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# Stimulated Neutrophils Evoke Signal Transduction to Increase Vascular Permeability in Rat Lungs

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Tanita, T., Song, C., Kubo, H., Ono, S., Sagawa, M., Sato, M., Matsumura, Y., Kondo, T. and Fujimura, S. *Stimulated Neutrophils Evoke Signal Transduction to Increase Vascular Permeability in Rat Lungs.* Tohoku J. Exp. Med., 1999, **189** (3), 213-225 —— The mechanisms by which stimulated neutrophils (PMNs) damage pulmonary vascular endothelium were investigated using twenty-four perfused lung preparations isolated from rats. We tested the ability of unstimulated and mechanically stimulated PMNs to adhere to pulmonary endothelial cells and, thereby, alter pulmonary vascular permeability (measured as the pulmonary filtration coefficient) and hemodynamics. To stimulate PMNs, they were gently agitated in a glass vial for 10 seconds. Perfusing lungs with the stimulated PMNs (stimulated group) elicited a 3-fold increase in the filtration coefficient as compared to lungs perfused with unstimulated cells (unstimulated group). This increase in filtration was completely blocked by preincubation of stimulated PMNs with CD18 monoclonal antibody (MoAb group). This increase in filtration coefficient was also completely blocked by GF109203X, a protein kinase C inhibitor (GF group). Pulmonary vascular resistance increased when the stimulated PMNs were injected to the isolated lungs. Although, preincubation of stimulated PMNs with CD18 MoAb successfully blocked and GF109203X partly blocked this increase in pulmonary vascular resistance. The accumulation of stimulated PMNs within the lungs, as assessed by myeloperoxidase (MPO) levels, was blocked by preincubation of stimulated PMNs with CD18 MoAb. However, GF109203X did not decrease MPO levels. These findings suggest that stimulated PMN-induced increases in pulmonary vascular filtration, resulted from endothelial cell injury caused by adhesion to the endothelial cells, evoke intracellular signaling within the endothelial cells. ——— adhesion molecules; GF109203X; neutrophils; protein kinase C; pulmonary vascular filtration 

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Acute respiratory distress syndrome is thought to be a typical pulmonary inflammation related to activation of neutrophils (PMNs) (Demling 1995). It is known that PMNs are activated during cardiopulmonary bypass (Elliott and Finn 1993) employed for thoracic or cardiac surgery, and therefore, they may play a role in the pulmonary injury associated with this procedure (Dreyer et al. 1995; Finn et al. 1996). Blood contact with synthetic surfaces during cardiopulmonary bypass causes a diffuse inflammatory reaction that includes PMNs activation (Gillinov et al. 1994). We postulated that during bypass, mechanical shear stress or contact to synthetic surfaces, such as membrane oxygenator, stimulates PMNs to increase the availability of adhesion molecules on their surfaces. Consistent with this notion, mechanical agitation for 30 seconds on a tube mixer induced an increase in the proportion of PMNs bearing receptors for C3b (C3b-R) (Naess et al. 1986), now classified as CD11b/CD18 (Mac 1), thus enabling them to more readily adhere to vascular endothelial cells. Once bound, PMNs may injure endothelial cells and increase pulmonary vascular resistance by releasing chemical mediators such as leukotriens, oxygen radicals and/or elastase.

Alternatively or in addition, the endothelial cell injury caused by activated PMNs may result from an interaction between PMN adhesion molecules and an endothelial cell signaling pathway mediating activation of protein kinase C (PKC). PKC is a key enzyme that controls intracellular signaling to regulate tight junction permeability (Fasano et al. 1995), or to regulate apoptosis by mitogen-activated protein kinase signaling cascade (Hall-Jackson et al. 1998). Several reports indicated that activation of PKC induced gap formation and barrier dysfunction of pulmonary artery endothelial cell monolayer (Patterson et al. 1995). If this signaling cascade play a role for regulation of pulmonary vascular permeability, and the primary sites for this inflammatory process are the endothelial cells, the effect should be inhibited by a PKC inhibitor. In this study, we addressed the question of whether stimulated PMNs increase pulmonary vascular permeability and/or resistance and, if so, whether intracellular signaling cascade mediate the increase. To answer these questions, we performed experiments on isolated rat lungs.

