EFFECTS OF PLATELET ACTIVATING FACTOR ON VARIOUS PHYSIOLOGICAL PARAMETERS OF NEUTROPHILS, ALVEOLAR MACROPHAGES, AND ALVEOLAR TYPE II CELLS

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INTRODUCTION

Platelet activating factor (PAF) is a glycerophospholipid (1-0-alkyl-2-acetyl-sn-glycerol-3-phosphoryl choline) which has been shown to mediate a broad range of biological activities.[1-3] Its pulmonary actions include contraction of pulmonary tissue,[4] secretion of leukotrienes from leukocytes,[5] airway constriction,[6] pulmonary edema,[7] and enhanced migration of neutrophils into the airspaces of the lungs.[8,9]

PAF can be released from several different cell types, such as, basophils, neutrophils and alveolar macrophages, in response to a variety of particulates or membrane stimulants which include zymosan, calcium ionophore, phorbol esters, chemotactic agents, and endotoxin.[10-13] Therefore, PAF may play an important role in the development of pneumoconioses by mediating pulmonary responses of lung cells to a variety of occupational dusts. To investigate this possibility, we determined the effects of PAF on several physiological parameters of neutrophils, alveolar macrophages, and alveolar type II epithelial cells.

METHODS

Isolation of Cells

Neutrophils were isolated from human blood by dextran settling and centrifugal elutriation.[14] Isolated neutrophils (93% pure) were resuspended in HEPES-buffered medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 5 mM glucose, and 1 mM $CaCl_2$; pH = 7.4). Cell number and volume were determined with an electronic cell counter equipped with a sizing attachment.

Rat alveolar macrophages were obtained by pulmonary lavage with Ca^{2+}, Mg^{2+}-free Hanks balanced salts solution.[15] Alveolar macrophages (94% pure) were resuspended in HEPES-buffered medium, counted, and sized electronically.

Rat alveolar type II cells were isolated by enzymatic digestion for 35 minutes at 37°C with 40 μ/ml type I elastase and 0.1% collagenase and purified by centrifugal elutriation.[16,17] Type II cells (92% pure) were resuspended in HEPES-buffered medium for measurement of membrane potential, oxygen consumption, and trypan blue exclusion. Type II pneumocytes were resuspended in 0.1 M NaCl plus 0.05 M HEPES (pH = 7.8) to measure cytochrome P450-dependent activities and aggregation. Cell size and number were determined electronically.

Measurement of Transmembrane Potential

Membrane potential of isolated cells in suspension was measured using a fluorescent probe, Di-S-C_3,[5] as described previously for neutrophils,[14] alveolar macrophages,[18] and type II cells.[19] Fluorescence was monitored at excitation and emission wavelengths of 622 and 665 nm, respectively. An increase in the fluorescence emission from the cell suspension indicated membrane depolarization.

Measurement of Respiratory Burst Activity

Release of reactive forms of oxygen by phagocytic cells was determined at 37°C by measuring the generation of chemiluminescence, secretion of hydrogen peroxide, or release of superoxide anion. Chemiluminescence from neutrophils (1×10^6 cells/5 ml of HEPES-buffered medium) was measured in the presence of 1×10^{-8} M luminol using a liquid scintillation counter operated in the out-of-coincidence mode.[20] Chemiluminescence from alveolar macrophages (3×10^6 cells/0.5 ml of HEPES-buffered medium) was measured in the presence of 1×10^{-5} M luminol using a Berthold 9505 Luminometer.

Hydrogen peroxide release from neutrophils or alveolar macrophages (1×10^7 cells/2.5 ml or 4×10^6 cells/3 ml, respectively) in HEPES-buffered medium containing 2.5 μM scopoletin, and 40 μg/ml horseradish peroxidase (type IX) was monitored fluorometrically at an excitation wavelength of 350 nm and an emission wavelength of 460 nm.[21,22]

Superoxide anion secretion from neutrophils or alveolar macrophages (1×10^7 cells/2.5 ml or 4.5×10^6 cells/6 ml, respectively) in HEPES-buffered medium was monitored spectrophotometrically at 550 nm as the reduction of 0.12 mM cytochrome C.[21,15]

Measurement of Cellular Viability

Oxygen consumption was measured at 37°C with an ox-

ygraph equipped with a Clark electrode. Type II cells (10^7 cells), neutrophils (6.5×10^6 cells), or alveolar macrophages (5×10^6 cells) were suspended in 1.7 ml of HEPES-buffered medium for these measurements.[16, 21,23]

Membrane integrity was determined by measuring the exclusion of trypan blue dye under light microscopy.[24]

Functional Measurements with Type II Cells

Cytochrome P450-dependent ethoxyphenoxazone dealkylase (EtOPhase) (EC 1.14.14.1) activity of type II cells was monitored at 36°C in a direct kinetic assay based upon the formation of a fluorescent product, resorufin, measured at an excitation wavelength of 530 nm and an emission wavelength of 585 nm.[17] NADPH was maintained at 0.5 mM by a glucose-6-phosphate dehydrogenase generating system.

