Platelet-Activating Factor Primes Neutrophil Responses to Agonists: Role in Promoting Neutrophil-Mediated Endothelial Damage

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During inflammation polymorphonuclear cells (PMNs) are exposed to agonistic stimuli including activated complement, kallikrein, arachidonic acid metabolites, monokines, and platelet-activating factor (PAF). We report that PAF not only directly activates PMNs but in miniscule quantities (10⁻¹⁵ mol/L) "primers" them as well, that is, permits PMNs to respond to subsequent stimuli that would be otherwise ineffectual. PAF priming of responses including superoxide generation, elastase release, and aggregation is time dependent and is maximal within five minutes. PAF need not be present during the subsequent exhibition of PMN agonists, but priming is inhibited by cold and is also inhibited by the PAF receptor antagonists BN 52021, L-652, and kadsurenone. An intact PAF molecule is required because lyso-PAF and methoxy-PAF do not prime PMNs. PAF priming of responses including superoxide generation, elastase release, and aggregation is time dependent and is maximal within five minutes. PAF need not be present during the subsequent exhibition of PMN agonists, but priming is inhibited by cold and is also inhibited by the PAF receptor antagonists BN 52021, L-652, and kadsurenone. An intact PAF molecule is required because lyso-PAF and methoxy-PAF do not prime PMNs. PAF priming is associated with both enhanced expression of the adhesive glycoprotein identified by OKM-1 antibody and an enhanced rise in intracellular calcium levels in response to the subsequent addition of agonists such as FMLP. PMNs primed with PAF and stimulated with either F-Met-Leu-Phe or phorbol esters are more effective in lysing and detaching cultured human endothelial cells—damage that can also be inhibited by the PAF antagonists. Because PAF is synthesized and exhibited on surfaces of endothelial cells perturbed by coagulation, we suggest that this lipid may potentiate otherwise trivial activators of marginated PMNs so that they become damaging to the PAF-synthesizing endothelium itself. If so, our studies suggest a possible therapeutic role for PAF inhibitors in excessive inflammatory states.

O UR LABORATORY has been interested in mechanisms by which neutrophils damage vascular endothelium in diverse inflammatory syndromes, particularly in the adult respiratory distress syndrome (ARDS). Neutrophils stimulated by inflammatory mediators such as activated complement adhere to the endothelial surface; adherent cells release toxic oxygen metabolites and lysosomal proteases that, in turn, can damage vascular endothelium. In conditions in which ARDS occurs such as polytrauma, neutrophils are presumably flooded with a variety of stimuli produced by damaged tissues that might modulate their activities. In fact, we and others have reported potent stimulatory effects on polymorphonuclear cell (PMN) function of activated complement, kallikrein, arachidonate metabolites, monokines, and bacterial peptides. Recent reports that endothelium, when exposed to thrombin, synthesizes platelet-activating factor (PAF) prompted our interest in this compound because coagulation activation is a common feature of severe inflammation.

PAF (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphocholine), a lipid produced by a variety of activated cells including basophils, monocytes, endothelial cells, and neutrophils themselves, was initially recognized by its capacity to aggregate and degranulate platelets in vitro. Belying its name, PAF probably has more important actions in inflammation; at concentrations far lower than needed for platelet effects, PAF activates neutrophils and causes chemotaxis, aggregation, superoxide release, and degranulation; moreover, PAF in vivo induces pulmonary sequestration of neutrophils, hypotension, bronchoconstriction, and increased vascular permeability.

Because PAF may play a critical role in inflammation, others have begun searches for PAF antagonists to act as potential antiinflammatory agents. Several such—either PAF analogues or PAF receptor antagonists—are now available. Many of the latter have been isolated from herbal medicinals used by the Chinese for centuries to relieve chest discomfort. Shen has described a powerful PAF receptor antagonist, kadsurenone, from the Chinese herbal plant Haifenteng; a similar receptor antagonist prepared by this group is a furanoid, code-numbered L652-731. In addition, investigators have isolated from seeds and leaves of the Ginkgo biloba tree or Ya-chiao-pan another potent PAF receptor antagonist, BN52021, which has been found to inhibit all biologic activities of PAF so far studied.