## Materials and Methods

### *Isolation of PMNs*

Human PMNs were isolated from heparinized blood obtained from a healthy adult who had no infectious foci and was taking no anti-inflammatory medications. Blood samples were layered over Polymorphprep® (Nycomed Pharma, Oslo, Norway) and centrifuged at 500 g for 35 minutes at room temperature, and the band of PMNs was harvested using a Pasteur pipette. The PMN fraction was resuspended in modified Hanks' balanced salt solution (Sigma, St. Louis, MO, USA) without calcium, magnesium or bicarbonate, and again centrifuged at 400 g for 10 minutes. The cells were finally resuspended in HBSS in order to restore

normal osmolarity and then counted using an automated cell counter (Coulter T-890; Coulter Electronics, Inc., Tokyo).

*Immunofluorescence*

The presence of adhesion molecules on the surface of the isolated PMNs was analyzed using an immunofluorescence technique. Stimulated and unstimulated PMNs ($\sim 10^6$) were incubated with 2.9 $\mu$g of anti-CD18 monoclonal antibodies (MoAb; YFC118.3; a gift from Department of Pediatric Oncology, Institute of Development, Aging and Cancer, Tohoku University, Sendai) for 20 minutes on ice. The immunofluorescence emitted from the PMNs was then analyzed using a flowcytometer on a CYTRON ABSOLUTE (Ortho Diagnostic Systems; Ravitan, NJ, USA).

*Isolated rat lung preparation*

Twenty-four adult male Sprague-Dawley rats ($252.6 \pm 72.3$ g) were used in this study. After anesthetizing each animal with pentobarbital sodium (50 mg/kg, ip), a tracheotomy was performed, and a 15-gauge luer stub adapter was inserted into the trachea (Clay Adams, Parsippany, NJ, USA). The carotid artery was then catheterized (PE 50; Clay Adams), 500 units/kg of heparin was injected and the rats were exsanguinated. At that point, we made a sternum-splitting incision and opened the pericardium. The pulmonary artery was catheterized by way of the right ventricle using polyethylene tubing (PE 200; Clay Adams) connected to silastic tubing (O.D. 4.65 mm, I.D. 3.35 mm; Dow Corning, Midland, MI, USA). The aorta was then ligated, and the left ventricle catheterized. We flushed the lungs with 50 ml of saline until the effluent became clear, and the lungs turned white. The venae cavae were then ligated, and the heart and lungs were removed en bloc.

After recording the weight of the preparation, including the heart, lungs and catheters, we placed it in a Plexiglas box (B-345; Lustro Ware, Tokyo) and suspended it from a counterbalancing bar attached to a force displacement strain gauge transducer (FT pick up TB-611T; Nihon Kohden, Tokyo) (Fig. 1). The trachea was connected to a compressed-air source so that the airway pressure could be continuously maintained at 2.0 cm $H_2O$. The catheters into the pulmonary artery and the left ventricle were connected to arterial and venous reservoirs, respectively. The reservoirs were immersed in a water bath and warmed to 37°C, saturated with mixed gas containing 30% $O_2$, 5% $CO_2$, and 65% $N_2$, and could be individually set at various heights to yield desired vascular pressures.

For this study, the lungs were kept in an isogravimetric state under zone 3 conditions (pulmonary arterial pressure > pulmonary venous pressure > alveolar pressure). The heart and lungs were perfused with Krebs-Henseleit buffer containing 6% bovine serum albumin (fraction V; Sigma), and a constant pressure-flow system was used throughout the experiments. Pulmonary venous

