Vol. 128, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1985 Pages 297-304

ACTIVATION OF NADPH OXIDASE IN HUMAN NEUTROPHILS. SYNERGISM BETWEEN fMLP AND THE NEUTROPHIL PRODUCTS PAF AND LTB_4

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Received February 27, 1985

The induction of the respiratory burst in human neutrophils by combinations of fMLP and either PAF or LTB4 was studied. Pretreatment with PAF (0.0001 to 10 uM), which by itself did not elicit the burst, greatly enhanced the rate and extent of fMLP-induced superoxide production. A synergism of a different kind was observed with the reversed stimulus sequence: Pretreatment with fMLP made the neutrophils capable to respond to PAF with superoxide production. A moderate enhancement of the fMLP response was also obtained following pretreatment with LTB4. The response of the cells to LTB4, however, was not influenced by fMLP, and no synergism was observed between the two neutrophil products PAF and LTB4.

The results of this study demonstrate a marked synergism between fMLP and PAF and suggest that PAF may function as an amplifier of the respiratory burst response of stimulated neutrophils.

Among the products of stimulated neutrophils, two are of particular interest because of their ability to act as stimuli on the cells from which they are released. They are platelet-activating factor, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine (PAF) (1-3), and leukotriene B_4 (LTB_4) (4, 5). Experiments in vitro have shown that chemotactic and phagocytic stimuli induce the release of PAF and LTB_4 from human neutrophils (6-10). In vivo, at sites of bacterial invasion, it is therefore to be expected that neutrophils which respond to chemotactic molecules are also exposed to the endogenous stimuli, PAF and LTB_4. We have studied the respiratory burst of human neutrophils in response to combinations of the chemotactic peptide N-formyl-methionyl-leucyl-

This study was supported by grant No. 3.353-0.82 from the Schweizerischer Nationalfons.

Abbreviations: fMLP, formyl-methionyl-leucyl-phenylalanine; PAF, platelet-activating factor; LTB_4, leukotriene B_4

0006-291X/85 $1.50

phenylalanine (fMLP) and either PAF or LTB_4, and have observed a marked synergism between the exogenous and neutrophil-derived bioactive compounds.

METHODS

PAF (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphorylcholine) was kindly provided by Dr. R.N. Saunders, Sandoz Ltd., Hanover, NJ, USA. In some experiments, PAF from Bachem AG, Bubendorf, Switzerland was used. The same supplier was the source of lyso-PAF (1-O-hexadecyl-sn-glycero-3-phosphorylcholine) and fMLP. LTB4 was a gift of Dr. J. Rokach, Merck Frosst Canada Inc., Pointe Claire-Dorval, Que., Canada.

The preparation of the neutrophils and the measurement of superoxide production have been described (11). PBS (11) containing 2.5 mg/ml bovine serum albumin (BSA) was used throughout as incubation medium. Where indicated (Ca^{++}-free conditions), medium without added $CaCl_2$ and $MgCl_2$ and containing 1 mM EGTA was used. 10 mM stock solutions of fMLP (DMSO), PAF and lyso-PAF (0.9% NaCl containing 2.5 mg/ml BSA) and LTB4 (methanol) were prepared and diluted with medium shortly before use. In some experiments, neutrophils were treated with cytochalasin B (5 ug/ml) 5 min before stimulation.

RESULTS

PAF combined with fMLP. Fig. 1 shows the production of superoxide by human neutrophils stimulated with PAF followed by fMLP and viceversa. When

