Platelet-activating factor contributes to acute lung leak in rats given interleukin-1 intratracheally

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Lee, Young M., Brooks M. Hybertson, Hyun G. Cho, Lance S. Terada, Okyong Cho, Alexander J. Repine, and John E. Repine. Platelet-activating factor contributes to acute lung leak in rats given interleukin-1α intratracheally. Am J Physiol Lung Cell Mol Physiol 279: L75–L80, 2000.—Lung lavage fluid of patients with acute lung injury (ALI) has increased levels of interleukin-1 (IL-1) and neutrophils, but their relationship to the lung leak that characterizes these patients is unclear. To address this concern, we investigated the role of the neutrophil agonist platelet-activating factor [1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (PAF)] in the development of the acute neutrophil-dependent lung leak that is induced by giving IL-1 intratracheally to rats. We found that PAF acetyltransferase and PAF activities increased in lungs of rats given IL-1 intratracheally compared with lungs of sham-treated rats given saline intratracheally. The participation of PAF in the development of lung leak and neutrophil accumulation after IL-1 administration was suggested when treatment with WEB-2086, a commonly used PAF-receptor antagonist, decreased lung leak, lung myeloperoxidase activity, and lung lavage fluid neutrophil increases in rats given IL-1 intratracheally. Additionally, neutrophils recovered from the lung lavage fluid of rats given IL-1 intratracheally reduced more nitro blue tetrazolium (NBT) in vitro than neutrophils recovered from control rats or rats that had been given WEB-2086 and then IL-1. Histological examination indicated that the endothelial cell-neutrophil interfaces of cerium chloride-stained lung sections of rats given IL-1 contained increased cerium perhydroxide (the reaction product of cerium chloride with hydrogen peroxide) compared with lungs of control rats or rats treated with WEB-2086 and then given IL-1 intratracheally. These in vivo findings were supported by parallel findings showing that WEB-2086 treatment decreased neutrophil adhesion to IL-1-treated cultured endothelial cells in vitro. We concluded that PAF contributes to neutrophil recruitment and neutrophil activation in lungs of rats given IL-1 intratracheally.

acute lung injury; neutrophils

INTERLEUKIN (IL-1) and neutrophils appear to be closely linked to the pathogenesis of acute lung injury (ALI). First, IL-1 and neutrophils are increased in lung lavage fluid of ALI patients. Notably, there is a net proinflammatory IL-1 activity in the lungs of patients with acute respiratory distress syndrome (ARDS) despite the fact that both IL-1 and its antagonists are released at sites of inflammation (29). Second, treatment with IL-1 increases neutrophil adhesion and microvascular permeability in in vitro models of ALI (30). Third, instilling IL-1 intratracheally causes lung neutrophil recruitment and produces a neutrophil-dependent lung leak in rats. These findings suggest a relationship between IL-1 and neutrophils in ALI; IL-1 itself, however, is not a potent neutrophil chemoattractant or activator, and, accordingly, the intermediate mechanisms contributing to this relationship between IL-1 and lung leak are unclear. Although a number of possible explanations exist, a prior report showed that giving IL-1 intratracheally increased the activity of phospholipase A2 (PLA2) in the lung (19), suggesting that lipid mediators might contribute to IL-1-induced ALI. Moreover, platelet-activating factor (PAF) and PLA2 levels are increased in the lung lavage fluid of patients with sepsis or ARDS (13, 26, 35), and PAF increases the release of superoxide anion (O2−) during neutrophil respiratory burst (3, 14, 42). Because PAF is one of the downstream products of the lysophospholipids produced by PLA2 during inflammatory reactions (32), we postulated that PAF contributes to IL-1-induced lung injury involving neutrophils, and, in the present study, we investigated the role of PAF in the development of lung neutrophil recruitment and activation and lung leak in rats given IL-1 intratracheally.

METHODS

Sources of reagents. Recombinant IL-1 (endotoxin level < 1 endotoxin unit/mg) was purchased from R&D Systems (Minneapolis, MN), frozen in aliquots, and thawed daily before use. 125I-labeled bovine serum albumin ([125I]-BSA) was obtained from ICN Radiochemicals (Irvine, CA), 51Cr, [3H]selenotinin binoxalate, and [acetyl-3H]CoA were obtained from NEN Life Science Products (Boston, MA). WEB-2086 was donated by Boehringer Ingelheim. WEB-2086 (apafant) is a...
well-characterized and widely used hetrazepine derivative that acts as a potent PAF-receptor antagonist (6, 36). All other reagents were purchased from Sigma (St. Louis, MO).

