changed. A two-component weight increase was observed: an initial rapid weight gain due to recruitment and distension in the vascular bed and a slow weight gain attributed to filtration through the microvasculature. $Q_f$ after the elevation of P$_{la}$ can be estimated as lung weight gain during the period of slow weight gain (dW/dt). P$_{aw}$ was measured again 7 min after the elevation of P$_{aw}$. K$_f$ was calculated by dividing dW/dt by the change in P$_{do}$ and was then normalized to the rabbit body weight.

Myeloperoxidase Activity Measurement of the Lung

To estimate the number of intrapulmonary neutrophils, neutrophil myeloperoxidase (MPO) activity in the left lung was measured after the isolated perfused lung experiment. The lungs were frozen in liquid nitrogen immediately after the last measurement of $K_f$ and kept frozen at $-80^\circ$C until lyophilization. The frozen lungs were crushed into small pieces and suspended in 15 ml of distilled water and then homogenized using a tissue grinder (CON-TORQ, Eberbach, Ann Arbor, MI) on ice. The homogenate was shell-frozen quickly and lyophilized for $>24$ h to complete dryness. Lyophilized lungs were stored at $-80^\circ$C until the measurement of MPO activity. About 20 mg of lung sample were suspended in 2 ml of 50 mM potassium phosphate buffer (pH 6.0) with 0.5% hexadecyltrimethyl ammonium bromide. The suspension was centrifuged at 40,000 g for 15 min, and the supernatant was collected for MPO analysis. MPO activity was determined spectrophotometrically by measuring the rate of o-dianisidine oxidation at 25°C at pH 6.0 and expressed by the amount of enzyme reducing 1 µmol peroxide/min. MPO activity was calculated as follows (3, 8)

$$\text{MPO} = \frac{13.5(\Delta \text{OD/min})}{\text{mg dry lung wt}}$$

where OD is optical density. Wet-to-Dry Weight Ratio of the Lung

After the last measurement of $K_f$, right lungs were weighed to obtain a wet weight. The lungs were then dried completely in a microwave oven to obtain the dry weight. Wet-to-dry weight ratios (W/D) were then calculated.

Experimental Protocols

Baseline $K_f$ was measured after the lungs remained isogravimetric for 10–30 min. Fifteen minutes after the measurement of baseline $K_f$, X (Sigma Chemical, St. Louis, MO), dissolved in distilled water (20 mM), was added to perfusate to a final concentration of 430 µM. Three minutes later, XO (Sigma Chemical) was injected slowly, over 1 min, into the perfusate reservoir to a final concentration of 10 mU/ml. $K_f$ (experimental $K_f$) was measured again 60 min after XO administration. These amounts of X and XO were shown to increase $K_f$ nearly twofold in our study (Fig. 1). Lower concentrations of X had a tendency to increase experimental $K_f$ but not significantly compared with its baseline $K_f$. When we used 500 µM, most lungs were not isogravimetric after addition of the X/XO, and we could not measure the experimental $K_f$. Thus 430 µM X was determined. Finally, the right lung

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Figure 1: Effect of concentration of xanthine on changes in baseline and experimental capillary filtration coefficient ($K_f$). Values are means ± SE; n, number of rabbit lungs. Experimental $K_f$ was measured 60 min after addition of xanthine oxidase (XO) (10 mU/ml) to perfusate containing various concentrations of xanthine. *Different from baseline ($P < 0.01$).
was weighed for W/D determination, and the left lung was prepared for measurement of lung MPO activity.

To evaluate the role of neutrophils in X/XO-induced lung injury in isolated buffer-perfused lungs, the following interventions were performed in five randomly divided groups.

Vehicle + X/XO group (n = 7). Ten minutes before addition of X/XO, 1 ml of vehicle (sterile saline) was added to the perfusate reservoir. This group served as an oxidant injury control group.

Depleted + X/XO group (n = 7). To examine the role of neutrophils in the X/XO-induced increase in Kf, circulating and intrapulmonary neutrophils were depleted before addition of X/XO. The rabbits were sedated with a 0.2 ml sc injection of acepromazine maleate (Aveco, Fort Dodge, IA), and mechlorethamine (2 mg/kg iv; Sigma Chemical) was injected to deplete the circulating neutrophils 4 days before the experiments. Mechlorethamine was freshly prepared in saline at a concentration of 1 mg/ml. According to a preliminary study, the circulating neutrophil count was <100/mm3 on day 4 compared with 1,500–2,000/mm3 on day 0, whereas platelets were reduced from 350,000 to 136,000/mm3 and erythrocytes were reduced by 3%. The rabbits were prepared as described for the vehicle + X/XO group, except neutrophils were depleted.

