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

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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 neutrophile-intact 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 circulating XO after 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-phenyalanine 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 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 performed 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 (Intergra, 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 en 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 (Ppa) were measured by pressure transducers (model 23i, 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, 10⁵/mm³ leuko-

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Hakim et al. (15). Double-occlusion pressure (P do) was measured by the double-occlusion method of measurement of microvascular hydrostatic pressure. Few lungs that were not isogravimetric within 30 min of period of 20 min after the start of invasive procedures. A stabilization was increased by 8–9 cmH2O, resulting in increased Pmv; the elevation of P la.

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 do was measured by using the stable portion of the P pa and P la traces during occlusion.

Measurement of Kf

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

\[ Q_1 = K_f \left( P_{mv} - P_i - \sigma (I_{mv} - I_{i}) \right) \]

where Q1 is net flux of fluid across the microvascular wall, Pmv and Pi are the microvascular and interstitial pressures, respectively, Imv and Ii 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 Q1 was ~0, P do was measured. P la was increased by 8–9 cmH2O, resulting in increased Pmv, 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. Q1 after the elevation of P la can be estimated as lung weight gain during the period of slow weight gain (\( \Delta W/t \)). P aw was measured again 7 min after the elevation of P la. Kf was calculated by dividing \( \Delta W/t \) 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 Kf and kept frozen at ~80°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°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)

\[ \text{MPO (units/mg dry lung)} = \frac{13.5(\text{OD/min})}{\text{mg dry lung wt}} \]

where OD is optical density.

Wet-to-Dry Weight Ratio of the Lung

After the last measurement of Kf, 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 Kf was measured after the lungs remained isogravimetric for 10–30 min. Fifteen minutes after the measurement of baseline Kf, 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. Kf (experimental Kf) was measured again 60 min after XO administration. These amounts of X and XO were shown to increase Kf nearly twofold in our study (Fig. 1). Lower concentrations of X had a tendency to increase experimental Kf but not significantly compared with its baseline Kf. When we used 500 µM, most lungs were not isogravimetric after addition of the X/XO, and we could not measure the experimental Kf. Thus 430 µM X was determined. Finally, the right lung
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 $K_r$, 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 i.v; 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/mm$^3$ on day 4 compared with 1,500 to 2,000/mm$^3$ on day 0, whereas platelets were reduced from 350,000 to 136,000/mm$^3$ 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, J apan), 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 an infusion of ONO-5046 at a rate of 10 mg·kg$^{-1}$·h$^{-1}$ inhibited the leukotriene B$_4$-induced increase in $K_r$, W/D, and perfusate neutrophil elastase activity in rabbit lungs (34) and that the 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). Tx$\alpha_2$ 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, theTx synthesis inhibitor OKY-046 was added to the reservoir to achieve an initial perfuse 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 $\text{TXB}_2$ concentration in isolated blood-perfused rabbit lungs (35).

Vehicle + vehicle group (n = 5). Five minutes after measurement of baseline $K_r$, 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, $K_r$ was measured again as experimental $K_r$. 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 $P < 0.05$.
Thus administration of ONO-5046 and neutrophil depletion attenuated lung edema induced by X/XO administration. No significant difference was noted between the OXY-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 Kt (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 Kt 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 Kt 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 group (6.86 ± 0.14 vs. 5.82 ± 0.04, P < 0.01). W/D was significantly lower in the OKY-046 + 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).
neutrophils in X/XO-induced oxidant lung injury as

The objective of this study was to determine the role of neutrophil depletion or OKY-5046 attenuated these indexes of injury; 2) OKY-046 or OKY-5046 attenuated transient P\textsubscript{pa} 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.

We showed that 430 µM X and 10 mU/ml XO caused a 77% increase in K\textsubscript{f} (Fig. 1). This result is in agreement with the data of Barnard and Matalon (4), who demonstrated that 500 µM X and 5 mU/ml XO caused a twofold increase in K\textsubscript{f} in isolated buffer-perfused rabbit lungs and that the K\textsubscript{f} 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 K\textsubscript{f}. The objective of this study was to determine the role of neutrophils in X/XO-induced oxidant lung injury as measured by K\textsubscript{f} and W/D. In the present study, experimental K\textsubscript{f} and W/D were significantly lower in the depleted + X/XO 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 MPO. The neutrophil-replete lungs showed responsiveness to X/XO as measured by increases in permeability and edema (data not shown). These results indicate that intrapulmonary neutrophils play a major role in the X/XO-induced lung injury in our model. However, transient P\textsubscript{pa} increases after administration of X/XO were not suppressed in the depleted + X/XO group. This means that oxidant-induced vasoconstriction is independent of intrapulmonary neutrophils.

On the other hand, to investigate whether an inhibition of the pressor response induced by X/XO would attenuate the oxidant lung injury, the Tx synthesis inhibitor OKY-046 was used. Tx has been shown to be a major mediator of purine + XO-induced vasoconstriction in isolated saline-perfused rabbit lungs (30). Paterson et al. (23) demonstrated that OKY-046 prevented ischemia-reperfusion-induced Tx generation and circulating neutrophil H\textsubscript{2}O\textsubscript{2} production in the rat lower thoracic ischemia-reperfusion model. In the present study, OKY-046 significantly reduced the X/XO-induced pressor response (Fig. 4). This means that Tx is a major mediator of transient P\textsubscript{pa} increase after administration of X/XO in our model also. However, OKY-046 failed to attenuate increases in K\textsubscript{f} and W/D (Figs. 2 and 3). Thus we considered that X/XO-induced lung injury was caused by some action of intrapulmonary neutrophils other than X/XO-induced Tx generation. The possible cellular source of Tx in the lung is considered to be the pulmonary vascular endothelium, macrophages, neutrophils, and/or platelets. The main source of Tx is unclear in our study, but neutrophils were not the source, because 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 K\textsubscript{f} 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 P\textsubscript{pa} 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 TXB\textsubscript{2} and 6-ketoprostaglandin F\textsubscript{1α} concentrations as well as the increase in P\textsubscript{pa} 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

![MPO activity](image-url)
ONO-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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