Endotoxins Stimulate Neutrophil Adhesion Followed by Synthesis and Release of Platelet-activating Factor in Microparticles*

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Lipopolysaccharides and triacyl-cysteine-modified proteins of Gram-negative and positive organisms are potent endotoxins. Animal models show that the receptor for platelet-activating factor (PAF) is responsible for many of the deleterious effects of endotoxin, where regulated, localized PAF production localizes the inflammatory response. In contrast, biologically active analogs of PAF (PAF-like lipids) are generated by oxidative attack on phospholipids by chemical reactions that are unregulated and unlocalized. The identity and distribution of the PAF receptor ligand in endotoxemia is unknown. We found human polymorphonuclear leukocytes (PMNs) were a significant source of PAF receptor agonists after stimulation by either class of endotoxin. Production of PAF receptor agonists required that the PMN adhere to a surface, and adhesion (and therefore accumulation of PAF-like bioactivity) in response to endotoxic stimulation was delayed for several minutes. PAF-like oxidized phospholipids were found by mass spectroscopy, but biosynthetic PAF accounted for most of the phospholipid agonists arising from endotoxic stimulation. A significant portion of the PAF made by PMNs was secreted, in contrast to its near complete retention by other inflammatory cells. Endotoxic stimulation induced a respiratory burst with the production of superoxide and the formation and shedding of microparticles. Free and microparticle-bound PAF appeared in the media, and blocking microvesiculation with calpeptin blocked PAF release. The released material activated platelets, and platelets co-aggregated with endotoxin-stimulated PMNs. Adherent PMNs therefore behave differently than suspended cells and are a significant source of free PAF after endotoxic exposure. Leukocytes can couple endotoxic challenge to the widespread circulatory and inflammatory effects of endotoxin.

Neutrophils (PMNs)† play a critical role in endotoxic shock and death, because rendering animals neutropenic prior to lipopolysaccharide (LPS) challenge reduces mortality (1, 2).

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¶ The abbreviations used are: PMNs, polymorphonuclear leukocytes; LPS, lipopolysaccharide; PAF, platelet-activating factor; fMLP, formyl-methionylleucylphenylalanine; HBSS/A, 0.5% human serum albumin in Hanks’ balanced salt solution; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; PamCys, N-palmitoyl-S-[2,3-bis(palmitoyloxy)-2RS-propyl]/(R)-cysteine; MS, mass spectrometry.

However, this behavior of PMNs is not observed in vivo. LPS by itself is only a poor agonist for isolated PMNs but instead primes isolated PMNs to a state responsive to subsequent exposure to other, more complete agents (3).

PAF was initially described as a platelet agonist (4) but is now recognized as an agonist for all elements of the inflammatory or innate immune system. Elucidation of the molecular structure of this lipid agonist (5, 6) showed PAF to be 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, a modified ether phospholipid. PAF receptor ligands have a critical role in the response to endotoxin, because increasing sensitivity to PAF by overexpressing its receptor worsens the effects of endotoxin (7, 8). Conversely, suppressing this inflammatory axis by pharmacologic blockade of the PAF receptor reduces endotoxic inflammation (9, 10).

Activation of inflammatory cascades by PAF is a central component of host response to LPS endotoxemia (11), and this observation extends to Gram-positive organisms (12) even though they do not contain LPS. LPS is not the only endotoxin that affects mammals, and both Gram-negative and Gram-positive bacteria contain large numbers and large amounts of endotoxic lipoproteins. These proteins contain an N-terminal cysteine residue that is modified by the addition of an ether-linked diacylglyceride and, typically, an N-terminal fatty acyl amide. Proteins and peptides with this N-terminal lipid modification are highly potent inflammatory agonists (13, 14).

The identity of cells that make and secrete PAF in response to endotoxins is not established. Neutrophils (PMNs) are a prolific source of PAF but only when treated with a pharmacologic Ca2+ ionophore such as A23187 (15). Biologic agonists such as fMLP or opsonized zymosan stimulate the formation of just a few percent of this amount of PAF, and LPS is even less effective than this (15, 16). Endothelial cells, monocytes, and even platelets synthesize PAF when appropriately stimulated, but again, LPS is ineffective as an agonist for this response.