ONO-5046 + X/XO group (n = 7). To examine the role of neutrophil elastase in this injury, ONO-5046 (10 mg/kg; Ono Pharmaceutical, Osaka, Japan), a selective neutrophil elastase inhibitor, was added to the reservoir 10 min before addition of X/XO. ONO-5046 was dissolved in 1 ml of normal saline. This dosage was based on reports that infusion of ONO-5046 at a rate of 10 mg·kg−1·h−1 inhibited the leukotriene B4-induced increase in Kf, W/D, and perfusate neutrophil elastase activity in rabbit lungs (34) and that the concentration of ONO-5046 in the perfused blood was unchanged before and after an experiment using isolated blood-perfused rabbit lungs after a bolus administration of ONO-5046 (19).

OKY-046 + X/XO group (n = 7). TxA2 has been shown to play a major role in pulmonary vasoconstriction due to generated oxygen metabolites in isolated perfused rabbit lungs (30). To examine whether Tx participates in the pressor response and permeability change after administration of X/XO, the Tx synthesis inhibitor OKY-046 was added to the reservoir to achieve an initial perfusate concentration of 0.1 mM 10 min before addition of X/XO. OKY-046 was dissolved in 1 ml of normal saline. This dosage of OKY-046 was based on a previous report from this laboratory which showed that pretreatment with 0.1 mM OKY-046 prevented a phorbol myristate acetate-induced increase in perfusate TxB2 concentration in isolated blood-perfused rabbit lungs (35).

Vehicle + vehicle group (n = 5). Five minutes after measurement of baseline Kf, 1 ml of saline was added to the reservoir, and 10 min later 4.3 ml of distilled water were added to the reservoir. Sixty minutes after the administration of distilled water, Kf was measured again as experimental Kf. This group served as a time control group.

Statistical Analysis

Values are means ± SE unless otherwise indicated. Comparisons between baseline and experimental values were assessed using a Student’s paired t-test. A one-way ANOVA was used to compare values between groups, and differences between groups were determined using the Student-Newman-Keuls test. Differences were considered significant when P < 0.05.

### RESULTS

#### Baseline and Experimental Hemodynamic Measurements

Table 1 shows hemodynamic values of Ppa, Pdow, P Laur, and PVR at baseline (just before measurement of baseline Kf) and the experimental time period (60 min after addition of X/XO). No significant differences were noted in any parameters between the five groups at each time point or between time points within each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Ppa (cmH2O)</th>
<th>Pdow (cmH2O)</th>
<th>Pal (cmH2O)</th>
<th>PVR (cmH2O·l⁻¹·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle + X/XO</td>
<td>7</td>
<td>10.7 ± 0.7</td>
<td>7.8 ± 0.5</td>
<td>5.1 ± 0.1</td>
<td>55.7 ± 7.4</td>
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<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Experimental</td>
<td></td>
<td>10.8 ± 0.11</td>
<td>7.8 ± 0.4</td>
<td>5.1 ± 0.1</td>
<td>54.3 ± 10.5</td>
</tr>
<tr>
<td>Dep + X/XO</td>
<td>7</td>
<td>10.3 ± 0.6</td>
<td>8.0 ± 0.6</td>
<td>5.1 ± 0.2</td>
<td>51.4 ± 4.8</td>
</tr>
<tr>
<td>Baseline</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td></td>
<td>9.4 ± 0.6</td>
<td>7.5 ± 0.4</td>
<td>5.3 ± 0.2</td>
<td>40.7 ± 4.0</td>
</tr>
<tr>
<td>ONO-5046 + X/XO</td>
<td>7</td>
<td>9.6 ± 0.3</td>
<td>7.0 ± 0.3</td>
<td>4.9 ± 0.2</td>
<td>47.9 ± 5.0</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Experimental</td>
<td></td>
<td>9.2 ± 0.6</td>
<td>7.0 ± 0.2</td>
<td>5.0 ± 0.2</td>
<td>42.1 ± 6.6</td>
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<td>OKY-046 + X/XO</td>
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<td>10.1 ± 0.5</td>
<td>7.8 ± 0.4</td>
<td>5.3 ± 0.1</td>
<td>48.6 ± 4.5</td>
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<td>Baseline</td>
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<tr>
<td>Experimental</td>
<td></td>
<td>9.9 ± 0.6</td>
<td>7.5 ± 0.2</td>
<td>5.1 ± 0.2</td>
<td>47.9 ± 5.3</td>
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<tr>
<td>Vehicle + Veh</td>
<td>5</td>
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<td>7.0 ± 0.1</td>
<td>5.3 ± 0.2</td>
<td>43.0 ± 4.4</td>
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<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Experimental</td>
<td></td>
<td>9.4 ± 0.4</td>
<td>7.3 ± 0.3</td>
<td>5.5 ± 0.2</td>
<td>39.0 ± 3.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rabbit lungs. Veh, vehicle (saline or distilled water); X, xanthine; XO, xanthine oxidase; Dep, neutrophil depleted; ONO, ONO-5046; OKY, OKY-046; Ppa, pulmonary arterial pressure; Pdow, double-outlet pressure; Pal, left atrial pressure; PVR, pulmonary vascular resistance.