The enhancement or amplification of a neutrophil response to a given stimulus that is due to prior exposure of the cell to a different agonist has been referred to as "priming." Several biologic mediators, presumably relevant to ARDS, including endotoxin, interleukin-1 (IL-1) and tumor necrosis factor have been demonstrated to prime PMN responses. Others have reported enhanced PMN responses to stimuli after preexposure to PAF. Because PAF is generated by both inflammatory cells and endothelial cells in response to diverse mediators such as thrombin, we reasoned that PAF might prime responses of marginated PMNs to otherwise-weak neutrophil agonists and thereby promote neutrophil-mediated endothelial damage. If so, we wondered whether PAF antagonists might inhibit such enhanced endothelial damage. The present studies validate this hypothesis by demonstrating that PAF amplifies neutrophil superoxide production and elastase release and aggregates PMN adhesion to endothelium and subsequent endo-
thelial injury. Furthermore, PAF antagonists inhibit these amplified responses, which suggests a role for these agents in excessive inflammatory states.

**METHODS**

**Materials.** PAF (dl-α-phosphatidylcholine, β-acetyl-γ-O-hexadecyl or L-α-phosphatidylcholine, β-acetyl-γ-O-alkyl: P1402 and P9525), phorbol myristate acetate (PMA), dl- or lysophosphatidylcholine, γ-O-hexadecyl (lyso-PAF), ferricytochrome C (horse heart type), f-Met-Leu-Phe (FMLP), superoxide dismutase, dimethylsulfoxide (DMSO), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co (St Louis). Hanks' balanced salt solution (HBSS) and medium 199 were obtained from GIBCO (Grand Island, NY). Sodium $^{35}$Cr and $^{115}$In oxine were obtained from Amersham Corp (Arlington Heights, IL). Fura-2 was obtained from Molecular Probes (Eugene, OR). L652-731 (L-652) and kadsurenone were a kind gift of Dr John Chabala (Merck, Sharp, and Dohme, Rahway, NJ). BN52021 and methoxy-PAF were kind gifts of Dr Pierre Braquet (Institut Henri Beaufour, Paris). OKM-1 antibody was obtained from Ortho Diagnostic Systems (Westwood, MA). All materials were endotoxin free as tested by the limulus amoebocyte assay.

**Preparation of PMNs.** Human volunteer blood samples (40 mL) were drawn (after receiving informed consent under the guidelines of the Committee on the Use of Human Subjects in Research at the University of Minnesota into a plastic syringe containing 20 mL hydroxyethylstarch (Hespam; American Hospital Supply Corp, Irvine, CA) and 200 units preservative-free heparin (Upjohn Co, Kalamazoo, MI). The mixture was allowed to sediment at room temperature and the supernatant collected and centrifuged at 400 g for five minutes. The pellet was then resuspended in 0.2 mL ice-cold HBSS containing 100 mg/dL glucose. Residual erythrocytes were lysed in 15 mL ice-cold water, and after 25 seconds isotonicity was restored by the addition of 5 mL 3.6% sodium chloride. The suspension was centrifuged at 20,000 g for 30 minutes at 4°C. The resulting PMNs (>95% with <1% platelet contamination) were washed and resuspended in 10 mL HBSS. Viability was assessed by trypan blue exclusion and exceeded 95%.

**Endothelial cell cultures.** Human umbilical vein endothelial cells (HUEC) harvested from umbilical cords by collagenase digestion as previously described were grown to confluent monolayers and used as primary cultures.