**Administration of IL-1.** Male Sprague-Dawley rats weighing 300–350 g (Sasco, Omaha, NE) were fed a normal diet and acclimated to Denver’s altitude for at least 10 days before study. Initially, each rat was anesthetized with inhaled methoxyflurane. Subsequently, the trachea was exposed, a Teflon catheter was inserted into the trachea, and 50 ng of IL-1 (in 0.5 ml of sterile saline) were rapidly insufflated along with two 3-ml puffs of air. Sham-treated rats received identical anesthesia and surgery but were given only sterile saline intratracheally. Immediately after intratracheal instillation of IL-1 or saline, some of the rats were injected intravenously with the PAF-receptor antagonist WEB-2086 (8 mg/kg). After the neck incision was closed and sutured, the rats were allowed to awaken and were then returned to their cages with free access to food and water.

**Measurement of PAF.** Right lungs were homogenized in 10.0 ml of cold methanol with 50 mM acetic acid. After 4.0 ml of water and 5.0 ml of chloroform were added, the samples were vigorously mixed, and then the mixture was allowed to stand for 30 min at room temperature. Next, 5.0 ml of 0.1 M sodium acetate solution and 5.0 ml of chloroform were added, the mixture was centrifuged at 2,000 rpm for 5 min at room temperature, and the organic layer was collected. The aqueous phase was washed twice with 1.0 ml of chloroform, and the chloroform layers were added to the originally collected chloroform solution. The pooled chloroform fraction was evaporated under N2, and the solute was redissolved in 0.4 ml of 0.25% aqueous BSA solution. For the PAF assay (24, 40), platelets were isolated from 60 ml of whole human blood collected from normal donors by venipuncture. Whole blood was mixed with acid citrate-dextrose solution (41 mM citric acid, 75 mM trisodium citrate, and 130 mM glucose) in a 6:1 ratio and centrifuged at 1,600 rpm for 20 min at room temperature. The platelet-rich plasma was incubated with 1.0 μCi/ml of [3H]serotonin binoxalate for 15 min, then sedimented at 250 g for 15 min at 37°C. The platelet-containing pellet was washed with 5.0 ml of Tyrode gel solution containing 0.1 mM EGTA (pH 6.5) and with Tyrode gel solution supplemented with 0.25% BSA (pH 6.5), and then the pellet was resuspended in 1.0 ml of Tyrode gel solution with 0.25% BSA (pH 7.4). Next, 100 μl of the [3H]serotonin-incorporated platelet suspension were incubated with 0.1 ml of the PAF-containing extracts isolated from the lung tissue. The reaction was stopped by addition of 50 μl of 8 mM EDTA. After centrifugation at 12,000 g for 30 s, 100 μl of the supernatant were added to the scintillation cocktail, and the radioactivity was measured in a scintillation counter. Total intracellular serotonin available for release was determined by measuring [3H]serotonin in samples of labeled platelet suspensions treated with 100 μl of 0.2% Triton X-100. Release values were calculated as percentage of total cellular serotonin and converted into the concentration of PAF released with a PAF standard curve.

**Measurement of PAF acetyltransferase.** PAF acetyltransferase activity was measured in lung tissue homogenized in buffer (0.25 M sucrose, 1 mM EDTA, 1 mM diithiothreitol, 25 mM NaF, 0.1 M potassium phosphate, and 1% BSA, pH 8.0) at 4°C, followed by sonication for 90 s at maximum power (43). The sonicated lung homogenate was mixed with reaction buffer (0.1 M Tris-HCl, pH 6.9, with 50 μCi of [acetyl-3H]CoA, 2 mg of lyso-PAF in 2.0 ml of ethanol, 1 mM diithiothreitol, 25 mM NaF, and 0.1% BSA) before incubation at 37°C for 15 min in a water bath. After being vortexed with a 7% BSA solution, a 30% TCA solution was added to the samples and centrifuged at 750 g for 2 min. Resuspension of pellets was done with 4.0 ml of 7% TCA solution for another centrifugation at 750 g for 2 min. After a second centrifugation, the supernatants were discarded, and the pellets were resuspended with 1.0 ml of 0.1 M potassium phosphate solution in 1% SDS (pH 8.0). For the scintillation counting, 0.2 ml of sample solution and 4.0 ml of scintillation cocktail were mixed and counted for 1 min.