#### Table 1: Hemodynamic profiles at baseline and experimental time periods in isolated perfused lungs

X/XO-Induced Kf Increase and Lung Edema

Baseline and experimental Kf are shown in Figs. 1 and 2. Increasing concentrations of X in the perfusate resulted in increases in vascular permeability related to dose of the products of X/XO in this preparation (Fig. 1). X at 430 µM was used, since it resulted in a significant change in permeability without altering the isogravimetric state of the preparation. The X/XO administration caused a significant increase in microvascular permeability as measured by Kf, compared with baseline in the vehicle + X/XO group (P < 0.01), and experimental Kf was significantly higher in the vehicle + X/XO group than in the vehicle + vehicle group (0.020 ± 0.002 vs. 0.011 ± 0.001 g·min⁻¹·cmH2O⁻¹·kg⁻¹, P < 0.01). No significant difference was noted in the vehicle + vehicle group between baseline and experimental Kf measurements (0.010 ± 0.001 and 0.011 ± 0.001 g·min⁻¹·cmH2O⁻¹·kg⁻¹, respectively). Experimental Kf were significantly lower in the depleted + X/XO and ONO-046 + X/XO groups (0.012 ± 0.001 and 0.014 ± 0.003 g·min⁻¹·cmH2O⁻¹·kg⁻¹, respectively) than in the vehicle + X/XO group. In the ONO-5046 + X/XO group, experimental Kf was significantly higher than baseline Kf (P < 0.01), whereas experimental Kf in the depleted + X/XO group was not. Experimental Kf had a tendency to be higher in the

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ROLE OF NEUTROPHILS IN OXIDANT LUNG INJURY

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depleted + X/XO and ONO-5046 + X/XO group than in the vehicle + vehicle group, although the differences were not significant. Experimental Kf was significantly higher in the OKY-046 + X/XO group (0.017 ± 0.002 g·min⁻¹·cmH₂O⁻¹·kg⁻¹) than in the vehicle + vehicle group (P < 0.05) and was not different from the vehicle + X/XO group.

X/XO administration induced lung edema, as shown by increased W/D of the lung (Fig. 3). W/D was significantly higher in the vehicle + X/XO group than in the vehicle + vehicle group (6.86 ± 0.14 vs. 5.82 ± 0.04, P < 0.01). W/D was significantly lower in the ONO-5046 + X/XO and depleted + X/XO groups (6.41 ± 0.09 and 6.30 ± 0.13, respectively) than in the vehicle + X/XO group (P < 0.01), although they were significantly higher than in the vehicle + vehicle group (P < 0.01).

Thus administration of ONO-5046 and neutrophil depletion attenuated lung edema induced by X/XO administration. No significant difference was noted between the OKY-046 + X/XO (6.94 ± 0.13) and vehicle + X/XO groups. W/D ratios were significantly higher in the OKY-046 + X/XO group than in vehicle + vehicle group (P < 0.01).