Aggregation of type II cells was monitored at 37°C using a Lumi Aggregometer. Increased aggregation was measured as increased light transmission.

Type II cells used for measurement of cytochrome P450 and aggregation were isolated from rats metabolically induced by pretreatment with β-naphthoflavone. For these assays, cells were suspended in 0.1 M NaCl and 0.05 M HEPES (pH = 7.8).

Statistical Analysis

Data are expressed as means ± standard errors of n experiments conducted with cells obtained from different preparations. Data were analyzed by a Student's t test with significance set at $p < 0.05$.

RESULTS

Platelet activating factor can initiate a wide variety of pulmonary responses.[1,4-9] However, details concerning the cellular mechanisms responsible for the activities of PAF are not fully defined. Therefore, this investigation characterized the actions of PAF on three types of lung cells, i.e., two types of pulmonary phagocytes (neutrophils and alveolar macrophages) and alveolar type II epithelial cells.

The effects of PAF on pulmonary phagocytes are summarized in Table I. PAF was a direct stimulant of neutrophils *in vitro*. PAF induced substantial depolarization of the plasma membrane which was rapid (peaking within 15 sec after addition of PAF) and transient (returning to the resting level within 2 min). The effect of PAF on the membrane potential (E_m) of neutrophils was dose-dependent, exhibiting a $K_{1/2}$ value of 2.5 μM. This PAF-induced depolarization was sodium-dependent, i.e., removal of extracellular sodium eliminated the effect. PAF (10 μM) was also a potent activator of neutrophils, i.e., it induced significant generation of chemiluminescence and release of hydrogen peroxide. As was the case for membrane depolarization, stimulation of the secretary activity of neutrophils by PAF was dependent on extracellular sodium. In contrast to the above responses, *in vitro* treatment of neutrophils with PAF (10 μM) resulted in only a small increase in superoxide anion release and no significant elevation of oxygen consumption.

As with neutrophils, *in vitro* treatment of alveolar macrophages with PAF (12 μM) resulted in membrane depolarization. This response was rapid (peaking within 40 sec) and prolonged (not returning to resting Em). In contrast to neutrophils, *in vitro* treatment of alveolar macrophages with PAN (12 μM) did not activate the respiratory burst in these cells, i.e., there was little or no PAF-induced increase in chemiluminescence, hydrogen peroxide release, superoxide secretion, or oxygen consumption (Table I). However, PAF(12 μM) did potentiate activation of alveolar macrophages by zymosan (2 mg/ml), i.e., PAF increases zymosan-stimulated superoxide release by 36% (Figure 1) and zymosan-induced chemiluminescence by 55% (Figure 2).

The *in vitro* effects of PAF on isolated type II cells were also characterized. At levels of 12 μM or below, PAF did not affect membrane integrity or oxygen consumption, i.e.,

Table I

Effects of Platelet Activating Factor on Phagocytes

Cell Types	Em	Oxygen Consumption	Superoxide Release	Chemiluminescence	Hydrogen Peroxide Release
Neutrophils	transient depolarization	0	+	+++	+++
Alveolar Macrophages	prolonged depolarization	0	0	0	0

Maximal responses of neutrophils and alveolar macrophages after *in vitro* exposure to 10 μM or 12 μM PAF, respectively. The relative magnitude of enhancement is signified by +. No response is signified by 0. Data for each assay are taken from four separate experiments.

trypan blue exclusion was 81 ± 2% before and 82 ± 1% after PAF treatment while oxygen consumption levels were 0.23 ± 0.04 and 0.19 ± 0.03 nmoles O_2/min/10^6 cells, respectively. However, PAF (12 μM) did cause depolarization of type II cells which was rapid (peaking within 1 min) and prolonged. This depolarization exhibited dependence on extracellular sodium. PAF also enhanced the activity of cytochrome P450-dependent ethoxyphenoxazone dealkylase (EtOPhase). A maximum stimulation of 2.5 fold was noted at 10 μM PAF (Figure 3). Such activation was demonstrated in intact cells but not in sonicated preparations (Table II) or microsomes. The decline in P450 activity at higher levels of PAF may be due in part to PAF-induced aggregation of type II cells which was significant at PAF levels above 18 μM (Figure 4).