The PAF receptor responds to ligands other than PAF. Some of the fragmented phospholipids formed by an uncontrolled oxidative attack on polyunsaturated alkyl phospholipids (17, 18) activate the PAF receptor (19). These precursor polyunsaturated alkyl phospholipids are abundant in leukocytes, and LPS primes the oxidative burst of leukocytes that generates oxidizing free radicals. The enzymatic pathway that produces PAF is tightly controlled, whereas, in contrast, the formation of PAF-like lipids proceeds from uncontrolled oxidative chemical reactions.

There is a second important distinction between PAF and the PAF-like lipids that arise from oxidizing chemical reactions. PAF synthesized by endothelial cells in response to physiologic mediators remains cell-associated, but is translocated and displayed on the extracellular membrane surface; it is this focal expression that localizes the inflammatory response to areas of

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activated endothelium (20, 21). PAF produced by A23187-stimulated PMNs also remains cell-associated (15, 22), and in fact only monocytes are known to release the PAF they synthesize (23). In contrast, oxidatively fragmented phospholipids with PAF-like activity generated by endothelial cells exposed to lipid-soluble hydroperoxides are released into the surrounding medium (24). Cells exposed to this oxidative insult show several morphological adaptations with the formation and release of membranous blebs or microvesicles (24). Release of PAF or PAF-like lipids disrupts spatially localized physiologic signaling and has the potential to produce a disseminated systemic inflammatory response (21).

Here we show that PAF is produced by human neutrophils in response to endotoxic stimulation, either LPS or endotoxic lipopolysaccharides, but only after a delay, and only after the leukocytes become adherent. Adherent, endotoxin-challenged PMNs synthesized PAF but also released a significant portion of this PAF in association with microparticles shed from the cells. PAF synthesized and released from endotoxin-stimulated PMNs activated surrounding platelets, providing a molecular link between acute inflammation and thrombosis. Activated PMNs may therefore propagate and amplify the response to endotoxin through PAF-related mechanisms.

**MATERIALS AND METHODS**

**Chemicals and Reagents—**Chemicals and reagents were purchased from the following sources: sterile filtered HBSS, BioWhittaker Inc. (Walkersville, MD); sterile tissue culture plates, Falcon Labware (Lincoln Park, NJ); human serum albumin, Baxter Healthcare (Glendale, CA); endotoxin-free PBS, LPS (Escherichia coli O111:B4), and Rhizopus arrhizus phospholipase A₁, Sigma (St. Louis, MO); Rhodobacter sphaeroides lipids, Life Scientific Laboratories (Campbell, CA); aminopropyl columns, J. T. Baker Inc. (Phillipsburg, PA); 8-well borosilicate chambered coverslips, Nalgé Nunc International (Naperville, IL); FURA-2 AM, Alexa 594/wheat germ agglutinin, DAPI, and Alexa 488-phalloidin, Molecular Probes (Eugene, OR); recombinant human plasma PAF acetylhydrolase and hPAFR293 cells, Combinant Human Plasma PAF Acetylhydrolase and hPAFR293 Cells (Indianapolis, IN). PamCysSK₄ was synthesized by conjugating Ser-Lys-Ser-2-palmitoyl-SR[f2,3-bispalmitoyl-2RS]-propyl-[(R)-cysteine (PamCys) from Roche Applied Science (Indianapolis, IN). PamCysSK₄ was synthesized by conjugating Ser-Lys(S)، to PamCys at the Pepptide Synthesis facility of University of Utah (Salt Lake City, UT).

**PMN Isolation and Adhesion Assays—**Human serum was isolated from healthy human volunteers, pooled, and frozen until required. PMNs were isolated from healthy human donors by dextran sedimentation and centrifugation over Ficoll as described previously (25). For adhesion assays, PMNs were labeled with 111In-labeled oxine (25) and centrifuged over Ficoll as described previously (25). For this, subconfluent hPAFR293 cells were treated with Versene (Invitrogen, Carlsbad, CA) and resuspended in fresh culture media at 1 × 10⁶ cells/ml. The presence of PAF receptor ligands was tested with HEK293 cells stably transfected with a cDNA encoding the human PAF receptor. For this, subconfluent hPAFR293 cells were treated with Pam3CysSK₄ (Invitrogen, Carlsbad, CA) for 30 min before addition to the plates.