X/XO-Induced Pulmonary Vasoconstriction

The administration of X/XO induced a transient increase in Ppa within 2 min. Figure 4 shows peak Ppa increases (peak Ppa - baseline Ppa) after administration of X/XO in each group. In response to X/XO, the Ppa increase in the vehicle + X/XO group was 14.1 ± 2.3 cmH₂O and was significantly higher than in the vehicle + vehicle group (P < 0.01). The Ppa increases were significantly lower in the OKY-046 + X/XO and ONO-5046 + X/XO groups (3.6 ± 0.7 and 6.9 ± 1.6 cmH₂O, respectively) than in the vehicle + X/XO group. No significant differences were noted in the Ppa increase between the vehicle + X/XO and depleted + X/XO groups. After Ppa reached its maximum level, it gradually decreased to its baseline level before the measurement of experimental Kf (Table 1).

MPO Activity of the Lung

A previous study in our laboratory showed that the extent of the increase in pulmonary permeability caused by N-formyl-L-methionyl-L-leucyl-L-phenylalanine depended on the number of intrapulmonary neutrophils (29). We also found that addition of 1 × 10⁹ rabbit peritoneal neutrophils to the buffer in neutrophil-depleted lungs caused an increase in Kf after administration of X/XO, whereas addition of buffer (no neutrophils) or 5 × 10⁸ neutrophils did not (data not shown). Therefore, to investigate whether the number of intrapulmonary neutrophils could account for the differences in Kf and W/D between the experimental groups in the present study, MPO activity was measured. MPO activity was significantly lower in the depleted + X/XO

![Fig. 2. Effect of treatment with xanthine (X) and xanthine oxidase (XO) on changes in baseline and experimental Kf. Values are means ± SE; n, number of rabbit lungs. †Different from Kf in Veh + Veh group; ‡Different from Kf in Veh + X/XO group; §Different from Kf in OKY + X/XO, OKY-046 + X/XO. †Different from baseline Kf (P < 0.01); ‡Different from experimental Kf in Veh + Veh group: *P < 0.01; ‡P < 0.05. Different from experimental Kf in Veh + X/XO group: †P < 0.01; ‡P < 0.05.](http://jap.physiology.org/)
neutrophils in X/XO-induced oxidant lung injury. The objective of this study was to determine the role of neutrophils in X/XO-induced oxidant lung injury. We showed that 500 µM X and 5 mU/ml XO caused a twofold increase in Kf in isolated buffer-perfused rabbit lungs and that the Kf increase was attenuated by allopurinol or catalase. In our experiments, 500 µM X and 10 mU/ml XO often caused severe lung injury that prevented the measurement of the experimental Kf. The objective of this study was to determine the role of neutrophils in X/XO-induced oxidant lung injury as measured by Kf, and W/D. In the present study, experimental Kf and W/D were significantly lower in the X/XO group than in the vehicle + X/XO group, and MPO activity in the vehicle + X/XO group was not different from that in the vehicle + vehicle group. In a separate series of experiments, lungs from neutrophil-depleted rabbits were shown to be unresponsive to X/XO. To determine whether this absence of response was due primarily to neutrophils, we repleted the lungs with peritoneal-derived neutrophils to normal levels by 10.220.33.2 on October 1, 2017 http://jap.physiology.org/ Downloaded from
depleted + X/XO group. Neutrophil depletion did not attenuate the X/XO-induced pressor response. Neutrophil elastase has been shown to contribute to the pathogenesis of acute lung injury (17, 22, 26). Gossage et al. (14) demonstrated that neutrophil elastase inhibitor (SC-37698) attenuated the increase in lung lymph flow and the fall in systemic white blood cell count after an endotoxin challenge in sheep. Kubo et al. (21) also showed similar effects of ONO-5046 on endotoxin-induced lung injury in sheep. In these studies the protective effects of neutrophil elastase inhibition on lung injury seem to be due to the inhibition of neutrophil accumulation in the lung after endotoxin administration. In the present study, ONO-5046 significantly attenuated X/XO-induced Kf and W/D increases, but no significant difference was seen in MPO activity between the vehicle + X/XO and ONO + X/XO groups. Our results suggest that neutrophil elastase derived from activated intrapulmonary neutrophils in response to the oxidant stress is involved in lung injury without further accumulation of neutrophils in the lung. Interestingly, ONO-5046 attenuated the transient Ppa increase after X/XO administration (Fig. 4). As previously described, the pressor response in our model is independent of intrapulmonary neutrophils, and Tx plays a major role in the response. Therefore, the selective neutrophil elastase inhibitor ONO-5046 was expected to have no effect on the pressor response. Kubo et al. demonstrated in sheep that ONO-5046 attenuated the increase in TxB2 and 6-ketoprostaglandin F1α concentrations as well as the increase in Ppa 0.5–1 h after administration of endotoxin, although the main source of Tx was unclear.

