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Platelet-Activating Factor Modulates Endotoxin-Induced Macrophage Procoagulant Activity by a Protein Kinase C-Dependent Mechanism

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Macrophage procoagulant activity is an important mediator of extravascular fibrin deposition at sites of infection and appears to contribute to the pathogenesis of several infectious disease processes. Previous studies have shown that the inflammatory mediator platelet-activating factor was able to prime macrophages for induction of procoagulant activity by bacterial lipopolysaccharide. The present studies were designed to examine the mechanism of this priming effect. Platelet-activating factor (100 nM) primed macrophages for procoagulant activity generation in response to endotoxin at concentrations as low as 100 ng/ml and also following exposure to *Escherichia coli*, *Bacteroides fragilis*, and *Staphylococcus aureus*. The priming effect occurred following a pretreatment with platelet-activating factor for as short as 1 min, suggesting a rapid activation event. Two different doses of the calcium ionophore ionomycin were used to mimic the peak and sustained effects of platelet-activating factor on cytoplasmic calcium levels (1 μM and 100 nM, respectively). Neither dose was able to mimic the priming effect. However, extracellular calcium was necessary for induction of procoagulant activity and the priming effect. By contrast, the protein kinase C agonist phorbol myristate acetate reproduced the priming phenomenon observed for platelet-activating factor. In further support of the concept that protein kinase C activation mediated the effect of platelet-activating factor, the specific protein kinase C inhibitor staurosporine reversed the ability of platelet-activating factor to augment induction of macrophage procoagulant activity by endotoxin. These data suggest mechanisms by which inflammatory mediators within the microenvironment of infection might modulate the host response to bacterial pathogens.

Macrophage-mediated fibrin deposition via the expression of surface procoagulants (PCA) appears to play an important role in the pathogenesis of several infectious and inflammatory processes (3, 11, 12, 23). For example, PCA expression following infection with murine hepatitis virus type 3 correlates with the severity of disease in susceptible and resistant mouse strains (11, 23). Similarly, PCA has been postulated to mediate fibrin deposition on infected vegetations during bacterial endocarditis (12) and to be responsible for the fibrinopurulent exudates encountered during bacterial peritonitis (3). The proinflammatory effects of fibrin are mediated via several mechanisms. Both fibrinopeptide B and fibrin degradation products, generated during the deposition and degradation of fibrin, respectively, have been shown to have chemoattractant activity for phagocytic cells and to be capable of modulating their function (38, 40). In addition, bacterial sequestration within fibrin matrices promotes the development of persistent infection, by virtue of the ability of fibrin to impair clearance of bacteria by host cells (36, 37).

The local microenvironment established by virtue of the interaction between invading bacterial pathogens and host defense mechanisms contains a number of factors which might modulate the magnitude of the inflammatory response (29). These include cytokines such as interferon gamma, the interleukins, and tumor necrosis factor, arachidonic acid metabolites, such as the cyclooxygenase and 5-lipoxygenase products, and platelet-activating factor (PAF). PAF is a ubiquitous inflammatory mediator (31) which has been shown to mediate and/or contribute to the pathogenesis of several pathological processes, including endotoxin-induced lung injury and intestinal necrosis during endotoxemia (6, 17). Fibrin deposition is part of the pathological picture observed in each of these processes, suggesting that PAF contributes to the pathogenesis of these diseases by inducing local coagulation. In this regard, recent studies from our laboratory demonstrated the ability of PAF to augment the induction of PCA in macrophages in response to endotoxin (20). The purpose of the present studies was to investigate the cellular mechanisms underlying the priming effect of PAF on PCA production in macrophages.

MATERIALS AND METHODS

Animals. Six- to eight-week-old female Swiss Webster mice were obtained from Charles River Laboratories. Following delivery to our animal facility, animals were allowed to acclimate for 2 to 4 days prior to use in these studies. Animals were then maintained in colonies of no more than five mice per cage and fed mouse chow and water ad libitum.

Reagents. PAF (1-α-phosphatidylcholine-β-acetyl-γ-*O*-alkyl), phorbol myristate acetate (PMA), ionomycin, and indomethacin were obtained from Sigma Chemical Co., St. Louis, Mo.; staurosporine was from Calbiochem Corp., La Jolla, Calif.; RPMI 1640 medium was from Flow Laboratories, McLean, Va.; calcium- and magnesium-free Hank's balanced salt solution (HBSS), RPMI-Selectamine kit, and fetal calf serum were from GIBCO Laboratories, Grand Island, N.Y.; lipopolysaccharide B *Escherichia coli* O111:B4 (LPS) was from Difco Laboratories, Detroit, Mich.; and the acetoxymethylester of indo-1 was from Molecular Probes, Eugene, Oreg.

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PAF was dessicated with nitrogen gas and reconstituted in pyrogen-free sterile normal saline. LPS was reconstituted in pyrogen-free sterile water. PMA, ionomycin, and staurosporine were reconstituted in dimethyl sulfoxide and diluted in pyrogen-free sterile saline. Indomethacin was reconstituted fresh daily in absolute ethanol and diluted in pyrogen-free sterile saline. Fetal calf serum was heat inactivated at 56°C for 60 min and stored at 4°C until use. Brewer's thioglycolate medium (Difco) was solubilized in water, autoclaved at 125°C for 45 min, and stored in the dark at 22°C until uniformly green.

Growth and culture of bacteria. *Bacteroides fragilis* VPI 9032 was provided by Tracy Wilkins, Virginia Polytechnic Institute and State University, Blacksburg. Aliquots were maintained and frozen in thioglycolate medium without dextrose as previously described (35). Frozen cultures were thawed and 0.2 ml of bacterial suspension was inoculated into tubes containing 20 ml of minimal growth medium (35). The tubes were incubated for 24 h at 35°C in an anaerobic chamber (Forma Scientific, Marietta, Ohio) in which an atmosphere of 85% nitrogen, 10% hydrogen, and 5% CO_2 was maintained.

