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Synthesis of platelet-activating factor by human monocytes stimulated by platelet-activating factor

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The capacity of platelet-activating factor (PAF) to stimulate its own synthesis by human monocytes was examined. Adherent human monocytes of >85% purity were incubated with 100 fM to 10 nM of PAF in the presence of 20 µCi of [³H]acetic acid to radiolabel newly synthesized PAF. After incubation for 15 minutes to 15 hours, PAF was purified by high-performance liquid chromatography, and newly synthesized PAF was quantified by its radioactivity. PAF stimulated its own synthesis in a dose-related manner with a maximal twofold to threefold increase in synthesis at 10 pM to 100 pM of PAF. Maximal PAF synthesis occurred after incubation for 6 to 8 hours. There was a good correlation (r = 0.95) between PAF quantified by [³H]acetic acid incorporation and by rabbit platelet aggregation bioassay, indicating that the radioactive material is PAF. The protein synthesis inhibitor, cycloheximide, did not inhibit delayed PAF synthesis, indicating that delayed PAF synthesis does not require protein synthesis. PAF is metabolized rapidly in vivo. The capacity of PAF to stimulate its own synthesis would result in a prolonged effective half-life in vivo. This prolonged half-life could contribute to the capacity of PAF to induce prolonged inflammatory reactions in vivo. (J ALLERGY CLIN IMMUNOL 1991;87:715-20.)

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PAF is a structurally novel mediator of acute and subacute immunologic reactions. Administration of PAF induces delayed or persistent reactions in animals and humans.^{1,2} Intradermal injection of PAF in humans induces an acute wheal-and-flare reaction that resolves and is followed 3 to 6 hours later by an erythematous, indurated skin lesion.¹ Inhalation of PAF by humans induces acute bronchospasm that resolves within 1 hour and is followed by airway hypersensitivity to methacholine that may persist for weeks.² PAF is degraded rapidly by specific serum and cellular acetylhydrolases; thus, the half-life of PAF in vivo is only a few minutes.³ Given the short half-life of PAF, it is possible that prolonged biological effects of PAF result from production of secondary mediators induced by acute PAF administration. Several recent studies support this possibility. PAF stimulates IL-1 synthesis by human monocytes, the monocytic cell line THP-1, and rat splenic macrophages.⁴⁻⁷ PAF also stimulates TNF synthesis by human monocytes and alveolar macrophages.⁸⁻¹¹ PAF is a weak stimulus for IL-1 and TNF synthesis but has a much larger effect when it is combined with other stimuli, such as endotoxin or IFN- γ .⁴⁻¹¹ Another possible mechanism for inducing prolonged biologic effects is the stimulation of PAF synthesis by PAF itself. Autocrine stimulation of its own synthesis would result in an effective half-life that is much longer than the half-life of a few minutes that was calculated from the metabolism of exogenous, radiolabeled PAF. A recent publication demonstrates that PAF stimulates neutrophils to synthesize PAF.¹² We now present data demonstrating that PAF also stimulates human monocytes to synthesize PAF.

MATERIAL AND METHODS

The following materials were obtained as noted: PAF(1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine, Bachem Co., Torrance, Calif.), [³H]PAF (1-O-[1',2'-³H]alkyl-, 51 Ci/mmol) and [³H]acetic acid (80 Ci/mmol) (New England Nuclear, Boston, Mass.), 5 μ m of Ultrasphere HPLC column (4.6 mm by 15 cm, Beckman Instruments, Palo Alto, Calif.), HPLC grade solvents and culture plasticware (Fisher Scientific Co., Santa Clara, Calif.), cell-culture media (Applied Scientific, San Francisco, Calif.), and low endotoxin fetal bovine serum (Hyclone Laboratories, Logan, Utah). BN 52021 was a generous gift of Dr. Pierre Braquet (Institut Henri Beaufour, Paris, France). All other reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Stimulation of PAF synthesis

Newly synthesized PAF was quantified by incorporation of [³H]acetic acid into the sn-2 position.¹³ Human monocytes