**PMN Morphology by Confocal Microscopy—**PMNs at 2.5 × 10⁶/ml were incubated with an agonist for 30 min before addition to HBSSA at 1.1 × 10⁶ cells/ml. The presence of PAF receptor ligands was tested with HEK293 cells stably transfected with a cDNA encoding the human PAF receptor for analyzing their effect on HBSSA using confocal microscopy. PMNs were incubated with 111In-labeled PMNs detected by gamma counting to calculate the percentage of adherent cells expressed as a fraction of the total recovered cells.

**Lipid Extraction from Stimulated PMNs—**PMNs were incubated with the stated agonist in microcentrifuge tubes for suspended cells or over gelatin-coated tissue culture plates, an assay that measures β₂-integrin-dependent adhesion. Adherent cells were scraped from their substrate in medium and transferrin-bound carbon-coated nickel grids with chloroform to extract lipids by the method of Bligh and Dyer (26). The resulting total lipid extract was dried under N₂ and reconstituted with HBSSA (0.5% human serum albumin in HBSS). The reconstituted lipid extracts were sonicated and vortexed just before use. In some experiments the lipid extracts were treated with 1 μg/ml PAF acetylhydrolase for 3 h at 37 °C before analysis with 111In-labeled PMNs. Incorporation of [3H]Hæcate (Amersham Biosciences, Piscataway, NJ) into PAF was analyzed as described previously (15).

**Intracellular Ca²⁺ Measurements—**PAF-like activity was detected by monitoring rapid changes in intracellular Ca²⁺ levels as described previously (19). Briefly, PMNs (5 × 10⁶ cells/ml) were preincubated in the dark with the Ca²⁺-sensitive fluorescent dye FURA-2/AM (1 μM) at 37 °C for 45 min. PMNs were recovered by centrifugation, washed with fresh HBSSA three times, and resuspended in fresh HBSSA at 2.75 × 10⁶ cells/ml. The presence of PAF receptor ligands was tested with HEK293 cells stably transfected with a cDNA encoding the human PAF receptor. For this, subconfluent hPAFR293 cells were treated with Pam3CysSK₄ (Invitrogen, Carlsbad, CA) and resuspended in fresh culture media at 1.1 × 10⁶ cells/ml. The cells were incubated with 1 μM FURA-2/AM for 45 min at 37 °C before being recovered and washed with HBSSA by centrifugation as before. These cells were resuspended in HBSSA at 2.5 × 10⁶ cells/ml. Changes in intracellular Ca²⁺ were detected using a spectrophotometer with dual wavelengths and 380 nm and emission at 510 nm (27). These data are presented with 340/380 nm ratios on the y axis and a 5-min time period for the x axis.

**Separation of PAF-like Lipids and Mass Spectrometric Analysis—**Separation of PAF-like lipids by high-performance liquid chromatography was described previously (19). Briefly, PMNs (5 × 10⁶ cells/ml) were stimulated with PBS, A23187 (1 μM), LPS (5 μg/ml) with or without 2% pooled human serum, or 5 μg/ml PamCysSK₄, for 90 min before total lipids were extracted (26) from a total of 5 × 10⁶ PMNs. The total lipid extracts were condensed by rotavap (Buchi Labortechne, Flawil, Switzerland), dried under a flow of N₂ gas, and then reconstituted in CHCl₃. Phospholipids were isolated from neutral lipids and fatty acids by aminopropyl extraction columns (28) and then fractionated by reversed-phase high-performance liquid chromatography as described previously (19). Fractions eluting from the reversed-phase column were assayed after drying and resuspending an aliquot in HBSSA by analyzing their effect on FURA2-loaded PMNs as described above. Fractions containing PAF-like activity were pooled, dried under a flow of N₂ gas, resuspended in HBSSA at 37 °C, and then treated with 10,000 units of phospholipase A₁ from R. arrhizus in HBSSA at 37 °C overnight to remove diacyl lipids (29). Polar alkyl phospholipids were resolved from free fatty acids with aminopropyl extraction columns, and the recovered PAF-like lipids were reconstituted in methanol for analysis by electrospray ionization/MS/MS (19, 30) after introduction of a constant amount of [3H]PAF.