For addition to macrophages, the *B. fragilis* culture was removed from the anaerobic chamber, pelleted by centrifugation (1,800 × *g* for 20 min), washed twice in sterile normal saline (9.0 g/liter), and suspended in 20 ml of saline. For quantitation of viable bacteria, suspended *B. fragilis* cells were serially diluted and surface plated onto supplemented brain heart infusion agar (Difco). Surface colonies were counted after 48 h of incubation at 35°C in the anaerobic chamber.

Staphylococcus aureus and *E. coli* were maintained at 4°C on agar plates. A sterile loop was used to introduce organisms into tubes containing 20 ml of brain heart infusion medium. The tubes were incubated for 20 to 24 h at 37°C in a rocking water bath. Prior to addition to macrophages, *S. aureus* and *E. coli* were removed from the water bath, pelleted by centrifugation (1,800 × *g* for 20 min), washed twice in sterile saline, and suspended in 20 ml of saline. For quantitation of viable bacteria, suspended *E. coli* and *S. aureus* cells were serially diluted and surface plated onto nutrient agar (Difco). Surface colonies were counted after 48 h of incubation at 37°C.

Cell preparation. Peritoneal macrophages were harvested by peritoneal lavage 4 days following intraperitoneal injection of 2 ml of thioglycolate medium. Lavage fluid was centrifuged at 700 × *g* for 10 min. The supernatant was discarded, and the cell-containing pellet was resuspended in 5 ml of sterile water for 15 s to lyse any erythrocytes and then diluted to 45 ml with HBSS. The suspension was pelleted again, and the cells were resuspended in RPMI 1640 medium or calcium-free RPMI medium (prepared from a Selectamine kit, using a calcium-free balanced salt solution as a base) and then counted with a hemocytometer. The cell population was diluted to 2×10^6 cells per ml and aliquoted into polypropylene tissue culture tubes. The RPMI medium was supplemented with 10% fetal calf serum in all experiments except those studying the effect of different extracellular calcium concentrations. Use of this technique resulted in a cell suspension with a cell viability in excess of 95%, as measured by trypan blue exclusion, and a cell population of 80 to 90% macrophages, as measured by Wright's staining and nonspecific esterase.

Human mononuclear cells were collected from heparinized venous blood by centrifugation over Ficoll-Hypaque (400 × *g* for 20 min). The layer containing mononuclear cells was aspirated, resuspended in HBSS, and washed twice. This population contained ~30% monocytes, the remainder being lymphocytes, as assessed by Wright's staining.

Cell stimulation. (i) PAF priming. Cells were incubated with PAF (100 nM) from 1 to 30 min prior to stimulation. This concentration of PAF had been previously shown to produce optimal priming for LPS-induced PCA production by macrophages (12). Cells were then pelleted, resuspended in fresh supplemented medium, and stimulated with LPS (10 μg/ml) or the stated microorganism in various concentrations. After 4 h, cells were pelleted, resuspended in RPMI 1640 medium, and frozen for later PCA assay.

(ii) Response to ionomycin. Cells were incubated with ionomycin (1 μM or 100 nM) for 30 min prior to stimulation with LPS. Cells were then pelleted, resuspended in fresh supplemented medium, and stimulated with LPS (10 μg/ml). After 4 h, cells were pelleted, resuspended in fresh RPMI 1640 medium, and frozen for later PCA assay.

(iii) Response to PMA. Cells were incubated with PMA (10^{-9} to 10^{-6} M) for 30 min prior to exposure to LPS. They were then pelleted, resuspended in fresh supplemented RPMI, and stimulated with LPS. After 4 h, cells were again pelleted, resuspended in fresh RPMI 1640 medium, and frozen for later PCA assay. In some studies, cells were treated with the protein kinase C (PKC) inhibitor staurosporine (10 nM) for 5 min prior to being exposed to PAF (42).

(iv) Effect of indomethacin. Cells were incubated with indomethacin at concentrations ranging from 1 ng/ml to 10 μg/ml for 15 min prior to other manipulations. They were then treated with PAF or control medium for 30 min, pelleted, resuspended in fresh supplemented RPMI 1640 medium, and stimulated with LPS. Indomethacin at the desired concentration was present throughout the treatment period. After 4 h, cells were again pelleted, resuspended in fresh RPMI 1640 medium, and frozen for later PCA assay. Levels of prostaglandin E_2 were measured in the supernatants by radioimmunoassay according to the manufacturer's instructions (Amersham).

Measurement of PCA. PCA in freeze-thawed macrophages was determined by measuring their capacity to shorten the spontaneous clotting time of normal citrated human plasma in a one-stage clotting assay (22). In selected studies, PCA in freeze-thawed cells was compared with that in undisrupted cells. An 80-μl sample of freeze-thawed cells was added to 80 μl of citrated normal human platelet-poor plasma, and then 80 μl of 25 mM $CaCl_2$ was added to initiate the reaction. The time taken for the appearance of a fibrin gel at 37°C was recorded. Clotting times were converted to milliunits of PCA by comparison with a rabbit brain thromboplastin standard (Sigma Chemical Co.) in which 36 mg (dry weight) per ml was assigned a value of 100,000 mU of PCA. The induction of PCA from a baseline of 100 mU/2 × 10^6 macrophages to 750 mU/2 × 10^6 macrophages in cells stimulated by LPS for 4 h represented a shortening of the clotting time from 52 to 35 s. The assay was used over a range from 10 to 10,000 mU of PCA, this range being linear with normal plasma substrate. Previous studies have shown that PCA induced with *E. coli* LPS and the bacterial species tested has tissue factor-like activities, making comparison with a thromboplastin standard valid (35).