**Measurement of lung leak.** Four and one-half hours after intratracheal insufflation of IL-1, rats were given an intravenous injection of 1.0 μCi of 125I-BSA. Thirty minutes later, the lungs were perfused blood free, and 1.0 ml of blood was withdrawn from the right atrium. Radioactivity was determined with a gamma counter, and the lung leak index was calculated as the ratio of radioactivity in the right lung to radioactivity in 1.0 ml of blood.

**Measurement of lung MPO activity.** Lung myeloperoxidase (MPO) activity was measured in left lungs frozen at −70°C (7). Briefly, left lungs were thawed and then homogenized in 4 ml of potassium phosphate buffer (20 mM at pH 7.4), then centrifuged at 30,000 g for 30 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 4 ml of potassium buffer (50 mM, pH 6.0) with 0.5% hexadecyltrimethylammonium bromide. Lung homogenate was then sonicated for 90 s on ice at maximum power, incubated for 2 h at 60°C to inactivate tissue MPO inhibitors, and then assayed with o-dianisidine as the substrate.

**Measurement of lung lavage fluid neutrophils.** Lungs were lavaged by cannulating the trachea and instilling 8.0 ml of cold saline into the lungs, withdrawing and reinstilling 3 times, and then extracting as much fluid as possible. Approximately 6.0 ml of lavage were collected and centrifuged at 1,000 g for 10 min to sediment. The cellular pellet was resuspended with 1.0 ml of distilled water and 1.0 ml of Hanks’ balanced salt solution for a few seconds and centrifuged again. After the supernatant was discarded, the pellet was suspended in 0.5 ml of normal saline. With 0.2 ml of suspended solution, cytospin was performed for a differential count, and the cells were Wright stained. White blood cells were counted visually with a hemocytometer, and the fraction of neutrophils was calculated by the percentage of neutrophils in the differential count.

**Measurement of neutrophil nitro blue tetrazolium reduction.** Nitro blue tetrazolium (NBT) is converted into formazan, which appears as dark blue granules in the cytoplasm of activated neutrophils (28). Neutrophils were isolated from lung lavage fluid, mixed with NBT solution and bacterial extract, and then incubated at 37°C for 15 min. After incubation, the neutrophils were pelleted and Wright stained, and the percentage of formazan-positive neutrophils was assessed by light microscopy.

**Electron microscopy with cerium chloride staining.** To estimate H2O2 production, histochemical electron microscopy was performed with cerium chloride (8, 41), a staining method that allows the specific visualization of H2O2 production in activated neutrophils (5, 12). Briefly, lungs were isolated 6 h after intratracheal instillation of IL-1 or saline. Tissue samples were excised from the lungs, then incubated in cerium chloride medium containing 2.0 mM cerium(III) chloride, 10 mM 3-amino-1,2,4-triazol, 0.1 M Tris-maleate buffer, 7% sucrose, and 0.0002% Triton X-100 for 60 min. Subsequently, sections were then rinsed with 0.1 M Tris-maleate buffer to wash out unreacted cerium and washed again with 0.15 M sodium cacodylate-HCl buffer. Thereafter, tissues were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in osmium tetroxide, dehydrated in graded ethanol, and embedded in epoxy resin. Sections 60–
54 nm thick were cut with a diamond knife on a Sorval MT-7000 ultramicrotome and collected on grids coated with isopropylacetate. To maximize the contrast between the cerium chloride reaction product and the tissue, counterstain was not applied.

Assessment of neutrophil adhesion to endothelial cells. Neutrophil adhesion to endothelial cells was assessed with 54Cr-labeled human neutrophils and monolayers of bovine pulmonary artery endothelial cells in vitro (38). Human neutrophils were isolated from peripheral blood of normal donors by differential centrifugation. Bovine endothelial cells were incubated with IL-1 (100 ng/ml) with and without WEB-2086.