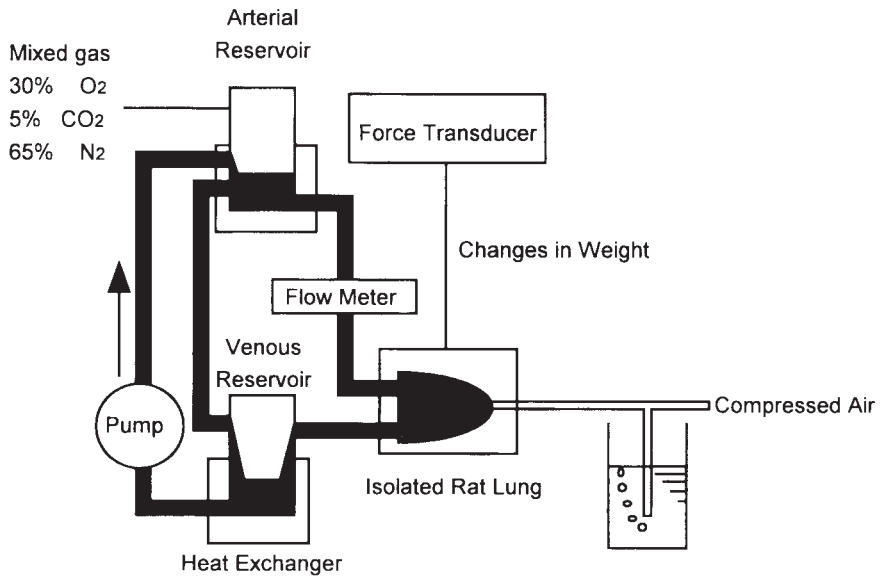


Fig. 1. Experimental setup for measuring pulmonary vascular permeability in isolated rat lungs. Rat lungs were placed in a Plexiglas box and perfused in an isogravimetric state under zone 3 conditions (pulmonary arterial pressure > pulmonary venous pressure > alveolar pressure). The perfusate was Krebs-Henseleit solution containing 6% bovine serum albumin. Weight gain was continuously monitored using a force transducer. Airway pressure (Paw), pulmonary arterial and venous pressures (Ppa and Ppv, respectively), and perfusate flow (Qp) were continuously monitored. Arterial and venous reservoirs were set at various heights to provide the desired vascular pressures.

and airway pressures (Pv and Paw, respectively) were adjusted to 2.5 and 2.0 cm $H_2O$, respectively, and pulmonary arterial pressure (Ppa) was adjusted so that the lungs were neither gaining nor losing weight.

*Experimental protocol*

The objective of these experimental protocols was to determine whether stimulated PMNs increase pulmonary vascular permeability and resistance and, if so, whether PKC in the endothelial cells mediates the increases. To accomplish this, 4 series of experiments were carried out in 4 groups of isolated rat lungs. In the first series of experiments (unstimulated group; $n=6$), 125 $\mu$l of saline was injected into the pulmonary artery; 30 minutes later, unstimulated PMNs (25 cells/$\mu$l) were injected. In the second series of experiments (stimulated group; $n=6$), 125 $\mu$l of Dimethyl Sulfoxide (DMSO; Sigma), a vehicle for GF109203X, an inhibitor of PKC (Sigma), was injected into the pulmonary artery, 30 minutes later, the perfusate was changed to that of new to avoid the effects of vehicle or tested agent to stimulated PMNs, and then stimulated PMNs (25 cells/$\mu$l) were injected. In the third series (GF group; $n=6$), we injected 50 nM of GF109203X, a PKC inhibitor, into the pulmonary artery. The perfusate was changed to that of new fluid more than 30 minutes later and then stimulated PMNs (25 cells/$\mu$l) were injected. In the fourth series (MoAb group; $n=6$), we injected saline

followed by stimulated PMNs (25 cells/$\mu$l) that had been previously incubated with CD18 MoAb. Stimulation of PMNs was achieved by shaking them gently in a glass vial for 10 seconds at room temperature ($20\pm2^{\circ}C$). In the MoAb group, stimulated PMNs were incubated with 2.9 $\mu$g of anti-CD18 MoAb (YFC118.3) per 106 PMNs for 20 minutes on ice. The PMN specimens were made up of $82.0\pm6.3\%$ neutrophils; the remaining cells were monocytes.

Before and after each injection of vehicle and again 90 minutes after injection of the PMNs (25 cells/$\mu$l of perfusate), we measured the filtration coefficient (K), an indicator of vascular permeability; to monitor the pulmonary hemodynamics, perfusate flow rate (Qp), Ppa and double occlusion pressure Pdo, which represents pulmonary capillary pressure, were measured (Townsley et al. 1986). As mentioned above, Ppv was set at 2.5 cm $H_2O$. After these measurements were obtained, either unstimulated or stimulated PMNs were injected into the pulmonary artery at the final concentration of 250 cells/$\mu$l of perfusate. The rat lungs were perfused with PMNs for $\sim$10 minutes and then flushed with 50 ml of saline to washout intravascular PMNs that had not adhered to endothelial cells. They were then stored in a deep freezer at $-80^{\circ}C$ until myeloperoxidase (MPO) measurements were made.