**PMN Morphology by Confocal Microscopy—**PMNs at 2.5 × 10⁶/ml in a volume of 200 μl were incubated with an agonist for the stated time in gelatin-coated 8-well borosilicate chambered coverslips. The adherent PMNs were washed three times with endotoxin-free PBS and fixed with 2% paraformaldehyde for 30 min at room temperature. Polymerized actin in the fixed cells was stained at 4 °C with Alexa 488-conjugated phalloidin, plasma membranes were stained with Alexa 594/wheat germ agglutinin at 10 μg/ml, and the nuclei were stained with DAPI at 5 μg/ml just before fluorescence microscopy.

**Isolation of Microvesicles from Endotoxin-stimulated PMNs—**Microvesicles were collected as described (31) with minor modifications. PMNs (5 × 10⁶ cells/ml) were stimulated with the stated agonist for 90 min at 37 °C in gelatin-coated P100 tissue culture plates (Falcon Labware, Lincoln Park, NJ). After incubation, media from each sample were centrifuged for 10 min at 500 × g at 4 °C to clear cells and debris. The supernatant from this low speed centrifugation was collected and sealed into Quick-Seal centrifuge tubes (Beckman Coulter Inc., Brea, CA) for centrifugation at 100,000 × g for 90 min at 4 °C to collect microvesicles (32). The resulting supernatant and cellular microvesicles were separately recovered, and their lipids were extracted and assayed as described above.

**RESULTS**

**LPS and Bacterial Lipopeptides Stimulate PMN Adhesion—**PAF causes PMNs to immediately begin to adhere to surfaces, in this case a gelatin-coated tissue culture well by a β₂-integrin-dependent process (33), with the maximal amount of adhesion being achieved by 30 min of stimulation (Fig. 1). LPS stimulation did not cause PMNs to immediately adhere to the surface, as shown by the near absence of adherent cells after 5 min of exposure, but after this delay PMNs exposed to LPS presented in solution began to adhere in a time-dependent fashion. In the absence of serum, and the LPS-binding protein and soluble CD14 it contains, the delay in the response to LPS was pro-
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longed and the number of PMNs that eventually adhered to the surface was significantly reduced. The major lipid-modified endotoxic protein of *E. coli* (14), the Braun lipoprotein, and a synthetic peptide based on its lipid-modified N terminus, Pam₃CysSK₄, stimulated PMN adhesion to the proteinized surface. The lipopeptide Pam₃CysSK₄ and the Braun lipoprotein induced the same delay before the PMNs began to adhere as found with LPS presented in serum, but we found (not shown) that both of these N-terminal lipid-modified molecules elicited the same response in the presence or absence of serum.

Endotoxins Stimulate PMN Spreading and Actin Filament Re-arrangement—PAF caused PMNs to rapidly spread onto the protein-coated surface and develop a polarized morphology (Fig. 2). Visualization of polymerized F-actin with phalloidin showed that nearly all of the spread PMNs have large amounts of bundled actin that was absent in suspended cells (shown on the upper right panel of Fig. 2). Initially the polymerized actin was distributed around the periphery of the cell but over time coalesced into a structure at the leading edge of lamellipodia. There were very few adherent PMNs after 5 min of treatment with LPS or Pam₃CysSK₄, and the few adherent cells present at this time contained little polymerized actin. However, as more cells began to adhere and spread, their content of polymerized actin increased and the distribution of this bundled actin after stimulation with either LPS or Pam₃CysSK₄ for 15 min was similar to PMNs treated with PAF for 5 min.