![Fig. 1. Lung platelet-activating factor (PAF; A) and PAF acetyltransferase (B) activity levels in rats given interleukin (IL)-1 intratracheally. Values are means ± SE; n, no. of determinations. Lungs from rats given IL-1 intratracheally had increased PAF and PAF acetyltransferase activity levels compared with those in sham-treated rats, P < 0.05. *Significantly different from sham treatment, P < 0.05.](image1)

![Fig. 2. Effect of WEB-2086 on lung leak in rats given IL-1 intratracheally. Values are means ± SE; n, no. of determinations. Lung leak of rats given IL-1 intratracheally was increased compared with that in sham-treated rats, P < 0.05. In comparison, the lung leak of rats given WEB-2086 and then IL-1 was decreased compared with that of sham-treated endothelial cells, P < 0.05. *Significantly different from IL-1 treatment, P < 0.05.](image2)

![Fig. 3. Effect of WEB-2086 on lung myeloperoxidase (MPO) activity (A) and lung lavage fluid neutrophils (B) in rats given IL-1 intratracheally. PMN, polymorphonuclear neutrophils. Values are means ± SE; n, no. of determinations. Lung MPO activity and lung lavage fluid neutrophils of rats given IL-1 intratracheally were increased compared with those in sham-treated rats, P < 0.05. In comparison, the lung MPO activity and lung lavage fluid neutrophils of rats given WEB-2086 and then IL-1 were decreased compared with IL-1-treated rats, P < 0.05. *Significantly different from IL-1 treatment, P < 0.05.](image3)

![Fig. 4. Effect of WEB-2086 on the neutrophil adherence to IL-1-treated endothelial cells in vitro. Values are means ± SE; n, no. of determinations. Neutrophil adhesion to IL-1-treated endothelial cells was increased compared with that in sham-treated endothelial cells, P < 0.05. In comparison, neutrophil adhesion to WEB-2086 and then IL-1-treated endothelial cells was decreased compared with that of sham-treated endothelial cells, P < 0.05. *Significantly different from IL-1 treatment, P < 0.05.](image4)
had been given saline intratracheally (Fig. 1A). Lungs from rats given IL-1 intratracheally also had increased PAF acetyltransferase activities compared with rats given saline intratracheally (Fig. 1B).

Effect of a PAF-receptor antagonist (WEB-2086) on the development of leak and neutrophil accumulation in lungs of rats given IL-1 intratracheally. Lungs from rats given IL-1 intratracheally 5 h previously had increased leak compared with rats given saline intratracheally (Fig. 2). In comparison, lungs from rats that were given IL-1 intratracheally and then treated with WEB-2086 had decreased leak compared with rats just given IL-1 intratracheally. Lungs from rats given IL-1 intratracheally had increased MPO activity (Fig. 3A) and lung lavage fluid neutrophils (Fig. 3B) compared with rats given saline intratracheally. In comparison, lungs from rats given IL-1 intratracheally followed by treatment with WEB-2086, had decreased MPO activities and lung lavage fluid neutrophil numbers compared with rats just given IL-1 intratracheally.

Effect of WEB-2086 on the adhesion of neutrophil to IL-1-treated endothelial cells in vitro. More neutrophils adhered to IL-1-treated than to saline-treated endothelial cells in vitro (Fig. 4). Endothelial cells treated with both WEB-2086 and IL-1 had decreased neutrophil adherence compared with endothelial cells treated with IL-1 alone.

Effect of WEB-2086 on the lung histology of rats given IL-1 intratracheally. When assessed with cerium chloride staining, lungs of rats given IL-1 intratracheally (Fig. 5B) had more cerous perhydroxide deposits (indicative of H$_2$O$_2$ formation) along neutrophil-endothelial cell interfaces than lungs of rats given saline intratracheally (Fig. 5A). In comparison, WEB-2086-treated rats subsequently given IL-1 intratracheally (Fig. 5C) had decreased neutrophil-endothelial cell H$_2$O$_2$ staining compared with rats given IL-1 intratracheally.

Fig. 5. Effect of WEB-2086 on the ultrastructure of lungs from rats given IL-1 intratracheally. Neutrophil-endothelial cell interface cerium chloride staining was increased in lungs of rats given IL-1 (B) compared with that in sham-treated rats (A). In comparison, cerium chloride staining was decreased in lungs of rats given both WEB-2086 and IL-1 (C) compared with that in lungs of rats given IL-1 alone (B). Micrographs are representative of multiple sections of 4 separate experiments. Original magnification, $\times 4,000$.

Fig. 6. Effect of WEB-2086 on the nitro blue tetrazolium (NBT) reduction of neutrophils from lung lavage fluid of rats given IL-1 intratracheally. Values are means $\pm$ SE; $n$, no. of determinations. Lung lavage fluid neutrophil NBT reduction in rats given IL-1 intratracheally was increased compared with that in sham-treated rats, $P < 0.05$. In comparison, lung lavage fluid neutrophil NBT reduction in rats given WEB-2086 and then IL-1 was decreased compared with in sham-treated rats, $P < 0.05$. *Significantly different from IL-1 treatment, $P < 0.05$. 