**Neutrophil priming with PAF.** L-PAF was stored at −20°C in chloroform, and DL-PAF was made to a 10 mmol/L solution in phosphate-buffered saline (PBS) and stored at −70°C. For experiments, an aliquot of the stock solution of DL-PAF was prepared in HBSS with 0.5% human albumin (American Red Cross, Washington, DC) and added to the final incubation mixture. An aliquot of the L-PAF dissolved in chloroform was evaporated under nitrogen and redissolved in HBSS with 0.5% human albumin to the required concentration. We measured the ability of PAF to prime neutrophil superoxide release, elastase release, aggregation, and adhesion by methods previously described. For example, the priming for superoxide release was assayed by adding PAF at various concentrations and for varying time intervals to $2 \times 10^6$ PMNs in HBSS at 37°C. In some experiments the cells were subsequently washed and centrifuged at 1,200 rpm for two minutes, the supernatant removed, and 1 mL HBSS added. Cytochrome c (75 μmol/L) was then added to each tube and was followed by a stimulus such as FMLP (10−7 mol/L) or PMA (100 ng/mL). The cells were incubated at 37°C for 15 minutes and thereafter placed on ice. The supernatant was removed after centrifugation for the measurement of cytochrome c reduction as previously described. Priming was performed by using both L-PAF and DL-PAF. No differences were seen when using either PAF preparation. When PAF inhibitors were used, they were added at the given concentrations for five minutes at 37°C before the addition of PAF. BN52021, L-652, and kadsurenone were dissolved in DMSO and then added to HBSS to result in a final concentration of DMSO not exceeding 0.1%. Control buffer contained HBSS with 0.1% DMSO. The control responses to FMLP or PMA were performed by adding HBSS with 0.5% albumin to the PMN suspension, washing as described earlier, and then stimulating with FMLP or PMA. In some experiments, cytochalasin B (5 μg/mL) was added to PMNs before stimulation with FMLP. A similar priming technique was used to measure the stimulated release of elastase by measuring the cleavage of a peptide substrate, MeO-Suc-Ala-Ala-Pro-Val-nitroanilide, as previously described. We arbitrarily defined 100% elastase release by the amount of elastolytic activity in the supernatant of PMNs (2 × 10^6/mL) treated with cytochalasin B (5 μg/mL) and stimulated with FMLP (10−7 mol/L) for 20 minutes at 37°C. Neutrophil aggregation and adhesion to endothelium were performed as previously described upon similarly primed PMNs.

**The effect of PAF priming on neutrophil CR3 expression.** Studies assessing the effect of PAF priming on neutrophil complement receptor type 3 (CR3) expression were performed as described by Nelson et al. Briefly, PMNs (5 × 10^6/mL) suspended in PBS containing 0.2% BSA were stimulated with PAF in various concentrations for 15 minutes at 37°C. Alternatively, in sequential agonist experiments 10−11 mol/L PAF (or HBSS as a control) with 0.5% human albumin was added to the PMNs for five minutes followed by stimulation with FMLP (10−7 mol/L) or buffer. At the end of the incubation the reaction was stopped by placing the PMNs on ice, adding anti-CR3 antibody (5 μL OKM-1), and mixing, and incubation at 4°C was continued for 30 minutes. After incubation, the cells were washed twice with cold PBS, treated with goat antimouse fluorescein-conjugated secondary antibody, incubated for 30 minutes at 4°C, again washed and treated with 1% paraformaldehyde, and stored in the dark at 4°C until analyzed by a Cytofluorograph 50H and 2150 computer (Ortho Diagnostic Systems). The flow cytometer was calibrated as previously described, and control studies were done to establish that staining was specific for the primary antibody used.

**Assay of PMN intracellular Ca2+.** We measured intracellular calcium levels by using Fura-2 as a fluorescent indicator of cytosolic free calcium. PMNs (1 × 10^6/mL) were loaded with Fura-2 (5 μmol/L), washed, and resuspended in HBSS (1 × 10^6/mL). The change in emission fluorescence (512 nm) over time after stimulation with FMLP and/or PAF was measured with a Spex Fluorolog Spectrofluorometer (Spex, Edison, NJ). The intracellular calcium concentration was calculated by measuring the ratio of the fluorescence intensity at excitation wavelengths of 340 nm and 380 nm as described by Grynkiewicz et al. The resting neutrophil intracellular calcium concentration was 40 ± 10 nmol/L.