PMNs Generate a Ligand for the PAF Receptor after Endotoxic Stimulation—We considered the possibility that the delay in adhesion when PMNs were stimulated by either endotoxin resulted from the need to synthesize a secondary, adhesion-promoting agonist. PAF rapidly stimulates PMN adhesion, so we investigated the effect of blocking the PAF receptor on the adhesion induced by each endotoxin. We found (Fig. 3A) that adhesion induced by LPS or Pam₃CysSK₄ was modestly reduced by competitive inhibition of the PAF receptor with BN52021, whereas nearly half of the adhesion induced by the Ca²⁺ ionophore A23187 was suppressed by interfering with PAF receptor signaling.

PAF was synthesized by PMNs in response to LPS or Pam₃CysSK₄ only after they had already adhered and spread (vide infra). Therefore, even the modest effect of PAF receptor antagonist on adhesion indicates that a portion of this adhesion-promoting phospholipid mediator must have been released from adherent cells to affect other cells in the incubation. This outcome would differ from the normal intracellular retention of PAF by suspended PMNs (15, 22), so we treated PMNs with the ionophore A23187, LPS, or Pam₃CysSK₄ and then recovered the media and extracted the lipids it contained. The lipids released from PMNs treated with each agonist, but not those of buffer-treated cells, stimulated fresh, quiescent PMNs to rapidly adhere to gelatinized wells (Fig. 3B). The adhesion-promoting effect of the material released from activated cells was completely lost by blocking the PAF receptor of the test cells with BN52021, and so the released lipid agonist(s) likely acted through the PAF receptor. Additionally, we found that treating the recovered lipids with recombinant PAF acetylhydrolase, a phospholipase A₂, that specifically hydrolyzes PAF and oxidatively fragmented phospholipids (34), also completely suppressed the pro-adhesive effect of the lipids released from endotoxin-treated PMNs.

We directly determined whether the PMN agonists generated by endotoxin-treated PMNs stimulated the PAF receptor by testing these lipid extracts on cells stably transfected with cDNA encoding the human PAF receptor (hPAFR293 cells). Untransfected 293 cells did not respond to PAF (Fig. 3C) but did show a Ca²⁺ flux after treatment with the Ca²⁺ ionophore A23187. hPAFR293 cells stably expressing the PAF receptor responded to PAF as expected but did not do so when directly challenged with LPS or Pam₃CysSK₄, showing that, in contrast to a previous report (35), these two endotoxins do not directly stimulate the PAF receptor. The hPAFR293 cells, however, did respond to the lipids extracted from PMNs exposed to LPS or Pam₃CysSK₄ for 60 min. The PAF receptor antagonist BN52021 blocked the response of hPAFR293 cells to the lipid agonists made by PMNs in response to each endotoxin, so the one or more activating lipids were not endotoxin carried over from the first incubation.

![Fig. 1. Temporal change in PMN adhesion in response to endotoxins.](image)

![Fig. 2. Spreading and actin polymerization in adherent PMNs in response to endotoxins.](image)
response to LPS or Pam3CysSK4 stimulation compared with the total amount of the three molecular species of PAF generated in PMNs treated or not with PAF-acetylhydrolase before the lipid extracts were tested for adhesion-promoting activity with 111In-labeled PMNs. The values represent the mean ± range of two samples for each condition. 

PMNs were stimulated with PBS, LPS, or Pam_cysSK4, for 60 min at 37 °C and incubated with the stated agonist for 5 min at 37 °C, and the fraction of adherent PMNs was then determined by gamma counting. The short adhesion time prevents a response from any endotoxin that might be carried over into the assay. The values represent the mean ± range of two samples for each condition. 

We examined the effect of activated PMNs on a different cell type, freshly isolated human platelets, so we could distinguish primary and secondarily activated cells and because PMN-derived PAF can foster this important intercellular interaction (19). After stimulation by the two endotoxins, PAF can be formed by oxidative fragmentation of alkyl phosphatidylinositol and other PMNs (15, 22)). PAF, as expected, stimulated formation of platelet-leukocyte aggregates (38). PAF, as expected, stimulated formation of platelet-leukocyte aggregates (Fig. 4A). We then pre-treated leukocytes with LPS or Pam_cysSK4, and then added freshly isolated human platelets. The combination of the two cells resulted in formation of large aggregates of leukocytes with the anucleate platelets. The co-incubated platelets, and activated PMNs increased the content of polymerized actin within the distinctively anucleate platelets (Fig. 4B). Neither LPS nor Pam_cysSK4 stimulation caused these changes when added to platelets alone, nor did PAF by itself cause the platelets to aggregate and then adhere to the immobilized gelatin in this assay system.

