Cyclooxygenase Metabolites Possibly Produced by Endothelial Cells Mediate the Lung Injury Caused by Mechanically Stimulated Leukocytes

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TANITA, T., UEDA, S., CHUN, S., HOSHIKAWA, Y., NODA, M., KUBO, H., SUZUKI, S., ONO, S. and FUJIMURA, S. Cyclooxygenase Metabolites Possibly Produced by Endothelial Cells Mediate the Lung Injury Caused by Mechanically Stimulated Leukocytes. Tohoku J. Exp. Med., 1997, 183 (3), 221–232 — To determine whether mechanically stimulated leukocytes increase pulmonary vascular permeability and resistance and, if so, whether cyclooxygenase metabolites mediate the increase, we assessed the effects of stimulated and unstimulated leukocytes, and of a cyclooxygenase inhibitor on pulmonary vascular permeability and resistance in isolated perfused lungs from Sprague-Dawley rats. Leukocytes were stimulated by gentle agitation in a glass container for 10 seconds. After baseline measurements were made, stimulated or unstimulated leukocytes were added to the perfusate. The effects of the cyclooxygenase inhibitor, meclofenamate, on the pulmonary vascular filtration coefficient and pulmonary vascular resistance were measured. In the rats that received stimulated leukocytes, the pulmonary vascular filtration coefficient and the vascular resistance were about 2.5 times and 3.3 times higher, respectively, than those in the rats that received unstimulated leukocytes. These increases were completely and partly blocked by meclofenamate. Histological examination indicated that meclofenamate did not prevent the adhesion of leukocytes to the pulmonary vascular endothelium. These findings suggest that mechanically stimulated leukocytes increase pulmonary vascular permeability and that cyclooxygenase metabolites produced by endothelial cells may injure the cells. ——— cyclooxygenase metabolites; pulmonary vascular permeability; isolated rat lung; leukocyte © 1997 Tohoku University Medical Press

Acute respiratory distress syndrome (ARDS) is thought to be a typical pulmonary inflammation related to activation of polymorphonuclear leukocytes

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(Demling 1995). A recent report indicated that leukocytes are stimulated during cardiopulmonary bypass (Elliott and Finn 1993) and thus could be a partial cause of pulmonary injury after cardiopulmonary bypass (Elliott and Finn 1993). We suspected that during bypass, mechanical shear stress could stimulate leukocytes by increasing the number of adhesion molecular on the surface of leukocytes, allowing the leukocytes to adhere to vascular endothelial cells and injure them. Adhesion of leukocytes may release chemical mediators, such as, leukotriens, oxygen radicals or elastase. Then they injure vascular endothelial cells and increase in vascular resistance. In support of mechanical shear stress increasing adhesion, mechanical agitation caused an increase in the proportion of leukocytes bearing receptors for C3b (C3b-R), which is known as CD11b/CD18 (Mac-1) (Naess et al. 1986).

If the increase in vascular permeability and/or resistance caused by mechanically stimulated leukocytes is blocked by cyclooxygenase metabolites, main sites for these inflammatory process may exist in the endothelial cells. Since, leukocytes are not main source for cyclooxygenase metabolites (Yoshimoto et al. 1986) and main arachidonate metabolites of leukocytes are leukotriens.

In this study, we asked whether stimulated leukocytes increase pulmonary vascular permeability and resistance and, if so, whether cyclooxygenase metabolites mediate the increase. To answer these questions, we did experiments on isolated rat lungs.

Materials and Methods

Preparation

We anesthetized 18 adult male Sprague-Dawley rats (420 ± 73 g) with pentobarbital sodium (50 mg/kg, ip). Then we performed a tracheotomy, inserted a 15-gauge luer stub adapter (Clay Adams, Parsippany, NJ, USA), inserted a catheter (PE 50; Clay Adams) into the carotid artery, and injected 500 units/kg of heparin to the rats. After exsanguinating the rat, we made a sternum-splitting incision, and opened the pericardium. We inserted plastic tubes (PE 200; Clay Adams) connected to silastic tubes (O.D. 4.65 mm, I.D. 3.35 mm; Dow Corning, Midland, MI, USA) into the pulmonary artery by way of the right ventricle and into the left ventricle. The aorta was ligated when the catheter was inserted into the pulmonary artery. We flushed the lungs with 50 ml of saline until the effluent became clear and the lungs turned white. Then we ligated the venae cavae and removed the heart and lungs en bloc.