**PMN-mediated endothelial cell detachment and killing.** The ability of PMNs to cause endothelial cell lysis and detachment was measured by using 51Cr-labeled HUECs grown to confluence in 24-well 2-cm² dishes as previously described.

**Statistics.** Results are expressed as means ± SEM. Statistical analysis used Student's t test.
RESULTS

PAF primes PMN respiratory burst and elastase release. PMNs were incubated or primed with various concentrations of PAF for five minutes followed by the addition of FMLP. As seen in Fig 1A, PAF alone at concentrations up to \(10^{-7}\) mol/L induces virtually no superoxide production (dotted line); likewise, FMLP \((10^{-7}\) mol/L), when added alone to neutrophils, provokes minimal superoxide \((5\text{ nmol}/10^6\text{ PMNs/15 min})\). If PMNs are first primed with minute amounts of PAF—as low as \(10^{-11}\) mol/L—and then stimulated with FMLP, however, large quantities of superoxide are produced (solid line). This priming for superoxide production was not limited to the agonist FMLP; PAF \((10^{-7}\) mol/L) pretreatment of PMNs also synergistically enhanced superoxide production when PMNs were later stimulated with PMA \((38 \pm 1\text{ nmol}/10^6\text{ PMN/15 min} v 46 \pm 1\text{ nmol}/10^6\text{ PMN/15 min with PAF priming, } P < .01)\). Other neutrophil functions are also primed by PAF, that is, PAF-primed, FMLP-stimulated neutrophils release much more elastase than cells exposed either to PAF or FMLP alone (Fig 1B). Preincubation with PAF \((10^{-8}\text{ mol/L})\) also enhanced PMN elastase release induced by PMA \((34\% \pm 2\% \text{ release with PMA v 52\% \pm 2\% with PAF plus PMA, } P < .01)\).

PAF priming of superoxide (and elastase) release is time dependent as shown in Fig 2. Pretreating PMNs with PAF enhances the response to subsequently exhibited FMLP, with a maximal increment in superoxide production noted after five minutes of preincubation; thereafter the priming effect deteriorates over the next 60 minutes. Moreover, PAF need not be present during subsequent FMLP stimulation to obtain an enhanced response; PAF, washed away from the PMNs before FMLP addition, still provokes PMN hyperresponsiveness. For example, PMNs treated with PAF \((10^{-8}\text{ mol/L})\) for five minutes and then stimulated with FMLP (with the PAF remaining in the reaction solution) produced 20 \(\pm\) 2 nmol/10^6 PMNs/15 min—virtually identical to PMNs pretreated with PAF and then washed before the addition of FMLP \((19 \pm 1\text{ nmol}/10^6\text{ PMNs/15 min})\).

PAF primes PMN adhesive responses. Preincubation of PMNs with PAF also enhances their adhesion to endothelium. Data shown in Table I demonstrate that priming of PMNs with PAF \((10^{-7}\text{ mol/L})\)—a dose that does not itself increase adhesion (line 2)—significantly enhances the adhesion of FMLP-stimulated PMNs (compare lines 3 and 4). Similarly, neutrophils primed with PAF and stimulated with PMA become significantly more adherent than cells treated with PMA alone (lines 5 and 6). Another adhesive phenomenon, PMN aggregation, is also synergistically enhanced: PAF \((10^{-8}\text{ mol/L})\) or FMLP \((10^{-7}\text{ mol/L})\) by themselves induce only modest PMN aggregation; in contrast, PMNs first treated with PAF for five minutes and then stimulated with FMLP aggregate to a much greater degree (Fig 3), and

| Table 1. PAF Primes Stimulated PMN Adhesion to HUEC |
|----------------|-----------------|----------------|
| PMN Treatment | PMN Adhesion (%) | \( P \) |
| PMN           | 17.91 ± 2.81    | .991          |
| PMN + PAF     | 17.54 ± 2.73    | .991          |
| PMN + FMLP    | 32.71 ± 4.93    | <.001         |
| PMN + PAF + FMLP | 69.74 ± 2.61 | <.001         |
| PMN + PMA     | 37.20 ± 3.92    | <.001         |
| PMN + PAF + PMA | 71.95 ± 3.07 | .991          |