Table I

| PAF        | C4-PAF | pg/10^6 cells | -fold change | pg/10^6 cells | -fold change |
|------------|--------|---------------|--------------|---------------|--------------|
| Buffer     | 9.0    | 1.0           | 5.1          | 1.0           |
| LPS        | 58.8   | 6.5           | 4.7          | 0.9           |
| Pam_cys    | 110.1  | 12.2          | 11.2         | 2.2           |
| A23187     | 1754.8 | 194.6         | 2.0          | 0.4           |

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Endotoxin-stimulated PMNs release PAF-like lipid(s) which might be carried over into the assay. The values represent the mean ± range of two samples for each condition. 

PMNs in Response to Endotoxin

Stimulated PMNs Release PAF-containing Microparticles—Cells such as endothelial cells (39) and PMNs (15, 22) retain the bulk of the PAF they synthesize, whereas other cells,
exemplified by monocytes (23) and eosinophils (40), release the PAF they make. The experiments shown in Fig. 4 show that a portion of the PAF generated by PMNs in response to endotoxic stimulation was released from the cell. We directly assessed whether adherent PMNs released the PAF they make by treating PMNs with Pam3CysSK4 for increasing periods of time and then separately recovering the cells and their media. We found (Fig. 5) that there was a time-dependent increase in cellular phospholipid agonists and that, after a further delay, a significant portion of this material was recovered in the media overlying the cells. As noted above, PAF accumulation and release required that the cells be adherent, because suspended cells did not generate PAF.

We questioned how endotoxin-treated PMNs came to release significant amounts of PAF and considered that the formation of oxidized phospholipids with PAF-like activity showed the membranes of endotoxin-treated PMNs to be oxidatively damaged. One consequence of severe cellular oxidation is the loss of membrane structure with the release of membrane microvesicles or “blebs” (24, 41) that might be a vehicle for PAF release. We visualized cells and microparticles by staining PMNs with Alexa 488-phalloidin after treatment with LPS or Pam3CysSK4 to find that many of the endotoxin-treated cells had ragged cellular edges and had developed distinct focal inclusions of polymerized actin (Fig. 6). Some cells also appeared to have lost their nuclear DNA, and there were small extracellular structures that contained just polymerized actin. We collected media from these cells, subjected it to high speed ultracentrifugation to recover microparticles, and analyzed the lipids we recovered for PAF activity. We discovered that the PAF released from endotoxin-stimulated PMNs was equally distributed between particles and soluble material (Fig. 6B). The shedding of microvesicles seems to arise from cytoskeletal alterations that are blocked by calpain inhibitors, including calpeptin (41, 42). We found that calpeptin effectively sup-

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**Fig. 4.** Endotoxin-stimulated PMNs activate quiescent platelets. PMNs were incubated at 37 °C for 60 min with buffer, PAF, LPS, or Pam3CysSK4, as before on gelatin-coated tissue culture plates. After 60 min, buffer or 10⁶ platelets were added, and the co-incubation was continued for 30 min. After this, the adherent cells were washed and fixed, and nuclei were stained with DAPI (blue), polymerized actin with Alexa 488-phalloidin (green), and plasma membranes with Alexa 594-wheat germ agglutinin (red) as described under “Materials and Methods.” The preparations were visualized by fluorescence microscopy at ×40 (A) or ×100 (B).
pressed the release of PAF, although not its synthesis and intracellular accumulation, from PMNs treated with either LPS (not shown) or Pam3CysSK4 (Fig. 6C).