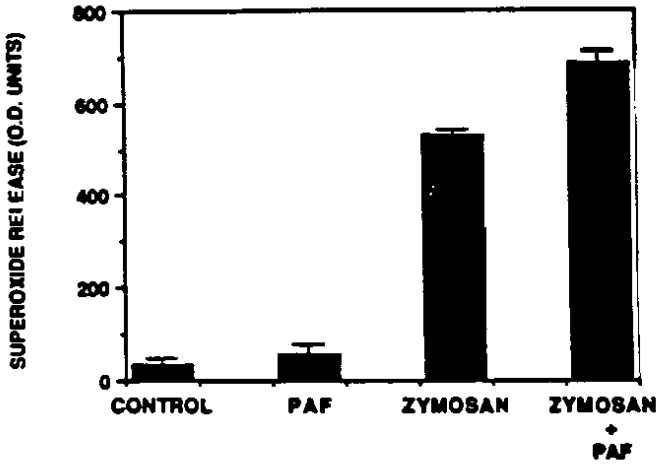

Figure 1. Effects of PAF on superoxide anion release from rat alveolar macrophages. Superoxide secretion at 37°C was monitored spectrophotometrically by measuring the reduction of cytochrome c over 30 minutes at a wavelength of 550 nm. Cells (4.5×10^6 cells/6 ml) were treated *in vitro* with 12 μM PAF and/or 2 mg/ml zymosan. Values are means ± standard errors of four different preparations.

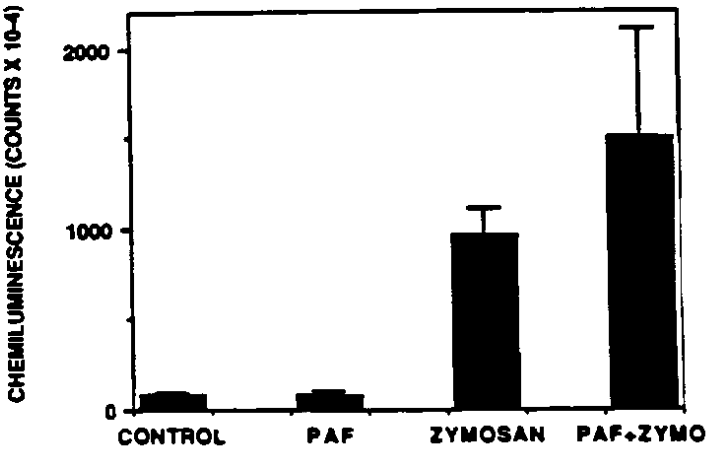

Figure 2. Effects of PAF on chemiluminescence generated from rat alveolar macrophages. Chemiluminescence was measured for 10 minutes at 37°C in the presence of 10^{-5}M luminol. Cells (3×10^6 cells/0.5 ml) were preincubated at 37°C in the presence or absence of 12 μM PAF for 15 minutes prior to addition of 2 mg/ml zymosan and measurement of chemiluminescence. Values are means ± standard errors of three different preparations.

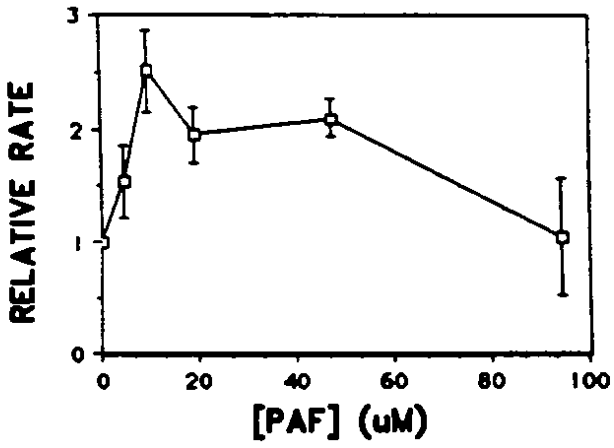

Figure 3. Effect of PAF on cytochrome P450-dependent EtOPhase activity in rat alveolar type II cells. Type II cells were obtained from β-naphthoflavone-treated rats. Cells ($1.6–2.5\times10^6$/ml) were suspended in 0.1M NaCl, 0.5 nM NADPH, and 0.05 M HEPES (pH = 7.8) at 36°C, a fluorescence baseline established, and the reaction initiated with 2.5 μM EtOPh. In the absence of PAF, EtOPhase activity of 3 separate preparations of type II cell was 1.06, 1.38, and 0.50 pmoles resorufin formed/min/10^6 cells, respectively. Data after addition of PAF are rates relative to these controls (means ± standard errors).