PMNs were pretreated with PAF \((10^{-7}\text{ mol/L})\) or buffer for five minutes and stimulated with either FMLP \((10^{-7}\text{ mol/L})\) or PMA \((100\text{ ng/mL})\) and PMN adhesion to endothelium measured as in Methods. Results are expressed as the mean ± SEM PMN adhesion of four experiments done in triplicate.
this was verified microscopically to be enhanced aggregation and not simply enhanced light transmission. 35

Mechanism of enhanced PMN responses due to PAF priming. Both adhesion and aggregation involve the expression of adherent surface glycoproteins (assayable by monoclonal antibodies such as OKM-1) that also function as receptors for iC3b (CR3 receptors). PAF alone increases the expression of these CR3 receptors in a dose-dependent fashion (Fig 4), and significant amplification occurs with very small doses (10^-12 mol/L), some three orders of magnitude less than that needed to produce perceptible PMN aggregation. Moreover, in ancillary experiments PAF (10^-12 mol/L) preincubation was found to amplify the increase in CR3 receptor expression provoked by 10^-7 mol/L FMLP by increasing the mean channel fluorescence by 50% compared with FMLP (10^-7 mol/L) alone.

Because receptor-ligand signal transduction in PMNs is thought to be mediated by changes in intracellular calcium levels and phosphatidylinositol turnover, we assayed with the calcium-sensitive probe Fura-2 the effect of priming upon FMLP-induced intracellular calcium accumulation. As shown in Fig 5, at very low concentrations of PAF (10^-11 to 10^-14 mol/L) no change in intracellular calcium levels occurs; however, if PMNs are first primed with these same tiny concentrations of PAF and then stimulated with FMLP a dose-dependent increase in intracellular calcium levels above that mediated by FMLP (10^-9 mol/L) alone is evident.

The order of addition of agonists is critical for maximal priming. As shown in Fig 6, the addition of PAF to PMNs for five minutes followed by washing and the subsequent addition of FMLP increases superoxide production by 19 nmol over either agonist alone (first bar, Fig 6). In marked contrast, no enhanced superoxide production is seen if the agonists are added in the reverse order (second bar). Moreover, priming by PAF requires an intact PAF molecule because lyso-PAF (which has a hydroxyl group instead of an acetate at position 2) or methoxy-PAF does not prime superoxide production in PMNs subsequently stimulated with FMLP (bars 3 and 4). Finally, others have shown that PAF, to exert its other known actions, must be bound by specific membrane receptors—2,36—the metabolism of such PAF is slowed by cold incubation (4°C). This also seems so for priming because PAF priming is prevented at cold
PAF priming enhances PMN-mediated endothelial lysis and detachment. We previously showed that neutrophil-mediated endothelial damage depends on close adhesion of PMNs to endothelial monolayers as well as oxidant production and protease release. Because PAF enhances PMN adhesion and aggregation as well as superoxide and elastase release, we suspected that such priming might aggravate endothelial cell lysis and detachment wrought by activated PMNs. In support, as shown in Table 2, we used concentrations (10⁻⁷ mol/L) of FMLP and PAF that when added alone to PMNs cause little endothelial lysis or detachment (lines 2 and 3). If PMNs are first primed with PAF and then stimulated five minutes later with FMLP, however, significantly enhanced endothelial cell lysis and detachment occurs (line 4). PMA (100 ng/mL), a more powerful stimulant of PMN responses, does cause PMN-mediated endothelial cell damage, even during the brief incubation shown in Table 2 (line 5); notwithstanding, PAF priming further aggravates this PMA-mediated damage (line 6).

Inhibition of PAF priming by PAF antagonists. The recently described PAF receptor antagonists BN52021, kadsurenone, and L652 all inhibit PAF priming of neutrophils to subsequent agonists and concomitantly abrogate PAF enhancement of PMN-mediated endothelial injury. These compounds in micromolar concentrations inhibit PAF-mediated incremental responses in several PMN functions, including PAF-primed neutrophil adhesion (Fig 7A), superoxide release (Fig 7B), and elastase release (Fig 7C). These antagonists did not inhibit FMLP-induced superoxide release, elastase release, or adhesion by themselves, but only the incremental PAF priming response (data not shown).