Measurement of cytosolic calcium ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was measured fluorometrically with the calcium-sensitive fluorescent dye indo-1 (15). The cells were preloaded by incubation in RPMI 1640 medium (HCO_3^- free) with 2 μg of the precursor acetoxymethylester of indo-1 per ml for 20 min at

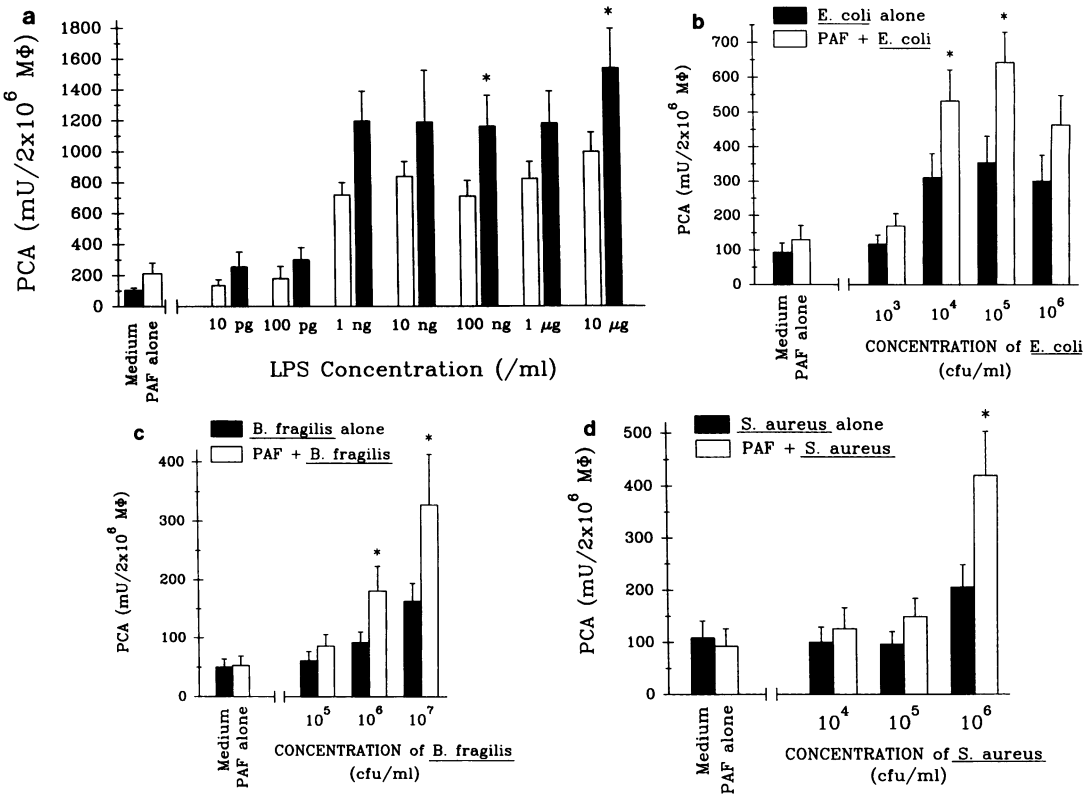

FIG. 1. Effect of PAF on induction of macrophage PCA by LPS or various bacterial pathogens. Macrophages were treated with PAF (100 nM) for 30 min at 37°C, washed, and then exposed to the designated stimulus for 4 h at 37°C. Cells were then freeze-thawed and assayed for PCA. (a) LPS; (b) *E. coli*; (c) *B. fragilis*; (d) *S. aureus*. Data represent the mean and standard error of 6 to 11 experiments, each done in duplicate. *, $P < 0.05$ versus stimulus alone. MΦ, macrophages.

37°C. This hydrophobic compound rapidly diffuses across the plasma membrane into the cytoplasmic space where the acetoxymethylester is cleaved by esterases, trapping the hydrophilic indo-1 in this compartment. Indo-1 then binds calcium in a 1:1 ratio, and upon binding, calcium exhibits a shift in its fluorescence spectrum that can be measured with a fluorometer. For measurement of $[Ca^{2+}]_i$, 10^6 cells per ml were suspended in control medium in a Perkin-Elmer LS-5 fluorescence spectrometer with an excitation wavelength of 331 nm and an emission wavelength of 410 nm. Slit widths of 3 and 15 nm were used for excitation and emission, respectively. PAF (100 nM) was then added to the cell suspension. Calibration was done with ionomycin to cause maximal fluorescence and Mn^{2+} to quench the fluorescent signal. Indo-1 has a K_d of 254 nM for calcium, and a ratio of F_{max}/F_{min} of 12 was used to calculate $[Ca^{2+}]_i$.

Endotoxin contamination. RPMI 1640 medium, HBSS, fetal calf serum, sterile water, sterile saline, and all other reagents were tested for endotoxin contamination by the standard *Limulus* amoebocyte lysate assay (Association of Cape Cod, Woods Hole, Mass.) and were found to contain <0.1 ng of endotoxin per ml, which constituted the lower limits of the test.

Statistics. Statistics were calculated by one-way analysis of variance and Student's *t* test with the Bonferonni correction. Data are expressed as the mean and standard error of the number of experiments indicated, each performed in duplicate.