*Measurements*

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

*Calculation of the filtration coefficient*

The filtration coefficient (K) was calculated as previously described (Tanita et al. 1996). Briefly, Ppa and Ppv were simultaneously increased by 3 cm $H_2O$ and the time-dependent increase in lung weight was measured. The gain in lung weight occurred in two phases; the standard interpretation of the weight gain curve is that there are an early gain due to increased vascular volume and a slower gain resulting from continuous filtration. The increase in vascular volume was completed within $<3$ minutes; consequently, this component could be discriminated from the filtration component by plotting the log of the weight gain as a function of time (Drake et al. 1980; Tanita et al. 1996). By using the method of least squares to fit a line to the later phase (data obtained from the last 7 minutes) and extrapolating back to time zero, we obtained the initial filtration rate. K was calculated by dividing the initial filtration rate by the applied microvascular pressure increment and normalizing to one gram of wet lung weight.

*Myeloperoxidase assay*

As an index of PMN accumulation in the lungs, we measured MPO using a luminescence technique (Uehara et al. 1991). The frozen rat lungs were thawed, a volume of 0.02% cethyltrimethyl ammonium bromide (Wako Chemicals, Tokyo) equivalent to 5 times the lung weight was added, and the lungs were homogenized on ice. The homogenates were centrifuged at 11 180 $\times$ g and 4°C for 15 minutes in a high speed, refrigerated centrifuge (HITACHI 18PR-3, Hitachi, Tokyo). The pellets were rapidly frozen in liquid nitrogen, placed on ice, and then homogenized by sonication (UD201, Tomy Seiki, Tokyo) at 30 W for 30 seconds (30 times of 0.5 seconds burst plus 0.5 seconds stand). Freezing and sonication of the pellets were repeated 7 times. The homogenates were centrifuged at 16 100 $\times$ g and 4°C for 15 minutes, and the supernatants were collected for assay. Initially, standard reagent (50 $\mu$M superoxide dismutase, 20 $\mu$l; 2 mM desferrioxamine, 20 $\mu$l; 50 mM KBr, 20 $\mu$l; 40 mM $H_2O_2$, 25 $\mu$l; 0.2 M acetate-buffer, 1 ml; distilled water, 765 $\mu$l; and 0.2 mM 2-methyl-6-[p-methoxyphenyl]-3, 7-dihydroimidazo[1, 2-a] pyrazin-3-one [MCLA, Tokyo Kasei Organic Chemicals, Tokyo], 100 $\mu$l) was injected into a luminescence reader (BLR301, Aloca, Tokyo), and the basal luminescence was characterized for 3 minutes. The luminescences of the test specimens (50 $\mu$l of supernatant) was then assayed.

*Statistical analysis*

Data are expressed as mean $\pm$ S.D. and were analyzed by analysis of variance and Bonferroni test. $p$ Values of $<0.05$ were accepted as significant.

## Results

*Immunofluorescence*

Comparison of stimulated and unstimulated PMNs by analysis of their immunofluorescence revealed that CD18 and CD11b were upregulated on the surfaces of stimulated PMNs (Fig. 2, left-hand panels). In contrast, CD11a and CD11c were unaffected by a mechanical stimulation (Fig. 2, right-hand panels).

*Pulmonary vascular permeability*

Addition of vehicle or unstimulated PMNs had no significant effect on baseline values for the filtration coefficient which was measured before and after injection of vehicle and after injection of PMNs (4.97 $\pm$ 1.14, 4.45 $\pm$ 0.87, 5.45 $\pm$ 3.23 mg $\cdot$ cm $H_2O^{-1} \cdot$ minutes$^{-1} \cdot g^{-1}$, respectively). On the other hand, after stimulated PMNs were injected, there was a 4-fold increase ($p<0.05$, vs. unstimulated group) in the filtration coefficient (5.88 $\pm$ 0.55, 5.55 $\pm$ 1.51, 23.52 $\pm$ 11.82 mg $\cdot$ cm $H_2O^{-1} \cdot$ minutes$^{-1} \cdot g^{-1}$, respectively) (Fig. 3). In the MoAb group, the increase in the filtration coefficient induced by stimulated PMNs was completely blocked by preincubation with antibodies against CD18 (5.92 $\pm$ 0.97, 5.41 $\pm$ 2.21, 5.57 $\pm$ 2.32