After recording the weight of the heart and lungs and catheters, we placed them in a plastic box (B-345; Lustro Ware, Tokyo) and suspended them in a plexiglas box from a counterbalancing bar, which was pivoted opposite the force displacement strain gauge (FT pick up TB-611T; Nihon Kohden, Tokyo) (Fig. 1). We connected the trachea to a compressed-air source and maintained the airway pressure at 2.0 cmH₂O. We connected the catheters from the pulmonary artery
Fig. 1. Experimental setup for identifying injury in isolated rat lungs. Rat lungs were placed in the plastic box, and perfused in an isogravimetric state under zone 3 conditions (pulmonary arterial pressure > pulmonary venous pressure > alveolar pressure). The perfusate was 6% bovine serum albumin dissolved in Krebs-Henseleit solution. Weight gain was continuously monitored using the force transducer. Airway pressure, pulmonary arterial and venous pressures, and perfusate flow were continuously monitored. Arterial and venous reservoirs were set at various heights to give any desired vascular pressures.

and the left atrium to the arterial and venous reservoirs, respectively. The reservoirs were saturated with mixed gas containing 30% O₂, 5% CO₂, and 65% N₂. The reservoirs could be individually set at various heights to give any desired vascular pressures. The arterial and venous reservoirs were immersed in a water bath and warmed to 37°C.

For the study, the lungs were kept in an isogravimetric state under zone 3 conditions (pulmonary arterial pressure > pulmonary venous pressure > alveolar pressure) (West et al. 1964). The heart and lungs were perfused in bovine serum albumin (fraction V; Sigma, St. Louis, MO, USA) dissolved in Krebs-Henseleit buffer to a concentration of 6%. A constant pressure-flow system was used throughout the series of experiments: Pulmonary venous and airway pressures (Ppv and Paw) were adjusted 2.5 and 2.0 cmH₂O, respectively, and pulmonary arterial pressure (Ppa) was adjusted so that the lungs were neither gaining nor losing weight.

Isolation of leukocytes

Autologous leukocytes were isolated from heparinized blood obtained from experimental rats when they were exsanguinated. Blood samples were centrifuged at 600 × g for 20 minutes, and the buffy coat was obtained and then the cell suspension was centrifuged at 600 × g for 20 minutes. The cells were resuspended in saline and counted using an automated cell counter (Coulter T-890; Coulter Electronics, Inc., Tokyo) and adjusted to 10⁷/µl.
Experimental protocol

To determine whether stimulated leukocytes increased pulmonary vascular permeability and resistance and whether cyclooxygenase metabolites mediate these increases, we performed three series of experiments in isolated rat lungs. In the first series (6 rats), we injected saline into the pulmonary artery followed more than 30 minutes later by unstimulated leukocytes. In the second series (6 other rats), we injected saline followed by stimulated leukocytes. In the third series (6 other rats), we injected meclofenamate (Conroy et al. 1991) followed by stimulated leukocytes. We stimulate leukocytes by shaking them gently in a glass container for 10 seconds at room temperature (20 ± 2°C). In these leukocytes specimens, neutrophils fraction was 12.3 ± 7.5%, and the remainder was monocytes. Before and after each vehicle was injected and again 90 minutes later after leukocytes were injected (final concentration of 300/μl of perfusate), we determined the following sets of variables: Filtration coefficient (K), an indicator of vascular permeability; flow rate and pulmonary arterial and double occlusion pressures (Dawson et al. 1982) for the calculation of pulmonary vascular resistance. Each set of variables was determined three times. The average of the three determinations was used as the value for the rat.

Measurements

Pulmonary arterial pressure (Ppa), pulmonary venous pressure (Ppv), and airway pressure (Paw) were continuously measured with pressure transducers (P23ID; Gould Inc., Santa Ana, CA, USA) and the perfusate flow was measured using electromagnetic flow meter (FF050 and MF-27; Nihon Kohden). The lung weight, Ppa, Ppv, Paw, and flow rate were continuously recorded on a polygraph (WT-687G; Nihon Kohden).