RESULTS

Effect of PAF on PCA stimulation. Figure 1 examines the ability of PAF to augment PCA induction in murine peritoneal macrophages by a range of LPS concentrations and also in response to various bacterial pathogens. Pretreatment with PAF enhanced PCA production following exposure to LPS at concentrations as low as 1 ng/ml, reaching statistical significance at concentrations greater than 100 ng/ml (Fig. 1a). PAF also primed cells for PCA induction in response to live *E. coli*, *B. fragilis*, and *S. aureus* (Fig. 1b to d). While PAF generally augmented the ability of all bacterial concen-

TABLE 1. Effect of different PAF priming durations on PCA induction[a]

Duration of PAF priming (min)	PCA (mU/2 × 10^6 cells)[b] with the following stimulus:	
	LPS	PAF-LPS
1	257 ± 54	547 ± 60[c]
5	275 ± 54	738 ± 202[c]
10	406 ± 86	930 ± 329[c]
30	356 ± 126	810 ± 174[c]

[a] Cells were exposed to PAF (100 nM) or medium for the indicated time, washed, and then treated with LPS (10 μg/ml) for 4 h. They were then frozen and saved for PCA assay. The PCA of cells exposed to PAF alone (100 mM) for 4 h was 72 ± 14 mU/2 × 10^6 cells, $n = 8$.

[b] Mean ± SEM of seven to eight experiments, each done in duplicate.

[c] $P < 0.05$ versus LPS alone.

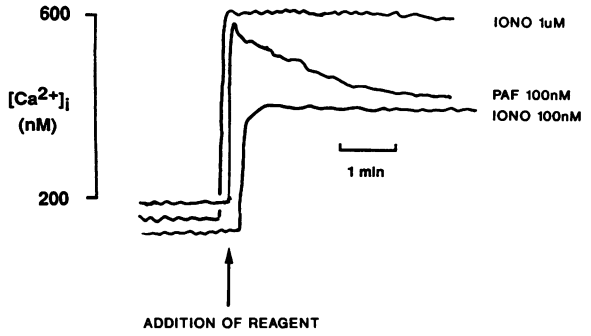

FIG. 2. Cytosolic calcium changes in response to PAF (100 nM) or ionomycin (IONO; 100 nM or 1 μM). The analog trace is representative of 12 similar experiments. Cytosolic calcium concentration is calculated as described in the Materials and Methods.

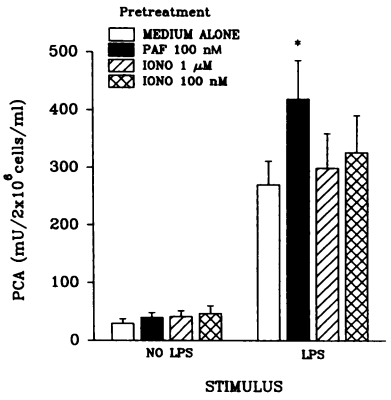

FIG. 3. Effect of ionomycin (IONO) on LPS-induced PCA. Cells were pretreated with ionomycin (100 nM or 1 μM) or PAF (100 nM) for 30 min at 37°C, washed, and then exposed to LPS for 4 h prior to assaying PCA. The data represent the mean and standard error of four experiments, each done in duplicate. *, $P < 0.05$ versus medium plus LPS.

trations to induce PCA, optimal priming was observed for *E. coli* at 10^4 to 10^5 CFU/ml, *B. fragilis* at 10^7 CFU/ml, and *S. aureus* at 10^6 CFU/ml.

The ability of PAF to prime human monocytes for LPS-stimulated PCA was also examined. As in peritoneal cells, PAF caused a significant augmentation of LPS-induced PCA ($P < 0.05$), without itself being stimulatory (medium, 8 ± 4 mU/10^6 cells; PAF alone, 13 ± 7 mU/10^6 cells; LPS, 2,026 ± 123 mU/10^6 cells; PAF plus LPS, 3,328 ± 653 mU/10^6 cells, $n = 3$ experiments, each performed in duplicate). All subsequent studies used murine peritoneal macrophages to investigate the mechanism of this phenomenon.

Time course of PAF priming. The duration of PAF exposure required to effect the priming response was examined (Table 1). Exposure of cells for as short as 1 min significantly enhanced PCA response to a subsequent 4-h incubation in LPS (10 μg/ml). Exposure of cells to PAF alone (100 nM) for 4 h did not induce PCA (72 ± 14 mU/2 × 10^6 cells, $n = 8$).

Role of calcium in PAF priming. The relatively brief period required for PAF to prime for LPS-induced PCA suggested that the effect was mediated via a rapid activation signal. PAF has been previously shown to stimulate a rapid rise in cytoplasmic calcium levels in macrophages (10, 20). As shown in Fig. 2, PAF (100 nM) induced a biphasic response in cytoplasmic calcium concentration characterized by an early peak to 494 ± 35 nM ($n = 12$) from a baseline of 196 ± 8 nM ($n = 12$); this was followed by a subsequent sustained, albeit slightly lower, plateau level (310 ± 31, $n = 12$). Studies performed in calcium-free EGTA-medium demonstrated that the early peak was predominantly derived from intracellular stores, while the sustained phase was dependent on extracellular calcium (data not shown).

To determine whether the PAF-induced rise in $[Ca^{2+}]_i$ was sufficient for its priming effect, we selected doses of the calcium ionophore ionomycin which were able to raise $[Ca^{2+}]_i$ to mimic the peak level (ionomycin, 1 μM; $[Ca^{2+}]_i$ = 638 ± 34 nM) and also the plateau level (ionomycin, 100 nM; $[Ca^{2+}]_i$ = 310 ± 31 nM; see Fig. 3). As demonstrated in Fig. 3, neither dose of ionomycin tested was able to reproduce the priming effect of PAF on LPS-induced PCA. These data support the notion that the PAF-induced rise in $[Ca^{2+}]_i$ was not solely responsible for its priming effect.