RESULTS

PAF levels in lungs of rats given IL-1 intratracheally. Lungs of rats insufflated with IL-1 5 h previously had increased PAF levels compared with lungs of rats that (100 $\mu$M) in full medium for 4 h before the addition of the neutrophils.

Analyses of significance. Data were analyzed with the use of a one-way analysis of variance with a Student-Newman-Keuls test of multiple comparisons or an unpaired t-test. A $P$ value of $<0.05$ was considered significant.
Our data is supported by reports indicating that IL-1 accompanied by PAF production. This interpretation of IL-1 increased lyso-PAF content in the lung and that activation of PLA2, which is also the rate-limiting enzyme for the production of lyso-PAF, was increased in lungs of patients with ALI (23, 25–27, 31).

Our initial finding was that PAF and PAF acetyltransferase activities were increased in lungs of rats given IL-1 intratracheally compared with lungs of rats given saline intratracheally. This was not surprising because IL-1 causes release of PAF from mouse alveolar macrophages and other cell types (36, 40). Furthermore, IL-1 increases PLA2 activity, which promotes PAF production (1, 19). PAF is synthesized by two pathways: the remodeling of lyso-PAF by PAF acetyltransferase and de novo synthesis (2, 34). The main source of PAF in the tissue is lyso-PAF produced by the activation of PLA2, which is also the rate-limiting enzyme for the production of lyso-PAF. As shown in our experimental data, lung PAF acetyltransferase activity was enhanced in lungs of rats given IL-1 intratracheally. These results suggest that PLA2 activation by IL-1 increased lyso-PAF content in the lung and that the increased activity of PAF acetyltransferase was accompanied by PAF production. This interpretation of our data is supported by reports indicating that IL-1 increases PLA2 activity and stimulates PAF production (17, 40) and that PAF plays a role in IL-1-mediated inflammation (33).

Our second finding was that treatment with WEB-2086, a PAF-receptor antagonist, decreased lung neutrophil accumulation, lung neutrophil activation, and lung leak in rats given IL-1 intratracheally. PAF is known for its ability to enhance the adherence, chemotaxis, and respiratory burst abilities of neutrophils (11, 16, 22, 37, 39, 42), and our present findings that WEB-2086 decreased lung neutrophil accumulation and lung leak after IL-1 instillation are consistent with PAF actions (36). Our parallel observation that WEB-2086 treatment also decreased IL-1-mediated increases in neutrophil adherence to endothelial cells in vitro further supports this as a possible underlying mechanism of action. This mechanism is in agreement with previous work (4) that also indicated that PAF antagonism decreased IL-1-stimulated adhesion of neutrophils to endothelial cells. In another related report (40), WEB-2086 treatment decreased intra-alveolar neutrophil migration and lung leak, but not lung neutrophil accumulation, in an immune complex-induced alveolitis model in rats.

Our histochemical electron microscopy studies revealed that 1) lungs from rats given IL-1 intratracheally had increased $\text{H}_2\text{O}_2$ production as assessed by cerium chloride staining in the interfaces of neutrophils and endothelial cells compared with lungs from rats given saline intratracheally and 2) that this increased cerium chloride staining of IL-1-insufflated rat lungs was inhibitable by WEB-2086. This is consistent with the observation that neutrophils recovered from IL-1-insufflated rats were increased compared with saline-insufflated rats and that WEB-2086 cotreatment decreased NBT reduction by neutrophils recovered from the lung lavage fluid of rats given IL-1 intratracheally. The findings are also consistent with the oxidative nature of the lung leak and the ability of antioxidants to decrease lung leak after intratracheal IL-1 instillation (10, 20, 21).

Taken in toto, this paper provides evidence that supports the premise that PAF contributes to IL-1-induced lung leak. Prior work by our laboratory (9, 15, 19) has already implicated tumor necrosis factor-α, cytokine-induced neutrophil chemoattractant, and PLA2 in IL-1-induced lung inflammation and injury. In addition, instilling PAF intratracheally into the lungs of rats produces a neutrophil influx and lung leak injury (Lee, Hybertson, and Repine, unpublished observations). The apparent and emerging complexity and redundancy of inflammatory mediators undoubtedly contributes to the severity of ALI and ARDS.

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REFERENCES


8. Hobson J, Wright J, and Churg A. Histochemical evidence for generation of active oxygen species on the apical surface of