Calculation of the filtration coefficient (K)

The filtration coefficient (K), an indicator of vascular permeability, was calculated as previously described (Tanita et al. 1996). In brief, both the pulmonary arterial and venous pressures were increased by 3 cmH₂O, simultaneously, and the lung weight gain was obtained. This weight gain in the lungs was expressed as two phases. The standard interpretation of the weight gain curve is that there is an initial vascular volume increment plus continuous filtration. The initial increment can be eliminated by making a semilogarithmic plot of weight gain over time and determining the slow component (Drake et al. 1980; Tanita et al. 1996). The total vascular volume increment was completed in less than 3 minutes. When we plotted the gains in weight of the lungs semilogarithmically as a function of time, the two phases of weight gain were revealed. By fitting a line to the later phase of weight gain (data obtained from the last 7 minutes) by the least squares method and extrapolating to time zero, we obtained
the initial filtration rate. The filtration coefficient, \( K \), was calculated by dividing the initial filtration rate by the applied microvascular pressure increment and normalizing to 1 g of wet lung weight.

**Calculation of the pulmonary vascular resistances**

The pulmonary arterial and venous resistances (\( R_a, R_v \)) and the pulmonary arterial and venous resistance ratio (\( R_a/R_v \)) were calculated as follows:

\[
R_a = \frac{(P_{pa} - P_{do})}{Q_p} \quad (1)
\]
\[
R_v = \frac{(P_{do} - P_{pv})}{Q_p} \quad (2)
\]
\[
\frac{R_a}{R_v} = \frac{(P_{pa} - P_{do})}{(P_{do} - P_{pv})} \quad (3)
\]

where \( P_{pa}, P_{pv}, \) and \( P_{do} \) are pulmonary arterial, pulmonary venous, and double occlusion pressures, respectively, and \( Q_p \) is the perfusate flow.

**Histology**

The lungs were perfused with 50 ml of saline followed by 20 ml of 10\% of buffered formalin at less than 15 cmH\(_2\)O of perfusion pressure after the all measurements had performed. Then they were immersed in 10\% of buffered formalin for 5 or 6 days. We obtained 5 sections from each experimental lung and dyed with Hematoxilin-Eosin.

**Immunofluorescence**

To determine whether stimulated leukocytes have adhesion molecules that could adhere to blood vessels, leukocytes, either unstimulated or stimulated, were stained using a direct immunofluorescence technique. Leukocytes (10\(^6\)) were incubated with 2.9 mg of anti-CD18 MoAb (YFC118.3) for 20 minutes on ice. Leukocytes were analyzed on an Ortho CYTRON ABSOLUTE (Ortho Diagnostic Systems; Ravitan, NJ, USA).

**Statistical analysis**

Data are expressed as mean ± S.D. and were analyzed by analysis of variance (ANOVA) with significance accepted as \( p < 0.05 \).

**Results**

**Pulmonary vascular permeability**

The filtration coefficient did not change from baseline after the addition of vehicle or unstimulated leukocytes (4.26 ± 0.86, 4.99 ± 1.11, 4.76 ± 2.01 mg · cm H\(_2\)O\(^{-1}\) · min\(^{-1}\) · g\(^{-1}\), respectively). However, after stimulated leukocytes were injected, the filtration coefficient increased by about 2.5 times (5.04 ± 0.84, 5.74 ± 1.30, 13.87 ± 7.93 mg · cmH\(_2\)O\(^{-1}\) · min\(^{-1}\) · g\(^{-1}\), respectively) (Fig. 2). The increase in the filtration coefficient induced by stimulated leukocytes was inhibited by meclofenamate (5.34 ± 1.37, 5.57 ± 1.40, 4.69 ± 1.79 mg · cmH\(_2\)O\(^{-1}\) · min\(^{-1}\) · g\(^{-1}\),
Fig. 2. Effect of meclofenamate on the filtration coefficient during lung injury induced by stimulated leukocytes. Values shown mean ± s.d. The value for stimulated leukocytes was significantly different from those for vehicle (*), for unstimulated leukocytes (†), and for meclofenamate (‡) (p < 0.05 in each case). □; unstimulated leukocytes group, ■; stimulated leukocytes group, ★; meclo group.

respectively) (Fig. 2).

**Pulmonary vascular resistance**

Pulmonary arterial and venous resistances were both increased by about 3.3 times after injection of stimulated leukocytes (Table 1). The increases reflected decreases in perfusate flow to about one-third of baseline. Pressures did not change. Pulmonary arterial and venous resistance ratio (Ra/Rv) was also unchanged. The increases in pulmonary arterial and venous resistances were not inhibited by meclofenamate.