Abbreviations used

IFN- γ :	Interferon- γ
IL-1:	Interleukin-1
TNF:	Tumor necrosis factor
PAF:	Platelet-activating factor, 1-o-alkyl-2-acetyl-sn-glycero-3-phosphocholine
HPLC:	High-performance liquid chromatography

were prepared by a modification of previous methods¹⁴ that yielded 10⁶ adherent monocytes of >85% purity in each well of 24-well plates. The adherent monocytes in each well were covered with 1 ml of RPMI 1640 containing 0.25 gm/dl of endotoxin-free bovine serum albumin (Boehringer Mannheim, Indianapolis, Ind.). Twenty microcuries of [³H]acetic acid in 5 μ l of RPMI 1640 were added to each well, and the plates were incubated at 37° C in a humidified atmosphere of 5% CO₂ per 95% air. After 30 minutes, 10 μ l of PAF suspended in RPMI 1640 containing 0.25 gm/dl of endotoxin-free bovine serum albumin or 10 μ l of buffer were added to replicate wells. The plates were then incubated for 5 minutes to 15 hours at 37° C in a humidified atmosphere of 5% CO₂ per 95% air. Cell viability was assessed by trypan blue dye exclusion and was >90% at all time points. At the end of the incubation, the culture fluids were removed, and each milliliter of fluid was mixed immediately with 2.2 ml of chloroform:methanol (1:1, vol/vol). Immediately after the culture fluids were removed, 0.5 ml of methanol was added to each well, the adherent cell ghosts were detached by scraping, and the methanol extract was transferred to glass tubes. Each well was rinsed with 0.5 ml of methanol, and the rinse fluid was added to the original extract. Sufficient chloroform, methanol, and water were added to the cell extracts to achieve final concentrations of 1.1, 1.1, and 1.0 ml, respectively. This process recovers >95% of [³H]PAF added to monocyte cultures (n = 3). Fifty micromoles of unlabeled PAF was added to each extraction mixture, after which the fluid and cell extracts were further extracted with chloroform:methanol as described.¹⁴

Quantitation of PAF synthesis

Each supernatant and cell extract was dried in a centrifugal evaporator and resuspended in 200 μ l of methanol that contained 5 μ g each of phosphatidylcholine and lysophosphatidylcholine as internal standards for elution times from HPLC. Twenty microliters of each sample was saved to quantify total radioactivity extracted. The remaining material was injected onto an Ultrasphere HPLC column that was developed with a linear gradient from 95% solvent A:4% solvent B to 92% solvent A:8% solvent B during 25 minutes at a flow rate of 1.5 ml/min.^{14,15} Solvent A was hexane:isopropanol:ammonium hydroxide (1:1:0.0005, vol/vol), and solvent B was water containing 0.5% (vol/vol) of ammonium hydroxide. The optical absorbance