Not surprisingly, BN52021, kadsurenone, and L652 also inhibit PAF-aggravated, neutrophil-mediated endothelial lysis (Fig 8A), probably by inhibiting enhanced production of superoxide and adhesion, and PAF-enhanced endothelial detachment (Fig 8B), probably by inhibiting enhanced neutrophil elastase release. Again, the PAF antagonists did not inhibit the agonist-stimulated, PMN-mediated endothelial injury itself, but only the increment induced by PAF priming (data not shown).

DISCUSSION

The present studies demonstrate that minuscule quantities of PAF markedly enhance neutrophil responses to subsequent activation by the agonists FMLP and PMA. The primed neutrophil responses include respiratory burst as evidenced by enhanced superoxide release, degranulation as measured by elastase release, and adhesion as measured by adherence of neutrophils to human endothelium as well as PMN aggregation. These findings essentially confirm recently reported observations of Ingraham et al and Gay et al. We now demonstrate for the first time that these PAF-primed neutrophils are also excessively damaging to cultured endothelial cells. Moreover, PAF-primed PMN responses and subsequent PMN-mediated endothelial damage were shown to be completely inhibited by the recently described PAF antagonists BN52021, L652, and kadsurenone.

We suggest that these results may provide new insights into mechanisms of vascular damage during inflammation. For instance, the circulating neutrophil is probably exposed to diverse inflammatory mediators that may modulate its function. We previously suggested that, in sepsis-associated ARDS, activated complement (C5a) may play a critical role in activating neutrophils to cause pulmonary endothelial cell damage. Although C5a is a powerful chemotaxin and...
promotes neutrophil adhesion and aggregation, it is a weak stimulus of the respiratory burst and exocytosis. This suggests that additional inflammatory agonists might be required to fully provoke PMNs and thus promote full-blown catastrophic ARDS. In fact, others have demonstrated that endotoxin, although a sluggish PMN agonist itself, primes neutrophil responses to other soluble stimuli. In addition, endotoxin induces from macrophages the release of the monokine IL-1, which can alone augment neutrophil adhesion to endothelium and also can induce PAF synthesis in endothelium. In this latter regard, evidence that PAF does indeed play an important etiologic role in endotoxic states: activation by inflammation of intravascular coagulation with production of thrombin. Thrombin, like IL-1, has been shown to induce PAF production in endothelial cells, and thrombin-exposed endothelium becomes an excessively adhesive substrate for neutrophils—perhaps by virtue of this production (Table I). Stimulated by these observations, we recently preliminarily reported that if PMNs are layered atop thrombin-treated endothelium they become hyperresponsive to otherwise ineffectual doses of agonists such as C5a, FMLP, or PMA. These amplified responses—including superoxide production, elastase release, and actual mediation of endothelial damage—can be specifically blocked by PAF inhibitors; this suggests that “cross talk” occurs between endothelial PAF stimulated by thrombin and marginated PMNs. These findings provide an interesting paracrine amplification of endothelial damage that may, for instance, accompany intravascular coagulation attending sepsis. That is, we propose that the small amounts of PAF derived from endothelial cells in the vicinity of activated coagulation potentiate otherwise trivial activators of marginated PMNs; these cells then become lethal to that same endothelium. If this construct is correct, inflammation-mediated vascular damage syndromes should be ameliorated by PAF inhibitors.
REFERENCES


39. Wickham NWR, Vercellotti GM, Yin HQ, Moldow CF, Jacob HS: Neutrophils are primed to release toxic oxidants by contact with thrombin-stimulated endothelium: Role of endothelial cell-generated platelet-activating factor. Clin Res 35:603, 1987 (abstr)
Platelet-activating factor primes neutrophil responses to agonists: role in promoting neutrophil-mediated endothelial damage

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