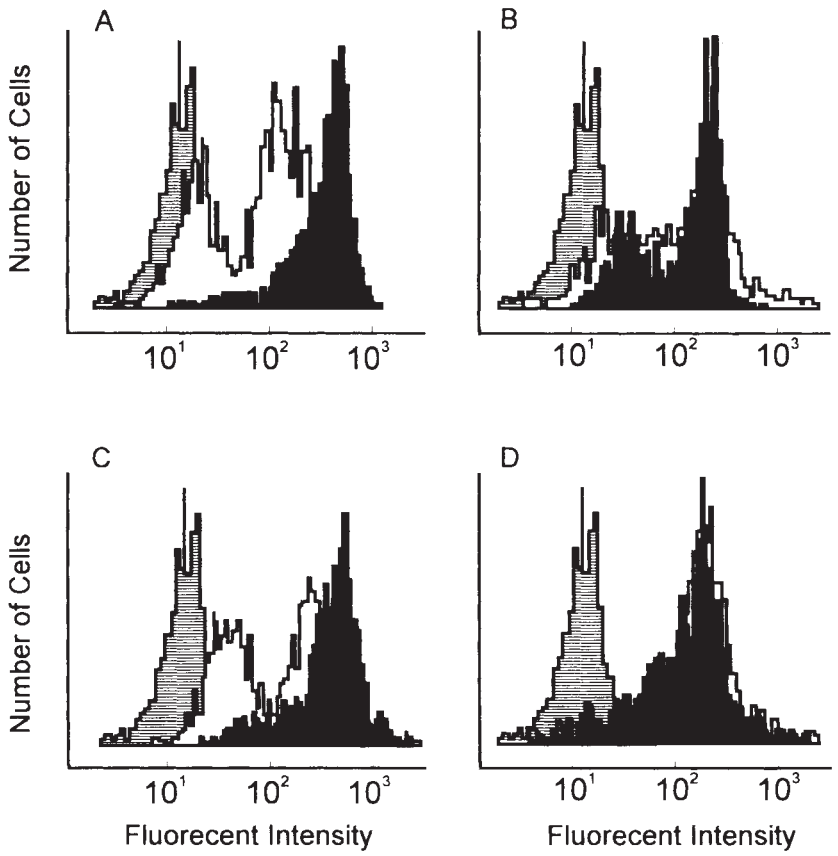


Fig. 2. Histograms drawing immunofluorescence reflecting expression of the $\beta 2$ integrins, CD18 (A), CD11a (B), CD11b (C) and CD11c (D), on the surface of the unstimulated PMNs (open histograms) and PMNs stimulated by being shaken in a glass vial (closed histograms); the hatched histogram represents a negative control.

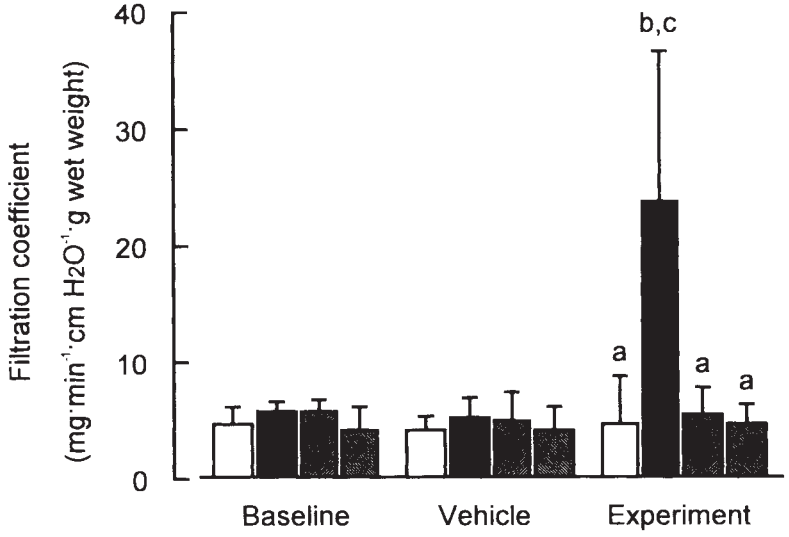


Fig. 3. Effects of anti-CD18 MoAb, and the PKC inhibitor, GF109203X, on the increase in pulmonary vascular permeability (filtration coefficient) elicited by stimulated PMNs. Values shown mean $\pm$ S.D. *a*, $p < 0.05$ vs. stimulated PMNs group; *b*, $p < 0.01$ vs. baseline; *c*, $p < 0.01$ vs. vehicle. □, unstimulated; ■, stimulated; ▨, MoAb; ▩, GF109203X.

mg•cm $H_2O^{-1}$•minutes$^{-1}$•g$^{-1}$, respectively) ($p<0.05$, vs. stimulated group). This increase was also inhibited by GF109203X, a PKC inhibitor, ($4.48\pm1.69$, $4.41\pm1.79$, $4.90\pm1.69$ mg • cm $H_2O^{-1}$ • minutes$^{-1}$ • g$^{-1}$, respectively) ($p<0.05$, vs. stimulated group).