DISCUSSION

Neutrophils are blood phagocytes which are recruited into the pulmonary air spaces following inhalation of foreign substances, such as, bacteria, virus, or dusts.[25] Alveolar macrophages are free lung phagocytes located on the surface of the small airways and the alveoli.[26] Upon exposure to microorganisms or occupational dust these phagocytes exhibit a respiratory burst releasing reactive oxygen species, such as, superoxide anion, hydrogen peroxide, and hydroxyl radicals.[27-29] Evidence indicates that dust exposure may cause hyperactivation of these phagocytes. The resultant secretion of reactive products may result in inflammation, cellular damage, and in extreme cases fibrosis or emphysema.[30,31]

In this investigation we evaluated the ability of platelet activating factor (a potentially important mediator of pneumo-

Table II

Effect of Sonication on the Responsiveness of Alveolar Type II Cell Cytochrome P450-Dependent Activity to Platelet Activating Factor

Additive[b]	P450-Dependent Activity[a] Cells	Sonicate[c]
None	16.6 ± 3.4	38.7 ± 5.6
BSA-HEPES	16.2 ± 2.4	38.6 ± 11.0
PAF (19 μM)	34.0 ± 6.4	34.2 ± 4.9

a) Specific activity expressed as pmoles resorufin formed/min/mg protein. Protein determined by the procedure of Lowry et al. (25). Data are means ± standard errors of two experiments.

b) Additive: None - 0.1M NaCl, 0.5 mM NADPH, and 0.05M HEPES (pH = 7.8); BSA-HEPES - 10 μl of 0.5% BSA in 0.01M HEPES (pH = 7.8) added to the above solution; PAF - 10 μl of PAF in 0.5% BSA and 0.01M HEPES (pH = 7.8) added.

c) Disrupted type II cells were obtained by pulse sonication (0.33 sec on, 0.67 sec off) of the cell suspension for 30 seconds at 3 Watts at 2°C.

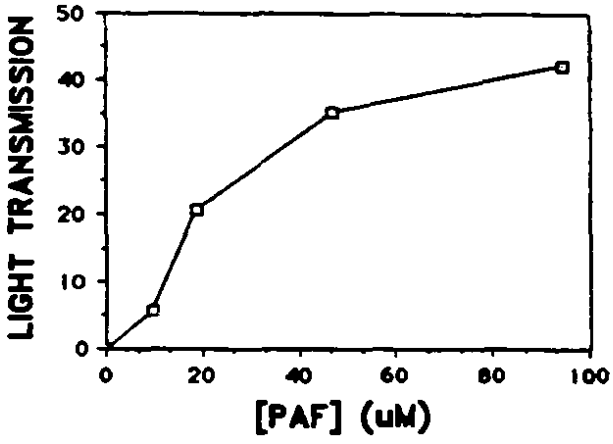

Figure 4. Effect of PAF on aggregation of rat alveolar type II cells. Type II cells were obtained from β-naphthoflavone-treated rats. Cells ($2.5–2.7 \times 10^6$ cells/ml) were suspended in 0.1M NaCl and 0.05M HEPES (pH = 7.8) at 37°C and aggregation monitored as light transmission. Data are means of two experiments.

conioses) to activate phagocytes. The data indicate that PAF depolarizes neutrophils by increasing membrane permeability to sodium. Such depolarization may trigger secretary activity in neutrophils.[20] Indeed, PAF does activate neutrophils to secrete hydrogen peroxide and generate chemiluminescence (Table I). However, activation of the respiratory burst is incomplete in neutrophils since PAF does not stimulate oxygen consumption and elevates superoxide release only slightly.

Although PAF depolarizes alveolar macrophages, it does not directly activate a respiratory burst (Table I). However, PAF treatment does prime the cells to be more responsive to subsequent exposure to particles (Figures 1 and 2). Since PAF may be released following dust exposure, the potentiating action of PAF could have important consequences in escalating the cycle of inflammation and tissue damage seen in certain occupational lung diseases.

Cytochrome P450-dependent monooxygenases are responsible for the metabolism of organic chemicals in pulmonary tissue.[32] We have shown that within the lung high levels of P450-dependent activities are found in alveolar type II cells.[17] Recent studies have suggested that endogenous factors released from phagocytes may depress P450-dependent activity in hepatocytes.[33-35] Since PAF is released from

phagocytes,[10-13] we tested its effect on P450-dependent activity of type II cells. In contrast to the hepatic system, PAF (a phagocyte-derived mediator) enhances P450-dependent activity of alveolar type II cells (Figure 3). This effect seems to be mediated through the cell membrane, since PAF fails to activate P450 in sonicated cells or microsomes (Table II). It is possible that PAF may alter membrane structures which translate into increased P450 activity. Action of PAF at the plasma membrane is supported by our evidence of PAF-induced changes in membrane permeability to ions and membrane potential. In addition, higher concentrations of PAF alter the membrane surface of type II cell sufficiently to cause aggregation (Figure 4).

In conclusion, PAF may be released from phagocytes following occupational exposures. This PAF would be inflammatory by directly activating neutrophils and potentiating the response of macrophages to particulates. In addition, xenobiotic metabolism by alveolar type II cells would be enhanced affecting the detoxication and/or activation of foreign compounds. The role which these cellular changes play in pneumoconioses remains to be defined.

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II

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