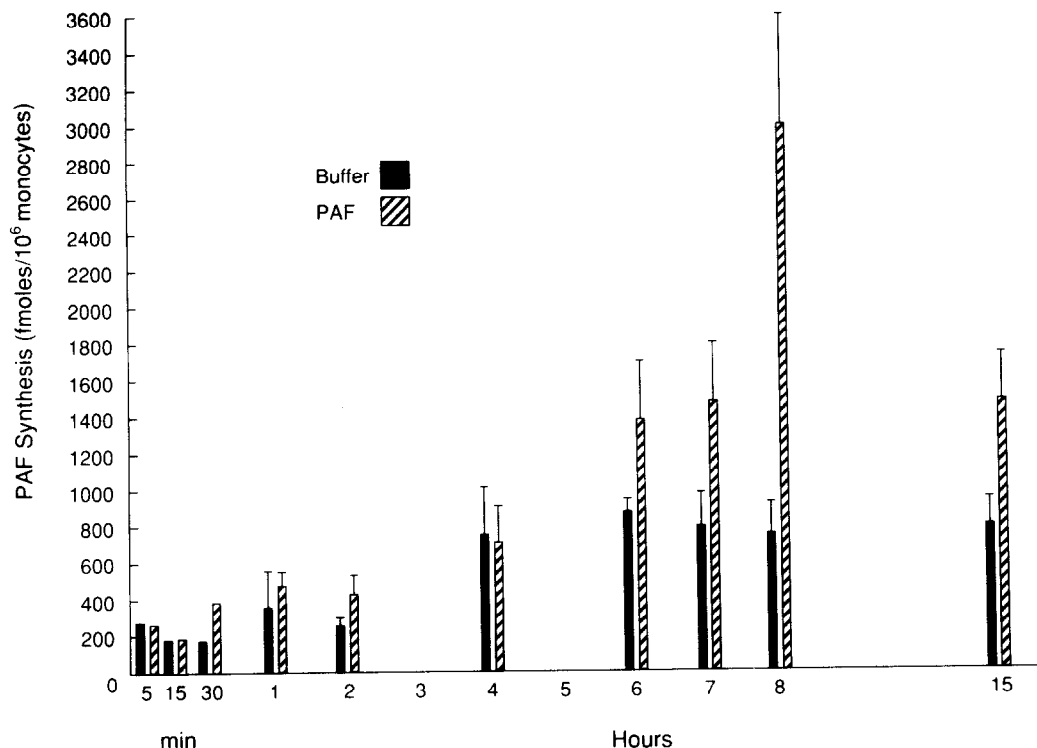


FIG. 1. Time course of synthesis of PAF by monocytes stimulated with PAF. Monocytes were incubated with 10 pM of PAF or buffer for the indicated times, after which PAF released into culture fluids and cell-associated PAF were quantified. These data are the mean $\pm$ SD total femtomoles of PAF per 10^6 adherent monocytes recovered from culture fluids plus cell extracts; $n = 4$ for 5, 15, and 30 minutes; $n = 8$ for 1 to 15 hours.

of the column eluate at 206 nm was monitored and recorded continuously. The eluate was collected in 30-second (0.75 ml) fractions that were mixed with 2 ml of Scintiverse (Fisher Scientific Co.), and the radioactivity was quantified by liquid scintillation counting. The efficiency of counting [^3H]PAF was $55\% \pm 4\%$ (mean $\pm$ SD; $n = 3$). On 16 consecutive days, the elution times of the purified standards were dipalmitoylphosphatidylcholine, 15.9 ± 0.6 min; [^3H]PAF, 22.8 ± 0.8 min; and lysophosphatidylcholine, 24.8 ± 0.6 min (mean $\pm$ SD). There were no differences in elution times between purified standards and standards mixed with monocyte extracts. The recovery of the [^3H]PAF standard from the column was $86\% \pm 3\%$ (mean $\pm$ SD; $n = 16$). PAF was quantified based on the specific activity of [^3H]acetic acid added to the monocyte cultures.

In some experiments, PAF was quantified by both incorporation of [^3H]acetic acid and by bioassay. In those experiments, unlabeled PAF was not added during the extraction procedure. The HPLC column eluates were divided in half and one half was used to quantify [^3H]acetic acid incorporation. The saved portions of the fractions that contained the peak of radioactivity were pooled, dried in a centrifugal evaporator, resuspended in water, lyophilized twice, and then resuspended in 0.2 ml of phosphate-buffered saline, pH 7.25, containing 0.1 gm/dl of human serum

albumin. PAF was then quantified by bioassay with rabbit platelet aggregation.¹⁴

RESULTS

Time course of PAF synthesis

Initial studies examined the capacity of 10 pM of PAF to stimulate monocytes to synthesize PAF during incubation for 5 minutes to 15 hours. PAF was equally distributed between culture fluids and cells at all times with the culture fluids containing $51.3\% \pm 19.1\%$ of the total amount of PAF recovered (mean $\pm$ SD; $n = 24$). The total quantity of PAF recovered from culture fluids plus cells after incubation for the different times is illustrated in Fig. 1. Adherent monocytes spontaneously produced small quantities of PAF with maximal synthesis occurring after incubation for 4 hours. PAF synthesis by monocytes incubated with 10 pM of PAF or vehicle were the same for the first 4 hours of incubation. After incubation for 5 to 15 hours, PAF stimulated PAF synthesis to levels two to three times the levels observed for control monocytes incubated with buffer ($p < 0.03$, Student's paired t test; $n = 25$). The quantity of PAF recovered at the