Previous studies have demonstrated that extracellular calcium is necessary for PCA induction by LPS (30). The effect of various extracellular calcium concentrations on LPS-induced PCA and on PAF priming was examined (Table 2). As previously shown, extracellular calcium was necessary for PCA production in macrophages. PCA was not produced by macrophages in the absence of extracellular calcium. Increasing extracellular calcium concentration caused a progressive increase in the amount of PCA induced by LPS and also in the ability of PAF to augment PCA production by LPS.

In the present studies, cells were frozen and thawed prior to assaying for PCA in order to disrupt the cell integrity, thereby revealing total cellular PCA. Without freeze-thawing, functional PCA activity was minimal. Recent studies have suggested that a rise in cytoplasmic calcium induced by A23187 could effect unmasking of functional PCA (4). To determine whether a physiological stimulus such as PAF

TABLE 2. Effect of extracellular calcium concentration on cytosolic calcium concentration and PCA production

Extracellular Ca^{2+}	Cytoplasmic calcium level (nM)[a]		PCA (mU/2 × 10^6 cells)[b]			
	Baseline	PAF stimulated	Medium	PAF	LPS	PAF-LPS
0	ND[c]	ND	4 ± 1	5 ± 1	3 ± 1	5 ± 1
200 nM	20 ± 18	20 ± 17	3 ± 1	3 ± 1	25 ± 11	36 ± 16
0.423 mM	236 ± 71	416 ± 180	24 ± 7	34 ± 9	189 ± 39	394 ± 85[d]
2.0 mM	282 ± 30	634 ± 100	155 ± 54	271 ± 127	526 ± 142	1,319 ± 204[d]

[a] $n = 4$, mean ± SEM.
[b] $n = 4$ to 6, mean ± SEM.
[c] ND, not determined.
[d] $P < 0.05$ versus LPS alone.

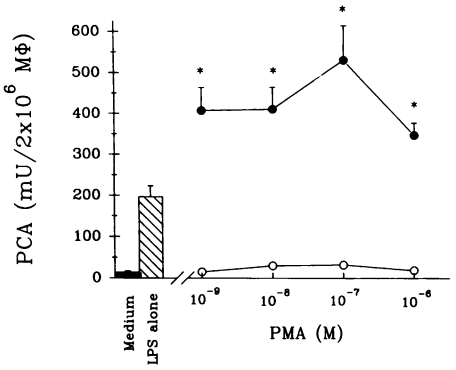

FIG. 4. Effect of PMA on LPS-stimulated PCA. Cells were pretreated with PMA (10^{-9} to 10^{-6} M) for 30 min at 37°C and then exposed to medium alone (○) or LPS (10 μg/ml) (●) for 4 h at 37°C, prior to freeze-thawing and assaying for PCA. The data represent the mean and standard error of six experiments, each done in duplicate. *, $P < 0.01$ versus LPS alone. MΦ, macrophages.

could mimic this effect, we assayed PCA in cells not exposed to freeze-thawing following exposure to PAF plus LPS. However, the PAF-induced rise in $[Ca^{2+}]_i$ was unable to mimic the previously observed effect of A23187 on LPS-induced PCA expression (PCA in undisrupted cells, 5 ± 1 mU/2 × 10^6; freeze-thawed cells, 579 ± 54 mU/2 × 10^6; n = 3 per group).

Role of PKC stimulation in PAF priming. In addition to its effect on $[Ca^{2+}]_i$, PAF has been shown to stimulate PKC via receptor-mediated stimulation of phosphatidylinositol hydrolysis with subsequent generation of the PKC agonist diacylglycerol (32). To investigate the hypothesis that PAF mediated its effect via stimulation of PKC, we examined the ability of the agonist PMA to augment LPS-induced PCA. Figure 4 demonstrates that a 30-min preexposure of cells to PMA at an optimal concentration of 100 nM was able to mimic the priming effect of PAF, while pretreatment of cells with dimethyl sulfoxide vehicle (0.1%) was without effect. Incubation of cells for 4 h in PMA alone (100 nM) also had no stimulatory effect on PCA (14 ± 3 mU/2 × 10^6 cells versus 18 ± 6 mU/2 × 10^6 cells, n = 6, medium versus PMA, respectively). In further support of the concept that PAF priming was mediated via PKC stimulation, the specific PKC antagonist staurosporine (10 nM) was shown to completely reverse the PAF effect (Fig. 5).

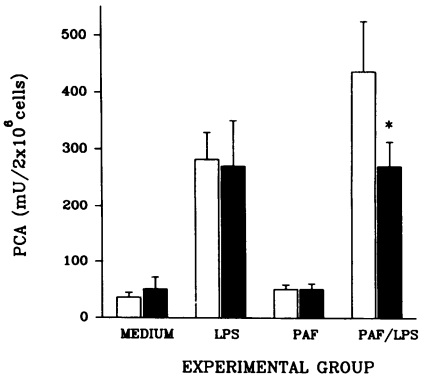

FIG. 5. Effect of the PKC inhibitor staurosporine on PAF priming of LPS-induced PCA. All groups were exposed to staurosporine (10 nM) or vehicle for 5 min prior to treatment. Staurosporine reverses PAF priming but has no effect on LPS-induced PCA. The data represent the mean and standard error of six experiments, each performed in duplicate. *, $P < 0.05$ versus PAF/LPS. □, no staurosporine; ■, 10 nM staurosporine.