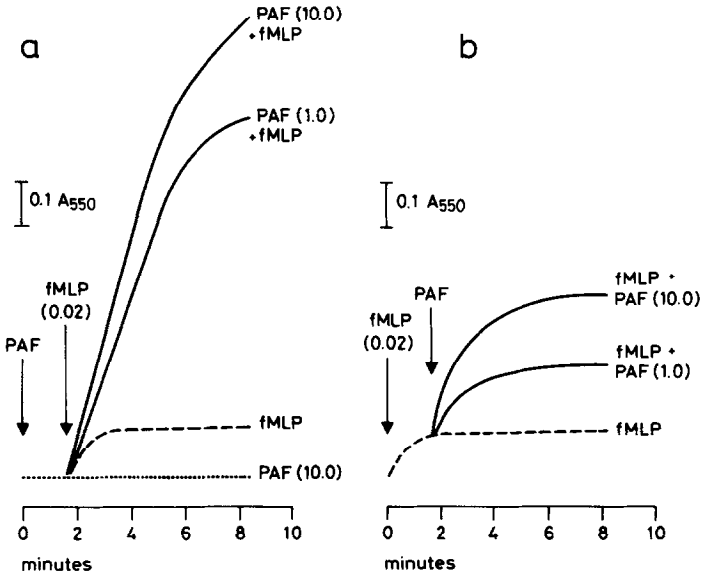

Fig. 1 Superoxide generation by human neutrophils stimulated with PAF and fMLP. (a) Pretreatment with PAF; fMLP (0.02 uM) was added 90 sec after PAF (1.0 or 10.0 uM). (b) Pretreatment with fMLP; PAF (1.0 or 10.0 uM) was added 90 sec after fMLP (0.02 uM). The responses to fMLP alone and PAF alone are represented by the broken and dotted lines respectively. Reproductions of the original tracings are shown here and in Figures 2, 3 and 4.

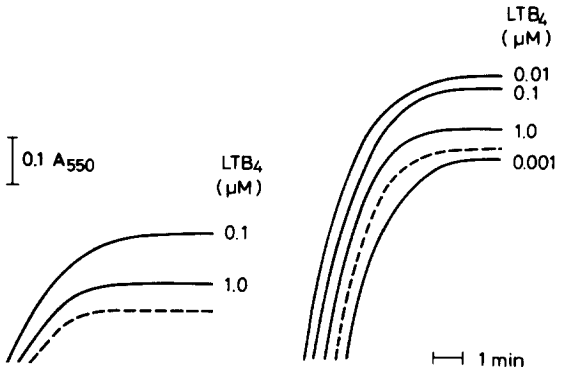

Fig. 2 Effect of pretreatment with LTB4 on fMLP-induced superoxide formation. LTB4 at the indicated concentrations was added 2 min before fMLP. Two experiments performed with either 0.02 uM fMLP (left) or 0.1 uM fMLP (right) are shown. The response to fMLP alone is represented by the broken line. Recordings were started after the addition of fMLP.

the cells were first exposed to PAF (Fig. 1a), generation of superoxide induced by the subsequent addition of fMLP was greatly enhanced. The magnitude of the effect observed becomes evident when one considers that PAF by itself was inactive, and fMLP, at the concentration used, had only a minimal effect.

When the sequence of stimulation was reversed (Fig. 1b), the response was less extensive. A remarkable effect was nevertheless obtained: The pretreatment with fMLP rendered the neutrophils susceptible to PAF. Interestingly fMLP-treated neutrophils remained responsive to PAF for several minutes after superoxide production elicited by fMLP had already ceased.

LTB_4 combined with fMLP. Analogous experiments were performed with LTB_4, and a synergism was also observed. However, as shown in Fig. 2, pretreatment with LTB_4 enhanced the response to fMLP to a moderate extent only. Furthermore, LTB_4 did not act in a concentration-dependent manner. In 9 experiments, the maximum increase of the response to fMLP varied between 1.3- and 2.4-fold and was obtained at 0.01 to 0.1 uM LTB_4. At higher concentration (1.0 uM), LTB_4 was less effective (Fig. 2). When the neutrophils were first treated with fMLP, no further generation of superoxide could be elicited with LTB_4 (0.01 - 1.0 uM) as the second stimulus.