**Histological findings**

Microscopy showed that lungs that received stimulated leukocytes had many leukocytes in the alveolar capillaries and perivascular cuffs around the pulmonary arterioles (Fig. 3). Lungs that received meclofenamate and stimulated leukocytes had the similar number of leukocytes (Fig. 4) but fewer perivascular cuffs.

**Immunofluorescence**

In the immunofluorescence study, CD18 on the surface of the stimulated leukocytes was higher than that of unstimulated leukocytes (Fig. 5).

**Discussion**

This study shows that stimulated leukocytes increase pulmonary vascular
Table 1. *Hemodynamic data*

<table>
<thead>
<tr>
<th></th>
<th>Perfusate flow (ml · min⁻¹)</th>
<th>Pulmonary arterial pressure (cm H₂O)</th>
<th>Double occlusion pressure (cm H₂O)</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Vehicle</td>
<td>Experiment</td>
</tr>
<tr>
<td>Unstimulated leukocytes</td>
<td>18.3 ± 3.7</td>
<td>15.7 ± 2.1</td>
<td>17.3 ± 4.7</td>
</tr>
<tr>
<td>Stimulated leukocytes</td>
<td>17.6 ± 4.5</td>
<td>15.0 ± 3.0</td>
<td>5.8 ± 3.4*†</td>
</tr>
<tr>
<td>Stimulated leukocytes + Meclo</td>
<td>14.2 ± 2.4</td>
<td>14.1 ± 3.5</td>
<td>6.8 ± 2.5*†</td>
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<tr>
<th></th>
<th>Pulmonary arterial resistance (Ra) (cm H₂O · ml⁻¹ · min⁻¹)</th>
<th>Pulmonary venous resistance (Rv) (cm H₂O · ml⁻¹ · min⁻¹)</th>
<th>Ra/Rv</th>
</tr>
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<tr>
<td></td>
<td>Baseline</td>
<td>Vehicle</td>
<td>Experiment</td>
</tr>
<tr>
<td>Unstimulated leukocytes</td>
<td>0.33 ± 0.10</td>
<td>0.35 ± 0.09</td>
<td>0.35 ± 0.09</td>
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<tr>
<td>Stimulated leukocytes</td>
<td>0.35 ± 0.13</td>
<td>0.35 ± 0.10</td>
<td>1.38 ± 0.83*†</td>
</tr>
<tr>
<td>Stimulated leukocytes + Meclo</td>
<td>0.37 ± 0.12</td>
<td>0.39 ± 0.20</td>
<td>0.86 ± 0.56</td>
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</tbody>
</table>

Values are means ± S.D. *Significantly different from the values measured before infusion of stimulated leukocytes. †Significantly different from the values measured after infusion of unstimulated leukocytes (p < 0.05).
Fig. 3. Number of leukocytes in the alveolar capillaries seen in one visual field (×1000). Significantly different from the value for the unstimulated leukocyte group (p < 0.05).

Fig. 4. Microscopic view of tissue from a lung that received stimulated leukocytes only (A: H-E staining, ×50) and from a lung that received both meclofenamate and stimulated leukocytes (B: H-E staining, ×50). In A, note the large number of leukocytes in the alveolar capillaries, and the perivascular cuff around the pulmonary arteriole. In B, note the large number of leukocytes but no perivascular cuff around the pulmonary arteriole.
permeability and resistance and that cyclooxygenase metabolites partly mediate these increases. In the rat lungs given stimulated leukocytes, permeability and resistance were 2.5 and 3.3 times greater than in rats given unstimulated leukocytes. Lungs that received stimulated leukocytes had many leukocytes in the alveolar capillaries and perivascular cuffs. Lungs that received meclofenamat and stimulated leukocytes had the similar number of leukocytes but fewer perivascular cuffs.

Indirect evidence that the increase in permeability was caused by stimulated leukocytes is that the filtration coefficient was increased but the double occlusion pressure did not change, so increased pressure was not the cause of increased permeability.

The mediator of the increased permeability induced by stimulated leukocytes may be the cyclooxygenase metabolite thromboxane A₂, or prostaglandin E₂ (Fujii et al. 1995). Thromboxane A₂ is one of the main mediators in endotoxin-induced lung injury (Salzer and McCall 1990; Schneidkraut and Carlson 1993), though it is not a mediator in lung injury induced by PMA (phorbol myristate acetate) or TNF (tumor necrosis factor) (Hocking et al. 1991; Wurtz et al. 1992). Where thromboxane A₂ or prostaglandin E₂ are produced is not clear, but they may be from leukocytes, from endothelium, or from interaction of the two. Leukocytes are not main source for cyclooxygenase metabolites, because main arachidonate metabolites of leukocytes are leukotrienes. Only a little cyclooxygenase metabolites are generated in leukocytes (Yoshimoto et al. 1986). In our experiment, leukocytes alone were stimulated and endothelial cells were not. However, the cause of the increase in the vascular permeability was cyclooxygenase products. A key to solve this conflict is the adhesion of the leukocytes.