Effect of indomethacin on macrophage PCA production. Both LPS and PAF are known to enhance synthesis of prostaglandin E_2 by macrophages (16, 21). Since the exogenous addition of prostaglandin E_2 has been demonstrated to impair PCA induction by a various stimuli, such as mouse hepatitis virus strain 3 (1), we hypothesized that inhibition of cyclooxygenase activity might unmask endogenous inhibition of PCA production by both LPS and PAF. However, indomethacin (10 μg/ml) had no effect on basal PCA production, LPS-stimulated PCA, or the ability of PAF to augment LPS-stimulated PCA. This concentration of indomethacin completely inhibited prostaglandin E_2 generation (data not shown).

DISCUSSION

Local fibrin deposition at sites of infection and inflammation is a consistent histological finding and appears to contribute to the pathogenesis of the underlying disease process. Defibrinogenation with ancrod has been shown experimentally to prevent intra-abdominal abscess formation (26), to lessen the severity of glomerulonephritis (8), and to abrogate the magnitude of lung injury following infusion of oleic acid (2). Similarly, the induration associated with delayed-type hypersensitivity reactions is absent in congenitally afibrinogenemic individuals and in patients treated with warfarin (9, 13). Several mechanisms may contribute to fibrin deposition at these inflammatory sites. Increased vascular permeability may expose contact factors of plasma to activators, such as collagen, in the extravascular space. In addition, cells of monocyte-macrophage lineage are known to express PCA on their cell surface in response to a variety of inflammatory stimuli, including endotoxin (24), immune complexes (34), and proteolytic products of the complement cascade (28), as well as several microbial pathogens (12, 35, 39, 43).

Within the inflammatory microenvironment, a wide range of mediator molecules may influence macrophage PCA induction. Lymphocyte-derived factors, such as macrophage PCA-inducing factor (14) and gamma interferon (27), have been shown to either directly stimulate or enhance PCA, while interleukin 4 downregulates it (41). By contrast, the macrophage products tumor necrosis factor and interleukin 1 are without effect on macrophage PCA, either alone or in combination with endotoxin (27). Our previous studies using the lipid inflammatory mediator PAF demonstrated that PAF was able to augment PCA response to LPS without itself having a stimulatory effect (12). This effect was specific for PAF in that it was inhibited by the PAF antagonist WEB 2086. The present studies were designed to further characterize this effect and investigate the underlying mechanisms.

The priming effect of PAF occurred following its exposure to cells for as little as 1 min, suggesting a rapid activation pathway for this phenomenon. Two PAF-induced events considered to be good candidates for this effect were the rise in cytoplasmic calcium and the phosphatidylinositol turnover with resultant stimulation of PKC activity (10, 32). The failure of ionomycin to mimic the effect of PAF, despite its ability to elevate $[Ca^{2+}]_i$ to comparable levels, argues against the hypothesis that the PAF-induced calcium rise was responsible for priming. By contrast, several lines of

evidence suggest that activation of PKC, independent of calcium changes, mediated this effect. First, exposure of cells to the PKC agonist PMA was able to reproduce priming of cells for LPS-induced PCA. This effect was not due to the dimethyl sulfoxide vehicle, since dimethyl sulfoxide alone was inactive. Second, inhibition of PKC activation using the specific antagonist staurosporine completely reversed the PAF priming effect. Finally, PMA was able to mimic PAF's effect without elevating $[Ca^{2+}]_i$, while staurosporine reversed PAF priming without inhibiting the PAF-induced calcium rise (data not shown). When considered together, these data indicate that the ability of PAF to prime cells for LPS-induced PCA is due to PKC activation and that a rise in cytosolic calcium induced by PAF is neither sufficient nor necessary for this effect. This mode of priming contrasts that observed for neutrophil-mediated endothelial damage, in which the PAF-induced rise in $[Ca^{2+}]_i$ has been shown to be responsible for the effect (45). Recent studies have shown that PAF stimulates arachidonic acid metabolism in Kupffer cells via PKC activation (7). The failure of indomethacin to block PAF priming suggests that cyclooxygenase products of arachidonic acid metabolism were not responsible for the effect. However, the possible contribution of various proinflammatory leukotrienes has not been evaluated. The exact mechanism by which PKC mediates the effect of PAF on PCA induction at the molecular level requires further investigation.

The macrophages used in the present studies were thioglycolate elicited and therefore do not represent a population of resting cells. However, the ability of PAF to effect priming for PCA induction in both human monocytes as well as rabbit alveolar macrophages (25a) suggests that the phenomenon is not dependent on the activational state of the monocyte-macrophage population studied.

LPS is a well-defined stimulus of several macrophage products. Previous studies have examined the signal transduction mechanisms involved in the induction of some of these mediator molecules. Expression of interleukin 1 gene products and biologic activities is mediated via stimulation of both PKC and calmodulin-dependent kinase (19), while tumor necrosis factor appears to be induced solely through the stimulation of PKC (5, 19). By contrast, the present studies demonstrate that LPS-induced PCA is not prevented by treatment with the PKC antagonist staurosporine. In addition, two different stimuli of PKC, PMA and PAF, were unable to stimulate PCA over the same time course that LPS induced PCA. When considered together, these data suggest that stimulation of PCA by LPS occurs via a PKC-independent mechanism in thioglycolate-elicited peritoneal macrophages.

The failure of stimuli of PKC to induce PCA in the present studies differs from findings reported by other investigators. Lyberg and Prydz (25, 33) demonstrated that PMA was able to stimulate PCA in adherent human monocytes. In addition, Janco and Morris (18) reported that both PMA and the chemoattractant formylmethionylleucylphenylalanine induced PCA in a mixed mononuclear cell population. The time course of PCA induction with these stimuli was delayed compared with that examined in the present studies. One further difference between these reports and the present studies may relate to the cell population being studied. Thioglycolate-elicited macrophages are contaminated by ~10% lymphocytes, while mononuclear cell populations consist of ~70% lymphocytes. Lymphocytes are known to augment the induction of macrophage PCA by several stimuli, including formylmethionylleucylphenylalanine (18, 22). This phenomenon may also be true for PMA-stimulated PCA. Similarly, the adherent monocyte population studied by Lyberg and Prydz (25, 33) may have been primed for subsequent stimulation by PMA during adhesion to the plastic culture dishes (44).