*Pulmonary vascular resistance*

Ppa and Pdo were not significantly affected by any of the treatment protocols (Table 1). However, in the stimulated group, Qp decreased and the pulmonary arterial, venous and the total resistances (Ra, Rv and Rt, respectiely) increased compared to their baseline values when stimulated PMNs were injected. Besides, in the stimulated group, there pulmonary vascular resistances (Ra, Rv and Rt) increase compared to the values of vehicle injection when stimulated PMNs were injected. In the GF group, Qp decreased and Rv was increased compared to the baseline values when stimulated PMNs were injected. Among Qp, Ra and Rt in the experimental conditions, values in the stimulated group were significantly lower (Qp) and higher (Ra and Rt) than values in the rest of groups, respectively (*e*, Table 1). Whereas, among Qp, Ra, Rv and Rt in the experimental condition, values in the GF group were significantly lower (Qp) and higher (Ra, Rv and Rt) than values in the unstimulated and MoAb groups, respectively (*f*, Table 1).

*MPO assay*

The Myeloperoxidase (MPO) levels measured in the stimulated group ($44.82\pm24.53$ kcpm) were significantly higher than those in the unstimulated group ($5.86\pm6.73$ kcpm) (Fig. 4) ($p<0.05$). This suggests that stimulated PMNs accumulated in the lungs to a significantly greater extent than unstimulated cells. The increased MPO seen with stimulated PMNs was completely blocked by preincubation with anti-CD18 MoAb ($5.16\pm4.53$ kcpm; MoAb group) ($p<0.05$), but they were unaffected by exposing cells to GF109203X ($31.93\pm11.98$ kcpm; GF group).

## Discussion

Little information about the increase in pulmonary vascular permeability caused by mechanically stimulated PMNs is currently available. In this study, we found that when injected into the pulmonary circulation, stimulated PMNs increase pulmonary vascular permeability possibly after first adhering to the endothelial cells lining the pulmonary arteries. Consistent with this idea, CD11b/18 (Mac 1) adhesion proteins on the surface of PMNs were upregulated by mechanical stimulation, and increases in vascular permeability were blocked when stimulated PMNs were treated with anti CD18 MoAb. We postulate, therefore, that stimulated PMNs injured vascular endothelial cells by attaching to their surfaces (Albelda et al. 1994). PMN-induced increases in vascular permeability were blocked when stimulated PMNs were treated with anti CD18 monoclonal

TABLE 1. *Effect of experimental protocols on selected hemodynamic parameters*

| | Ppa (cm $H_2O$) | Pdo (cm $H_2O$) | Qp (ml $min^{-1}$) | Ra (cm $H_2O$ $ml^{-1}$ $min^{-1}$) | Rv (cm $H_2O$ $ml^{-1}$ $min^{-1}$) | Rt (cm $H_2O$ $ml^{-1}$ $min^{-1}$) | Ra/Rv |
|---|---|---|---|---|---|---|---|
| Unstimulated PMNs | | | | | | | |
| Baseline | 11.4±1.1 | 6.1±0.5 | 14.8±4.7 | 0.39±0.13 | 0.27±0.12 | 0.66±0.24 | 1.53±0.50 |
| Vehicle | 11.2±1.3 | 5.9±0.5 | 15.0±4.6 | 0.37±0.13 | 0.25±0.09 | 0.62±0.20 | 1.56±0.51 |
| Experiment | 11.2±1.4 | 6.4±0.8 | 14.7±5.5[e,f] | 0.35±0.11[e,f] | 0.30±0.15[e,f] | 0.66±0.25[e,f] | 1.26±0.38 |
| Stimulated PMNs | | | | | | | |
| Baseline | 9.9±0.7 | 5.3±0.5 | 11.3±4.1 | 0.44±0.14 | 0.30±0.18 | 0.74±0.30 | 1.71±0.65 |
| Vehicle | 10.0±0.8 | 5.5±0.3 | 7.7±2.0 | 0.61±0.13 | 0.41±0.09 | 1.02±0.21 | 1.48±0.11 |
| Experiment | 9.8±0.6 | 5.5±0.3 | 3.8±1.2[b] | 1.21±0.38[b,d] | 0.89±0.38[b,c] | 2.09±0.75[b,d] | 1.42±0.21 |
| Stimulated PMNs + anti CD18 antibody | | | | | | | |
| Baseline | 10.5±0.9 | 6.1±0.4 | 12.9±3.8 | 0.38±0.17 | 0.30±0.08 | 0.68±0.24 | 1.26±0.25 |
| Vehicle | 10.6±1.1 | 6.2±0.5 | 12.8±3.6 | 0.38±0.16 | 0.31±0.10 | 0.69±0.25 | 1.20±0.20 |
| Experiment | 10.7±0.9 | 6.2±0.7 | 13.3±4.1[e,f] | 0.37±0.15[e,f] | 0.30±0.11[e,f] | 0.68±0.25[e,f] | 1.23±0.23 |
| Stimulated PMNs + GF109203X pretreatment | | | | | | | |
| Baseline | 10.7±1.5 | 5.2±0.7 | 11.0±3.0 | 0.55±0.24 | 0.25±0.07 | 0.80±0.27 | 2.25±1.18 |
| Vehicle | 11.1±1.1 | 6.0±0.5 | 8.7±2.1 | 0.60±0.15 | 0.42±0.12 | 1.03±0.24 | 1.49±0.46 |
| Experiment | 11.1±0.8 | 6.4±0.8 | 7.3±1.6[a,e] | 0.68±0.27[e] | 0.56±0.18[b] | 1.24±0.42[e] | 1.23±0.31 |