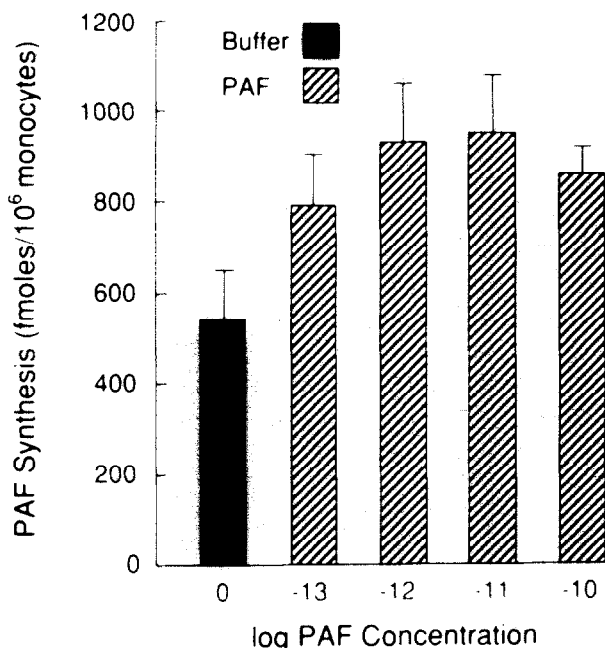


FIG. 2. Concentration dependence of the synthesis of PAF by monocytes stimulated by PAF. These data are from two experiments and are the mean femtomoles of PAF per 10^6 monocytes recovered from supernatants plus cells after incubation for 7 hours; $n = 3$ to 6.

later times was 50 to 100 times the quantity of PAF used as a stimulus, indicating that net PAF synthesis had occurred.

Dose dependence of PAF synthesis

The capacity of 100 fM to 10 nM of PAF to stimulate synthesis of PAF by monocytes was determined in two experiments (Fig. 2). PAF stimulated its own synthesis over a broad concentration range with maximal stimulation at 10 pM of PAF. The increase was statistically significant for 1 to 100 pM of PAF ($p < 0.05$, Student's paired t test).

Contribution of protein synthesis

Cytokines, such as IL-1, TNF, and IFN- γ , stimulate delayed synthesis of PAF by monocytes and endothelial cells, and the delayed synthesis requires protein synthesis.^{14, 16} To determine if protein synthesis is also required for delayed synthesis of PAF induced by PAF itself, monocytes were stimulated with 10 pM of PAF in the presence or absence of 10 μ g/ml of the protein synthesis inhibitor, cycloheximide. In two experiments, mean synthesis of PAF by 10^6 monocytes incubated with different combinations of stimuli and inhibitor for 7 hours was buffer, 880 fmol; 10 pM of PAF, 1500 fmol; and PAF plus cycloheximide, 2360 fmol. To confirm that cycloheximide inhibited protein

TABLE I. Quantitation of PAF synthesis by bioassay and incorporation of [^{3} H]acetic acid

Stimulus*	PAF synthesis (fmol/ 10^6 monocytes)	
	Bioassay	Radioassay
Buffer	40	220
PAF (pM)		
1	70	400
10	286	920
100	168	100

*Monocytes were incubated with the indicated stimuli for 7 hours. Total PAF in the cultures was extracted, after which the extracts were divided into two equal portions for quantitation of PAF by bioassay and radioassay. These data are the mean of replicate determinations.

synthesis by the monocytes, cell-associated IL-1- β was quantified by protein immunoblotting after monocytes were incubated for 4 hours with buffer or 10 pM of PAF without or with 10 μ g/ml of cycloheximide.⁶ PAF stimulated a twofold increase in IL-1 protein that was completely inhibited by cycloheximide.

Correlation of PAF quantified by bioassay and radioactivity

Seventeen samples (nine pellets and eight supernatants) were analyzed for PAF by both radioactivity and bioassay. Each sample contained platelet-aggregating material that eluted with retention times identical to the PAF standards. The specific PAF antagonists, BN 52021 (1 μ mol/L) and kadsurenone (4 μ mol/L), completely inhibited platelet aggregation by the samples, indicating that the aggregating material is PAF. There was a good correlation between the quantity of PAF determined by bioassay and radioassay ($r = 0.95$). The bioassay confirmed that PAF stimulates its own synthesis by monocytes (Table I).