**DISCUSSION**

We found that human leukocytes respond to LPS and to naturally occurring and synthetic endotoxic lipoproteins with functional changes that may influence inflammatory events in bacterial infection and sepsis. Human PMNs after stimulation with either of the two types of endotoxin adhered to a gelatin-coated surface and then rapidly spread onto the surface, accumulated polymerized actin, and developed lamellipodia. Eventually the cells lost this organized morphology and displayed ragged edges with the appearance of extracellular microparticles. PMNs synthesized and released significant amounts of PAF after they had undergone these ultrastructural changes. Unusual for PMNs, half or more of the newly made PAF was released from the cells in association with microparticles, a process that will elicit distal inflammatory responses.

Cells of the innate immune system typically respond quickly, but adhesion in response to either LPS (43) (Fig. 1) or the two Pam3Cys-containing molecules (Fig. 1) displayed a distinct hysteresis. PAF, in contrast, causes rapid and robust adhesion, with rapid spreading onto the surface accompanied by marked actin polymerization. We tested the hypothesis that PAF synthesis, and potentially its release, contributed to adhesion and spreading of endotoxin-treated PMNs, but PAF does not appear to have a significant role in these processes. First the temporal relationship between PAF and adhesion was incompatible with most of the cells having adhered by 30 min (Fig. 1) when we first began to find PAF in the supernatant of endotoxin-treated PMNs (Fig. 5A). Second, we tested whether PAF might act in an autocrine fashion, rather than on surrounding cells, by

**Fig. 5.** Time-dependent release of PAF from adherent PMNs after stimulation with Pam3CysSK4. PMNs were treated with Pam3CysSK4 for the stated times either as suspended or adherent cells. Total lipids were isolated from the cells or their overlaying media, dried, and reconstituted in HBSS/A, and PAF in the extracts was tested by bioassay using FURA-2AM-loaded PMNs as described under "Materials and Methods."

**Fig. 6.** Microvesicles shed from Pam3CysSK4-stimulated PMNs contain PAF. A, endotoxin-treated PMNs develop membrane blebs and small actin-rich particles. Adherent PMNs were stimulated with PBS, LPS, or Pam3CysSK4 for 90 min, washed, fixed, triple stained as in Fig. 4, and then visualized by fluorescence microscopy at a magnification of ×100. B, PMNs were treated with Pam3CysSK4 for 90 min before the media was collected, any unattached cells were cleared by centrifugation at 500 × g, and then microvesicles were recovered by centrifugation at 100,000 × g for 90 min. The lipids from this high speed pellet and the resulting supernatant were extracted and assayed using FURA-2AM-loaded PMNs as described under "Materials and Methods." C, calpain inhibition suppresses PAF release. PMNs were pretreated or not with 30 μM calpeptin for 30 min and then stimulated with either LPS (data not shown) or 5 μg/ml Pam3CysSK4 for 90 min. Total lipids in the media and from adherent PMNs were extracted, dried, and reconstituted in HBSS/A before the lipids from each sample were tested with FURA-2AM-loaded PMNs as before.
blockade of the PAF receptor with a competitive antagonist. This did diminish the number of adherent PMNs, but the effect was modest. It remains possible, however, that endogenously synthesized PAF has a role in promoting adhesion, because PAF is a far better ligand for the PAF receptor than are PAF receptor antagonists (44, 45) and we may not have effectively blocked the receptor with exogenously added antagonists. The inefficiency of PAF receptor blockade is also found in vivo, where PAF receptor antagonists have not been clinically effective even in situations where PAF and its receptor clearly have a role (11).

Adherent PMNs unlike those maintained in a suspended state synthesized PAF in response to LPS or PAMcCysSK4 (Table I), with a corresponding incorporation of [3H]acetate into PAF (not shown). The amount of PAF generated after endotoxin treatment was dwarfed by the amount of PAF made in response to A23187, being only 3% of this amount for LPS and double that for PAMcCysSK4. PMNs vigorously synthesize PAF when stimulated by A23187, but few other agonists result in any PAF production at all (15, 22). PAF production following fMLP stimulation is undetectable, unless cytochalasin B is included in the assay (22). This requirement is consistent for a role in the actin cytoskeleton in promoting the synthesis of PAF, and we observed a time-dependent increase in PMN polymerized actin content after endotoxic stimulation.