Values are expressed as mean ±S.D. Ppa and Pdo, pulmonary arterial and double occlusion pressures; Qp, perfusate flow rate; Ra, Rv and Rt, pulmonary arterial, venous and total vascular resistances. [a]$p<0.05$ vs. baseline. [b]$p<0.01$ vs. baseline. [c]$p<0.05$ vs. vehicle. [d]$p<0.01$ vs. vehicle. [e]$p<0.05$ vs. values in experiment of stimulated group. [f]$p<0.05$ vs. values in experiment of GF group.

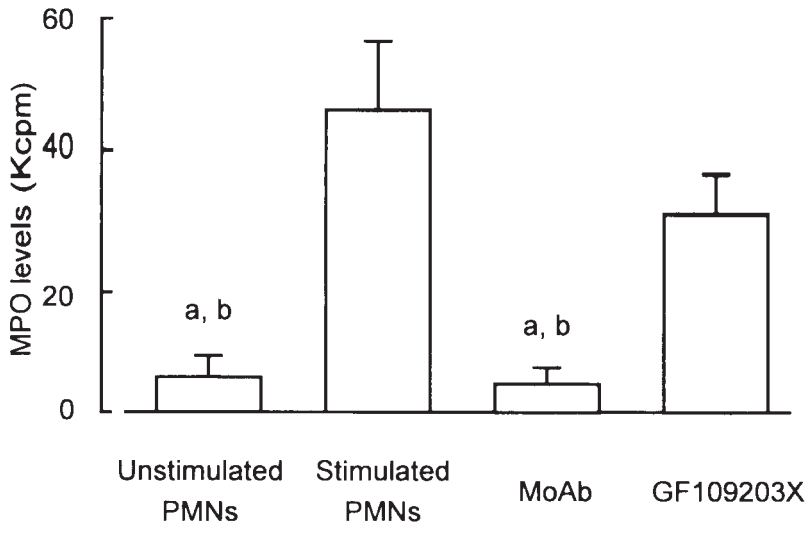


Fig. 4. MPO was measured in each experimental group. In lungs injected with stimulated PMNs, MPO levels were higher than in lungs injected with unstimulated PMNs or with stimulated PMNs incubated with anti-CD18 MoAb. On the other hand, GF109203X did not affect elevated MPO levels induced by stimulating PMNs. Values shown mean $\pm$ S.D. *a* and *b*, $p < 0.05$ vs. stimulated PMNs group and GF group, respectively.

antibodies.

Our analysis of cellular immunofluorescence showed that CD11b and CD18 were both upregulated on the surface of stimulated PMNs. CD11b/18 (Mac 1), a family of $\beta_2$ leukocyte integrins, is one of the principle glycoprotein-derived adhesion molecules that enable PMNs to firmly attach to endothelial cells. A previous report showed that agitation caused an increase in the proportion of granulocytes bearing receptors for C3b (C3b-R) and lymphocytes bearing receptors for Fc $\gamma$-R (Naess et al. 1986). C3b, which is also classified as Mac 1, uses ICAM-1 and/or ICAM-2 located on the surface of endothelial cells as counter ligands.