**DISCUSSION**

In the present study we demonstrated that 1) X/XO induced an increase in lung microvascular permeability and in lung edema, whereas pretreatment with neutrophil depletion or ONO-5046 attenuated these indexes of injury; 2) OKY-046 or ONO-5046 attenuated transient Ppa increases induced by X/XO, whereas neutrophil depletion did not; and 3) there were no significant differences in intrapulmonary neutrophil counts between the experimental groups, except in the depleted + X/XO group. Neutrophil depletion did not attenuate the X/XO-induced pressor response. Neutrophil elastase has been shown to contribute to the pathogenesis of acute lung injury (17, 22, 26). Gossage et al. (14) demonstrated that neutrophil elastase inhibitor (SC-37698) attenuated the increase in lung lymph flow and the fall in systemic white blood cell count after an endotoxin challenge in sheep. Kubo et al. (21) also showed similar effects of ONO-5046 on endotoxin-induced lung injury in sheep. In these studies the protective effects of neutrophil elastase inhibition on lung injury seem to be due to the inhibition of neutrophil accumulation in the lung after endotoxin administration. In the present study, ONO-5046 significantly attenuated X/XO-induced Kf and W/D increases, but no significant difference was seen in MPO activity between the vehicle + X/XO and ONO + X/XO groups. Our results suggest that neutrophil elastase derived from activated intrapulmonary neutrophils in response to the oxidant stress is involved in lung injury without further accumulation of neutrophils in the lung. Interestingly, ONO-5046 attenuated the transient Ppa increase after X/XO administration (Fig. 4). As previously described, the pressor response in our model is independent of intrapulmonary neutrophils, and Tx plays a major role in the response. Therefore, the selective neutrophil elastase inhibitor ONO-5046 was expected to have no effect on the pressor response. Kubo et al. demonstrated in sheep that ONO-5046 attenuated the increase in TxB2 and 6-ketoprostaglandin F1α concentrations as well as the increase in Ppa 0.5–1 h after administration of endotoxin, although the main source of Tx was unclear. ONO-5046 might have neutrophil-independent suppressive effects on Tx generation. However, we considered that the suppressive effect of

**DISCUSSION**

In the present study we demonstrated that 1) X/XO induced an increase in lung microvascular permeability and in lung edema, whereas pretreatment with neutrophil depletion or ONO-5046 attenuated these indexes of injury; 2) OKY-046 or ONO-5046 attenuated transient Ppa increases induced by X/XO, whereas neutrophil depletion did not; and 3) there were no significant differences in intrapulmonary neutrophil counts between the experimental groups, except in the depleted + X/XO group. Neutrophil depletion did not attenuate the X/XO-induced pressor response. Neutrophil elastase has been shown to contribute to the pathogenesis of acute lung injury (17, 22, 26). Gossage et al. (14) demonstrated that neutrophil elastase inhibitor (SC-37698) attenuated the increase in lung lymph flow and the fall in systemic white blood cell count after an endotoxin challenge in sheep. Kubo et al. (21) also showed similar effects of ONO-5046 on endotoxin-induced lung injury in sheep. In these studies the protective effects of neutrophil elastase inhibition on lung injury seem to be due to the inhibition of neutrophil accumulation in the lung after endotoxin administration. In the present study, ONO-5046 significantly attenuated X/XO-induced Kf and W/D increases, but no significant difference was seen in MPO activity between the vehicle + X/XO and ONO + X/XO groups. Our results suggest that neutrophil elastase derived from activated intrapulmonary neutrophils in response to the oxidant stress is involved in lung injury without further accumulation of neutrophils in the lung. Interestingly, ONO-5046 attenuated the transient Ppa increase after X/XO administration (Fig. 4). As previously described, the pressor response in our model is independent of intrapulmonary neutrophils, and Tx plays a major role in the response. Therefore, the selective neutrophil elastase inhibitor ONO-5046 was expected to have no effect on the pressor response. Kubo et al. demonstrated in sheep that ONO-5046 attenuated the increase in TxB2 and 6-ketoprostaglandin F1α concentrations as well as the increase in Ppa 0.5–1 h after administration of endotoxin, although the main source of Tx was unclear. ONO-5046 might have neutrophil-independent suppressive effects on Tx generation. However, we considered that the suppressive effect of

![Fig. 5. Myeloperoxidase (MPO) activity of lyophilized left lung. Values are means ± SE; n, number of rabbit lungs. *Different from MPO activity in other 5 groups (P < 0.01). No significant differences were noted between experimental groups, except Dep + X/XO group.](https://www.jap.physiology.org/content/doi/10.220.33.2/article/fig5)
ON-O-5046 on the pressor response could not account for the reduction of the lung injury in the ONO + X/XO group, because OKY-046 attenuated the pressor response but failed to attenuate the oxidant lung injury.