LPS induces an oxidative burst by PMNs (as does the Braun lipoprotein and PAMcCysSK4 (46), not shown), which was of sufficient magnitude to oxidatively attack membrane phospholipids. The double bonds of polyunsaturated fatty acids, whether the lipid is esterified or not, are susceptible to oxidative attack, and polyunsaturated fatty acids are preferentially esterified at the sn-2 position of phospholipids. Phosphatidylcholines with an sn-1 ether bond, the type of bond that confers much of the specificity of the PAF receptor for PAF (37), oxidize in parallel with their diacyl homologs (47), and PMNs are enriched with ether phosphatidylcholines containing the polyunsaturated arachidonyl residue (48). The biologically active PAF homologs 1-O-hexadecyl-2-(butanoyl or butenoyl)-sn-glycero-3-phosphocholine are oxidation products of 1-O-hexadecyl-2-archidonyl-sn-glycero-3-phosphocholine and are found in low density lipoprotein after, and not before, oxidation (19). We now find these oxidatively generated PAF analogs in PMNs after stimulation with LPS or PAMcCysSK4, and we take as evidence of a significant oxidative attack on the membrane phospholipids of PMNs. The PAF receptor displays strong preference for the acetyl residue of PAF, and these PAF analogs with sn-2 fatty acyl residues that are four carbons long are only 10% as potent as PAF (19). The oxidative burst of PMNs responding to LPS or endotoxic proteins is therefore sufficient to overcome cellular anti-oxidant protection mechanisms, but does not, in the end, generate the PAF bioactivity made and released from these cells.

PAF synthesized by PMNs in response to A23187 stimulation remains, for the most part, cell associated (16), whereas approximately half the PAF produced by cells exposed to PAMcCysSK4 was released from the cell (Fig. 5). One result of this is that cells stimulated by either type of endotoxin released as much PAF to their environment as did A23187-stimulated cells (Fig. 3B). The mechanism of this unusual release of PAF, we believe, comes from shedding of microparticles from endotoxin-treated cells, because the process of degranulation is insufficient to release PAF from stimulated PMNs (49). Adherent PMNs over time lost their characteristic morphology consistent with migrating cells to become cells with protruding spikes, punctate intracellular f-actin staining and indeed some cells appeared to have lost their nuclei (Fig. 6A). Phallolidin staining also showed the presence of small extracellular particles, which we recovered by ultracentrifugation. Stress reorganizes f-actin during apoptosis (50), as does cellular oxidant injury (51), and anti-oxidant intervention even attenuates membrane blebbing in vivo (52). Membrane bleb formation, whether in response to oxidative stress (41) or cell-specific agonists (42), is suppressed by interfering with calpain function, so finding that calpeptin effectively suppressed the release, but not the synthesis, of PAF from endotoxin-stimulated PMNs (Fig. 6C), so formation and release of membrane fragments accounts for all of the unexpected release of PAF from stimulated, adherent PMNs.

Transcellular activation by platelet (53) and leukocyte (54) microparticles is well established, and leukocytes from septic patients shed more microparticles than do leukocytes isolated from healthy controls (55). Membrane microparticles shed from PMNs stimulated with PAMcCysSK4 contained half of the total PAF released from the cells (Fig. 5C), and one consequence of this was the formation of platelet-leukocyte aggregates when these cells were present together (Fig. 4B). Animal experiments reveal a link between PAF signaling and endotoxic inflammation in a variety of models (11), and overexpression of the PAF receptor in transgenic animals increases mortality when they are challenged with LPS (7), whereas blockade of the PAF receptor with competitive antagonists is protective (11). PAF plays a critical role in physiologic inflammation, and clinical observations and animal models indicate PAF has a similar role in the pathogenesis of sepsis (56, 57). The release of PAF from endotoxin-stimulated PMNs identifies a molecular mechanism with the potential to propagate inflammatory events to distal organs in a systemic fashion but also to induce formation of occlusive platelet-leukocyte aggregates. Loss of localization and compartmentalization of potent inflammatory agents describes new points of dysregulation of a localized inflammatory response to sepsis.

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