The clinical significance of the observations reported in this manuscript remains undefined. However, the ability of PAF to augment macrophage-mediated fibrin deposition in response to both LPS and several bacterial pathogens suggests a further mechanism by which PAF might amplify the local inflammatory response. The use of PAF antagonists, such as WEB 2086, as a means to lessen this effect might potentially represent an adjuvant form of therapy in the management of various infectious and inflammatory disease processes, whose pathogenesis appears to be associated with macrophage-mediated fibrin deposition.

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REFERENCES

1. **Abecassis, M., J. A. Falk, L. Makowka, V. J. Dindzans, R. E. Falk, and G. Levy.** 1987. 16,16 Dimethyl prostaglandin E2 prevents the development of fulminant hepatitis and blocks the induction of monocyte/macrophage procoagulant activity after murine hepatitis virus strain 3 infection. J. Clin. Invest. **80:**881–889.
2. **Allard, M. F., C. M. Doerschuk, M. L. Brumwell, A. Belzberg, and J. C. Hogg.** 1988. Oleic acid-induced lung injury in rabbits: effect of fibrinogen depletion with arvin. J. Appl. Physiol. **64:**920–928.
3. **Almdahl, S. M., and B. Osterud.** 1986. Experimental gram-negative peritonitis: decreased thromboplastin activity in organs with a simultaneous rise of thromboplastin in blood monocytes and peritoneal macrophages. Res. Exp. Med. **186:**317–324.
4. **Bach, R., and D. B. Rifkin.** 1990. Expression of tissue factor procoagulant activity: regulation by cytosolic calcium. Proc. Natl. Acad. Sci. USA **87:**6995–6999.
5. **Bankey, P., B. A. Carlson, M. Ortiz, R. Singh, and F. Cerra.** 1990. Tumor necrosis factor production by Kupffer cells requires protein kinase C activation. J. Surg. Res. **49:**256–261.
6. **Chang, S. W., S. Fernyak, and N. F. Voelkel.** 1990. Beneficial effect of a platelet-activating factor antagonist, WEB 2086, on endotoxin-induced lung injury. Am. J. Physiol. **258:**H153–H158.
7. **Chao, W., H. Liu, D. J. Hanahan, and M. S. Olson.** 1990. Regulation of platelet-activating factor receptor and PAF receptor-mediated arachidonic acid release by protein kinase C activation in rat Kupffer cells. Arch. Biochem. Biophys. **282:** 188–197.
8. **Cole, E. H., M. F. X. Glynn, C. A. Laskin, J. Sweet, N. Mason, and G. A. Levy.** 1990. Ancrod improves survival in murine systemic lupus erythematosus. Kidney Int. **37:**29–35.
9. **Colvin, R. B., M. W. Mosesson, and H. F. Dvorak.** 1979. Delayed-type hypersensitivity skin reactions in congenital afibrinogenemia lack fibrin deposition and induration. J. Clin. Invest. **63:**1302–1306.
10. **Conrad, G. W., and T. J. Rink.** 1986. Platelet activating factor raises intracellular calcium ion concentration in macrophages. J. Cell Biol. **103:**439–450.
11. **Dindzans, V. J., E. Skamene, and G. A. Levy.** 1986. Susceptibility/resistance to mouse hepatitis virus 3 and macrophage procoagulant activity are genetically linked and controlled by two non-H2 linked genes. J. Immunol. **137:**2355–2360.
12. **Drake, T. A., G. M. Rodgers, and M. A. Sande.** 1984. Tissue factor is a major stimulus for vegetation formation in enterococcal endocarditis in rabbits. J. Clin. Invest. **73:**1750–1753.
13. **Edwards, R. L., and F. R. Rickles.** 1978. Delayed hypersensi-