DISCUSSION

These experiments demonstrate that PAF stimulates human monocytes to synthesize and release PAF. Two observations indicate that the material quantified as PAF by [^{3} H]acetic acid incorporation is indeed PAF. The material coelutes with PAF on HPLC and induces platelet aggregation that can be inhibited completely by selective PAF antagonists. There can be difficulty distinguishing stimulus and product in our studies. [^{3} H]acetic acid could exchange with the acetyl side chain of PAF used as a stimulus. The resulting

[^3H]PAF would then be considered newly synthesized PAF. Also, the bioassay cannot distinguish PAF, the stimulus, from PAF, the product. These two considerations cannot, however, account for most of the PAF identified in the monocyte cultures because the amount of PAF recovered was 50 to 100 times more than the stimulus. Thus, the bulk of the PAF identified in the monocyte cultures represents newly synthesized PAF.

Monocytes synthesize PAF spontaneously with maximal synthesis after 4 hours of incubation, perhaps because of adherence to plastic dishes. Stimulation of PAF synthesis by PAF follows a similar time course, and there is no net synthesis until 6 to 8 hours of incubation. Cytokines, such as IL-1 and TNF stimulate delayed synthesis of PAF by monocytes and endothelial cells.^{14, 16} In those cases, however, the delay results, at least in part, from the requirement for protein synthesis before delayed synthesis of PAF occurs. Proteins that might be involved in delayed PAF synthesis include enzymes in the biosynthetic pathway of PAF¹⁶ and protein cytokines, especially TNF.¹⁴ Delayed PAF synthesis induced by PAF does not require protein synthesis, however. Therefore, the mechanisms for enhanced synthesis are not known in this case.

PAF synthesis occurs by two distinct pathways: the remodeling pathway and the de novo pathway.^{18, 19} On the remodeling pathway, a phospholipase A2 cleaves the SN-2 ester bond of the PAF precursor, 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine, to yield lyso-PAF, which is then acetylated by an acetyltransferase to yield PAF. In the de novo pathway, the final step yielding PAF is the incorporation of phosphocholine into 1-O-alkyl-2-acetyl-sn-glycerol through the action of a DTT-insensitive CDP-cholinephosphotransferase. Quantitation of PAF by incorporation of [^3H]acetic acid into lyso-PAF only detects synthesis via the remodeling pathway. The good correlation between PAF measured by radioassay and bioassay suggests, however, that very little PAF is synthesized via the de novo pathway. The origin of lyso-PAF used by monocytes for PAF synthesis is somewhat ambiguous, since the albumin used in our cell cultures may contain substantial quantities of lyso-PAF. Whether this lyso-PAF contributes to PAF synthesis is unclear. Our experiments used low endotoxin albumin preparations to minimize the effects of endotoxin on PAF synthesis. These albumin preparations are not designed specifically to be fatty-acid free but should have low fatty-acid content.

The capacity of PAF to stimulate its own synthesis has been noted before in experiments with neutrophils.¹² The quantities of PAF produced by neutrophils stimulated by PAF are substantially more than the

quantities of PAF produced by monocytes in our studies. Regardless, the quantities of PAF produced by monocytes may be biologically relevant. The concentration of PAF in the monocyte cultures was approximately 500 pM to 2 nM (500 to 2000 fmol/ml). These concentrations of PAF are sufficient to stimulate monocyte cytotoxicity and synthesis of cytokines¹⁹ and to activate other cells, such as platelets. It appears to be a general property of immunologic mediators that they can stimulate their own synthesis. For example, IL-1 and TNF both stimulate their own synthesis.^{20, 21} These observations suggest that PAF, TNF, and IL-1 are autocoids whose synthesis is required for monocyte activation. Additional experiments are required to evaluate this possibility.

There are several possible mechanisms by which PAF can induce delayed or persistent inflammatory reactions. PAF stimulates delayed release of cytokines and itself. This delayed release of inflammatory mediators would be expected to produce prolonged inflammatory reactions. Not only does PAF stimulate synthesis of cytokines and itself, but cytokines also stimulate synthesis of PAF and themselves. These observations establish positive feedback loops in the synthesis PAF and cytokines by monocytes. These loops could be important to the amplification of the immune response, but they could, if they escape local regulation, lead to uncontrolled, sustained release of these potent factors and thereby result in diseases of inflammation.

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