Activated neutrophils are known as one of major sources of ROS (25). Therefore, it may be naturally assumed that ROS derived not only from X/XO but also from intrapulmonary neutrophils are involved in the X/XO-induced lung injury. Tsuji et al. (31) demonstrated that lipopolysaccharide-induced lung injury (increased permeability) and superoxide production were significantly reduced in neutrophil-depleted rat lungs. On the other hand, Barnard and Matalon (4) demonstrated in X/XO-induced lung injury in isolated buffer-perfused rabbit lungs that the lungs could remove large quantities of H$_2$O$_2$ but that this scavenging did not prevent the lung injury to the pulmonary microvasculature. In addition, we have determined that superoxide anion production from neutrophils stimulated in vitro by 50 µM X and 0.6 mU XO was only additive (basal superoxide production from neutrophils plus that from X/XO reaction) and was not amplified (data not shown). These studies suggest that ROS from intrapulmonary neutrophils do not play a major role in developing the X/XO-induced lung injury. ROS scavengers have been shown to reduce sepsis-induced (9), ischemia-reperfusion-induced (16), and X/XO-induced (10) lung injury in vivo. In isolated perfused lung, Yoshimura et al. (34) demonstrated that recombinant human superoxide dismutase attenuated leukotriene B$_4$-induced increases in $K_f$ and W/D. Barnard and Matalon demonstrated that X/XO-induced lung injury was prevented by allopurinol or catalase but not by superoxide dismutase and that H$_2$O$_2$ was primarily responsible for the X/XO-induced lung injury in isolated buffer-perfused rabbit lungs. We could not use these radical scavengers in our experiments, because they were expected to reduce generation of ROS not only from intrapulmonary neutrophils but also from the X/XO system.

We showed that intrapulmonary neutrophils played a major role in the X/XO-induced lung injury in isolated buffer-perfused rabbit lungs. However, experimental $K_f$ values had a tendency to be higher in the depleted + X/XO than in the vehicle + vehicle group (not significant), and the W/D ratios were significantly higher in the depleted + X/XO than in the vehicle + vehicle group. These results indicate that neutrophil depletion did not completely block the X/XO-induced lung injury and that a neutrophil-independent mechanism was partially involved in the X/XO-induced lung injury. The role of the neutrophils in the lung injury is complex, because it appears to be alteration of the response to X/XO. The mechanism of the lung injury is different: the dose of X/XO in our present study with neutrophils acts through neutrophil elastase, whereas a higher dose of X/XO acts by direct oxidant injury, because in our preliminary studies we found that 500 µM X could significantly increase $K_f$ in neutrophil-depleted lungs (data not shown). Our results were compatible with those of Anderson et al. (2), who demonstrated in rats that lipopolysaccharide- and N-formyl-neoleucyl-leucyl-phenylalanine-induced lung injury was neutrophil dependent and independent and that the nonneutrophil component of the lung injury appeared to be dependent on pulmonary XO. ROS generated from XO with purine have been shown, in vitro, to induce an increase in permeability of bovine aortic endothelial cells (7) and human umbilical vein endothelial cells (24). Thus a direct injury to the pulmonary vascular endothelium might be one of the mechanisms in the X/XO-induced lung injury, although the mechanism of neutrophil-independent lung injury was not directly assessed in our study. Other possible neutrophil-independent mechanisms might include activation of alveolar macrophages (5, 32) or mast cells in the lung (12). These hypotheses remain to be tested.

In summary, the present study indicates that, in isolated buffer-perfused rabbit lungs, activated intrapulmonary neutrophils play a major role in developing the X/XO-induced lung injury as measured by $K_f$ and W/D, that neutrophil elastase is involved in the injury, and that the X/XO-induced vasoconstriction is independent of intrapulmonary neutrophils.

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