Fig. 5. Changes in fluorescence, indicating differences in the expression of CD18 on the surface of the unstimulated leukocytes and leukocytes stimulated by being shaken in a glass container. Double (□) and solid (■) lines represent the surface expression of CD18 in unstimulated and stimulated leukocytes, respectively.
to the endothelial cells. Adhesion of leukocytes to the endothelial cells activates tyrosin kinase system in the endothelial cells. Then phospholipase C (PLC) is activated and inositol-1,4,5-triphosphate (IP3) and diacylglycerol are produced. Increase in intracellular calcium concentration, induced by IP3, activates phospholipase A2 (PLA2) which generate arachidonic acid from phospholipids (Glaser et al. 1993). We believe that this sequence was occured in the pulmonary vascular endothelial cells when we gave the stimulated leukocytes to the isolated rat lungs, and was blocked by meclofenamate when we pretreated the endothelial cells with it.

In our study, evidence that cyclooxygenase metabolites partly mediate the increase in pulmonary vascular permeability is that the cyclooxygenase inhibitor meclofenamate partly blocked the increase in pulmonary vascular resistance. Therefore, this increase in the vascular resistance was possibly caused by release of vasoactive substances such as thromboxanes (Chang 1994), or also possibly caused by embolization of leukocytes (Reynolds and McDonagh 1989).

The flow cytometrical study showed that CD 18 was upregulated on the surface of stimulated leukocytes, most of them were in the neutrophil fraction. CD 18, a family of leukocyte integrins, is one of the main adhesion molecules made from glycoprotein that induces firm adhesion of leukocytes to the endothelium. A previous report indicated that agitation caused an increase in the proportion of granulocytes bearing receptors for C3b (C3b-R), and of lymphocytes bearing receptors for Fc gamma-R (Naess et al. 1986). C3b is called Mac-1 (CD 11b / CD 18) and has ICAM-1 or ICAM-2 as counter ligands on the endothelial cells. Upregulation of CD 18 on the surface of leukocytes following agitation in a glass container might have caused an increase in adhesiveness to pulmonary vascular capillaries.

Meclofenamate did not prevent adhesion of leukocytes to the pulmonary vessels, whereas it blocked the increase in the pulmonary vascular permeability. Therefore, we conjectured that the stimulated leukocytes, being increased in adhesiveness, adhered to and injured the pulmonary vascular bed through cyclooxygenase metabolites. Adhesion of leukocytes to the endothelial cells may act as an initiator of lung injury, and these signals are transduced to PLA2 and then cyclooxygenase metabolites are produced, and finally they amplify lung injury.

ARDS is a typical inflammation related to activation of polymorphonuclear leukocytes. Stimulation of leukocytes are occurred by many causes, namely, sepsis or cardiopulmonary bypass. Sepsis may stimulate leukocytes via a chemical manner, and cardiopulmonary bypass may stimulate them via a mechanical manner, such as shear stress. One of merits of our method is to investigate interactions of leukocytes and endothelial cells without any chemical stimuli, such as, PMA, TNF and/or PAF (platelet activating factor). The mechanism of this mechanical stimulation is still unknown. Perfusate was circulated using rotary
pump, but unstimulated leukocytes did not injure the lungs, whereas stimulated leukocytes did. Contact of leukocytes to glass surface may shed off L-selectin on the surface of leukocytes and draw CD18 from cytoplasm (Fraticelli et al. 1996). One possibility of stimulation of leukocytes during cardiopulmonary bypass is contact of leukocytes to membrane of oxygenator. We did not confirm roles of platelets by giving stimulated platelets. Although, the concentration of platelets in the isolated leucocytes specimens might be very low, contamination of platelets might have affected to our results.

In conclusion, this study shows that stimulated leukocytes increase pulmonary vascular permeability and resistance and that cyclooxygenase metabolites which might be generated in the endothelial cells partly mediate these increases.

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References