tivity in man: effects of systemic anticoagulation. Science **200:**541–543.
14. **Gregory, S. A., R. S. Kornbluth, H. Helin, H. G. Remold, and T. S. Edgington.** 1986. Monocyte procoagulant inducing factor: a lymphokine involved in the T cell-instructed monocyte procoagulant response to antigen. J. Immunol. **137:**3231–3239.
15. **Grynkiewicz, G., M. Poenie, and R. Y. Tsien.** 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. **260:**3440–3450.
16. **Hartung, H. P.** 1983. Acetyl glyceryl ether phosphorylcholine (platelet-activating factor) mediates heightened metabolic activity in macrophages. FEBS Lett. **160:**209–212.
17. **Hsueh, W., F. Gonzalez-Crussi, and J. L. Arroyave.** 1987. Platelet activating factor: an endogenous mediator for bowel necrosis in endotoxemia. FASEB J. **1:**403–405.
18. **Janco, R. L., and P. J. Morris.** 1985. Regulation of monocyte procoagulant by chemoattractants. Blood **65:**545–552.
19. **Kovacs, E. J., D. Radzioch, H. A. Young, and L. Varesio.** 1988. Differential inhibition of IL-1 and TNF mRNA expression by agents which block second messenger pathways in murine macrophages. J. Immunol. **141:**3101–3105.
20. **Kucey, D. S., E. I. Kubicki, and O. D. Rotstein.** 1991. Platelet-activating factor primes endotoxin-stimulated macrophage procoagulant activity. J. Surg. Res. **50:**436–441.
21. **Kurland, J. I., and R. Bockman.** 1978. Prostaglandin E production by human blood monocytes and mouse peritoneal macrophages. J. Exp. Med. **147:**952–956.
22. **Levy, G. A., and T. S. Edgington.** 1980. Lymphocyte cooperation is required for amplification of macrophage procoagulant activity. J. Exp. Med. **151:**1232–1244.
23. **Levy, G. A., J. L. Leibowitz, and T. S. Edgington.** 1981. Induction of monocyte procoagulant activity by murine hepatitis virus strain 3 parallels disease susceptibility in mice. J. Exp. Med. **154:**1150–1163.
24. **Levy, G. A., B. S. Schwartz, and T. S. Edgington.** 1981. The kinetics and metabolic requirements for direct lymphocyte induction of human procoagulant monokines by bacterial lipopolysaccharide. J. Immunol. **127:**357–363.
25. **Lyberg, T., and H. Prydz.** 1981. Phorbol esters induce synthesis of thromboplastin activity in human monocytes. Biochem. J. **194:**699–706.
25a. **Maier, R. V., G. B. Hahnel, and J. D. Pagan.** 1990. Abstr. Annu. Meet. Surg. Infect. Soc. 1990, p. 50.
26. **McRitchie, D. I., M. J. Girotti, M. F. X. Glynn, J. M. Goldberg, and O. D. Rotstein.** 1991. Effect of systemic fibrinogen depletion on intraabdominal abscess formation. J. Lab. Clin. Med. **118:**48–55.
27. **Moon, D. K., and C. L. Geczy.** 1988. Recombinant IFN-γ synergizes with lipopolysaccharide to induce macrophage membrane procoagulants. J. Immunol. **141:**1536–1542.
28. **Muhlfelder, T. W., J. Niemetz, D. Kreutzer, D. Deebe, P. A. Ward, and S. I. Rosenfeld.** 1979. C5a chemotactic fragment induces leukocyte production of tissue factor activity. J. Clin. Invest. **63:**147–150.
29. **Nathan, C. F.** 1987. Secretory products of macrophages. J. Clin. Invest. **79:**319–326.
30. **Osterud, B., and T. Eskeland.** 1982. The mandatory role of complement in the endotoxin-induced synthesis of tissue thromboplastin in blood monocytes. FEBS Lett. **149:**75–79.
31. **Pinkard, R. N., J. C. Ludwig, and L. M. McManus.** 1988. Platelet activating factors, p. 139–168. *In* J. I. Gallin, I. M. Goldstein, and R. Snyderman (ed.), Inflammation: basic principles and clinical correlates. Raven Press, New York.
32. **Prpic, V., R. J. Uhing, J. E. Weiel, L. Jakoi, F. Gawdi, B. Herman, and D. O. Adams.** 1988. Biochemical and functional responses stimulated by platelet-activating factor in murine peritoneal macrophages. J. Cell Biol. **107:**363–372.
33. **Prydz, H., and T. Lyberg.** 1980. Effect of some drugs on thromboplastin (factor III) activity of human monocytes in vitro. Biochem. Pharmacol. **29:**9–14.
34. **Prydz, H., T. Lyberg, P. Deteix, and A. C. Allison.** 1979. In vitro stimulation of tissue thromboplastin (factor III) activity in human monocytes by immune complexes and lectins. Thromb. Res. **15:**465–474.
35. **Rosenthal, G. A., G. Levy, and O. D. Rotstein.** 1989. Induction of macrophage procoagulant activity by *Bacteroides fragilis*. Infect. Immun. **57:**338–343.
36. **Rotstein, O. D., and J. Kao.** 1988. Fibrinolysis using recombinant tissue plasminogen activator prevents intraabdominal abscesses. J. Infect. Dis. **158:**766–772.
37. **Rotstein, O. D., T. L. Pruett, and R. L. Simmons.** 1986. Fibrin in peritonitis. V. Fibrin inhibits phagocytic killing of *Escherichia coli* by human polymorphonuclear leukocytes. Ann. Surg. **203:**413–419.
38. **Senior, R. M., W. F. Skogen, G. L. Griffin, and G. D. Wilner.** 1986. Effects of fibrinogen derivatives upon the inflammatory response. Studies with human fibrinopeptide B. J. Clin. Invest. **77:**1014–1019.
39. **Sinclair, S. B., O. D. Rotstein, and G. A. Levy.** 1990. Disparate mechanisms of induction of procoagulant activity by live and inactivated bacteria and viruses. Infect. Immun. **58:**1821–1827.
40. **Stecher, V. J., and E. Sorkin.** 1972. The chemotactic activity of fibrin lysis products. Int. Arch. Allergy **43:**879–886.
41. **Szabo, G., and C. L. Miller-Graziano.** 1990. Induction and regulation of monocyte procoagulant activity. Transplantation **50:**301–309.
42. **Tamoaki, T., H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto, and F. Tomita.** 1986. Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase. Biochem. Biophys. Res. Commun. **135:**397–402.
43. **Van Ginkel, C. J. W., L. Thorig, J. Thompson, J. I. H. Oh, and W. G. Van Aken.** 1979. Enhancement of generation of monocyte tissue thromboplastin by bacterial phagocytosis: possible pathway for fibrin formation on infected vegetations in bacterial endocarditis. Infect. Immun. **25:**388–395.
44. **Van Ginkel, C. J. W., W. G. Van Aken, J. I. H. Oh, and J. Vreeken.** 1977. Stimulation of monocyte procoagulant activity by adherence to different surfaces. Br. J. Haematol. **37:**35–45.
45. **Vercellotti, G. M., H. Q. Yin, K. S. Gustafson, R. D. Nelson, and H. S. Jacob.** 1988. Platelet activating factor primes neutrophil responses to agonists: role in promoting neutrophil-mediated endothelial damage. Blood **71:**1100–1107.