Increasing the availability of CD18 on the surface of PMNs by agitation in a glass vial would be expected to cause an increase in adhesiveness of PMNs. When we injected stimulated PMNs into isolated rat lungs, the pulmonary vascular permeability increased. This elevation in permeability, however, was completely blocked by preincubation of stimulated PMNs with anti-CD18 MoAb. In addition, MPO levels in the MoAb group were not different from the levels in the unstimulated group and were, therefore, low compared to those of the stimulated PMNs group. The ability of anti-CD18 MoAb to block the effects of stimulated PMNs confirms the importance of PMN adhesion to endothelial cells via CD18. Previous report indicated that anti-CD11a or anti-CD11b MoAb partly diminished the increase in the lung permeability caused by cobra venom factor (Mulligan et al. 1993). Both lymphocyte function-associate Ag-1 (LFA-1) and Mac 1 were assembled with subunits of $\beta 2$ integrins (CD11a and CD11b, respectively) and CD18 as a common subunit. Since anti-CD18 MoAb may block both LFA-1 and Mac 1, it could completely blocked the elevation in permeability.

We found that GF109203X, a PKC antagonist, blocked the PMN-induced increase in the vascular permeability. The dependence on PKC activation indicates that one or more intracellular signal transduction pathways must be involved. Phobol myristate acetate (PMA), a PKC agonist, can upregulate CD11b on the surface of PMNs (Jung et al. 1994; van Kessel et al. 1994), and PMNs activated by PMA induced pulmonary vascular injury (Perry and Taylor 1988; Perry et al. 1990). However, PMA may affect endothelial permeability by itself (Garcia et al. 1995; Nagpala et al. 1996; Huang and Yuan 1997), we decided not to use PMA for stimulating PMNs. In our study, Mac-1 has been upregulated on the surface of PMNs by mechanical stimulation, and the pulmonary vascular endothelial cells have been treated by a PKC antagonist. That is, the signal transduction system, related to PKC, only in the pulmonary vascular endothelial cells was blocked. The MPO levels treated with GF109203X were significantly higher than those in the unstimulated or MoAb groups. Therefore, when stimulated PMNs adhered to the pulmonary endothelial cells, in which the PKC related signal transduction system was blocked, the pulmonary vascular permeability did not increase. As mentioned above, GF109203X, a PKC antagonist was washed out prior to injection of PMNs to the pulmonary artery, signal transduction systems in the PMNs were not blocked by GF109203X. Therefore, these consecutive phenomena, resulted in the increase in the vascular permeability, might occurr in the pulmonary vascular endothelial cells.

Activated protein tyrosine kinase in turn activates phospholipase C which hydrolyzes phosphatidyl inositol 1, 4, 5 triphosphate forming inositol 1, 4, 5 trisphosphate (IP3) and 1, 2-diacylglycerol-second messengers that respectively increase $[Ca^{2+}]_i$ and activate PKC. It is known that PKC has important functions in the regulation of endothelial permeability and in the maintenance of endothelial integrity (Yamada et al. 1990). For instance, PKC phosphorylates cytoskeletal proteins in cultured bovine pulmonary artery endothelial cells resulting in barrier dysfunction (Stasek et al. 1992). The PKC activation would be expected to result in increased phosphorylation of myosin light chain and actin rearrangement (Zhao and Davis 1998).

Injection of stimulated PMNs increased vascular resistance in both the stimulated group and in the GF group where activation of PKC was blocked. Agitation of PMNs may also reduce flexibility of PMNs and then thought to be stuck to the pulmonary capillary. Several reports (Erzurum et al. 1992; Doherty et al. 1994) indicated the relation of Mac 1 up-regulation and stiffness of the PMNs. However, values of Qp and total resistances in the GF group were significantly higher (Qp) and lower (Ra and Rt) than those in the stimulated group, respectively. Therefore, stimulated PMNs likely increased vascular resistance in two ways: They occluded capillaries by adhering to their walls (Wurtz et al. 1992) and they elicited PKC activation leading to vascular smooth muscle contraction. In the present study, effects on endothelium were not isolated from

those on vascular smooth muscle. To address that question, examinations using cell culture system is necessary.

In conclusion, stimulated PMNs increase pulmonary vascular permeability and resistance, and intracellular signaling cascade partly mediates the increase.

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