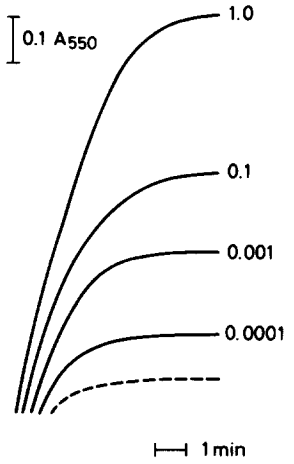

Fig. 3 Concentration dependence of the potentiating effect of PAF on fMLP-induced superoxide production. PAF at the indicated concentrations (uM) was added 1 min before fMLP (0.01 uM). The response to fMLP alone is represented by the broken line.

Combination of PAF and LTB_4. Neutrophils were also exposed to combinations of PAF and LTB_4 at various concentrations. Independent of the order of addition of the two compounds, this treatment consistently failed to induce a respiratory burst. When applied alone, LTB_4 at 1.0 uM induced a slight response, which, however, was weaker than that observed with 0.01 uM fMLP. At lower concentrations LTB_4 was inactive as was PAF up to 10.0 uM. On the other hand, both stimuli induced a weak but significant respiratory burst in cytochalasin B-pretreated neutrophils, confirming previous observations by others (2, 12, 13).

More on the synergism between PAF and fMLP. Fig. 3 illustrates the enhancement of the neutrophil response to fMLP by pretreatment with different concentrations of PAF. Already at 0.0001 to 0.001 uM (1/100 to 1/10 of the fMLP concentration used) PAF exhibited remarkable activity. Table I summarizes the results obtained in a series of similar experiments. The effect of PAF was already highly significant ($p < 0.01$) at the 0.1 nM level. With increasing PAF concentrations the rate and extent of fMLP-dependent superoxide formation were enhanced by factors of 2 to 4.4 and of 2.8 to 15.0, respectively. In control experiments the effect of the inactive metabolite lyso-PAF

Table I. Effect of PAF pretreatment on fMLP-induced superoxide formation by human neutrophils

PAF (uM)	Cytochrome c reduction				
	Maximum rate (a)	(RI)	Total during first 5 min (b)	(RI)	n
0	1.47 ± 0.57	(1.0)	1.28 ± 0.54	(1.0)	11
0.0001	2.87 ± 1.37	(2.0)	3.60 ± 1.93	(2.8)	5
0.001	3.50 ± 1.09	(2.4)	5.06 ± 1.94	(4.0)	9
0.01	4.11 ± 1.04	(2.8)	6.30 ± 2.71	(4.9)	11
0.1	4.75 ± 1.11	(3.2)	9.43 ± 3.63	(7.4)	11
1.0	5.94 ± 1.33	(4.0)	16.57 ± 5.22	(12.9)	11
10.0	6.49 ± 1.52	(4.4)	19.15 ± 2.75	(15.0)	3

The cells were exposed to PAF at the indicated concentration, and 1 min later 0.02 uM fMLP was added. Means ± SD. Statistical comparison (one-tailed t-test) with the respective controls (no PAF) shows that the effect of PAF was significant ($p < 0.01$ or better) at all concentrations.
(RI) Relative increase
(a) nmol/min per 10^6 cells
(b) nmol per 10^6 cells during the first 5 min following addition of fMLP

was tested. A slight enhancement of the fMLP response was observed at the highest concentration only (10 uM). This effect, however, was clearly smaller than the one obtained in the same experiment with 0.0001 uM PAF.

The influence of extracellular calcium was also examined (Fig. 4). When calcium was omitted, the response to fMLP alone was markedly reduced, and the same was true for the enhancing effect of PAF.

Fig. 5 summarizes the results of experiments in which the time between the addition of PAF and fMLP was varied. As shown, the enhancement of fMLP-induced superoxide generation was not critically dependent on the time of preincubation with PAF. Simultaneous addition of PAF and fMLP to the neutrophil suspension was as effective as the preincubation with PAF for 1, 5 or even 10 min.

DISCUSSION

This study demonstrates a two-way synergism between PAF and fMLP in the stimulation of the respiratory burst of human neutrophils. PAF was found to prime the cells for the subsequent response to the chemotactic peptide: With-

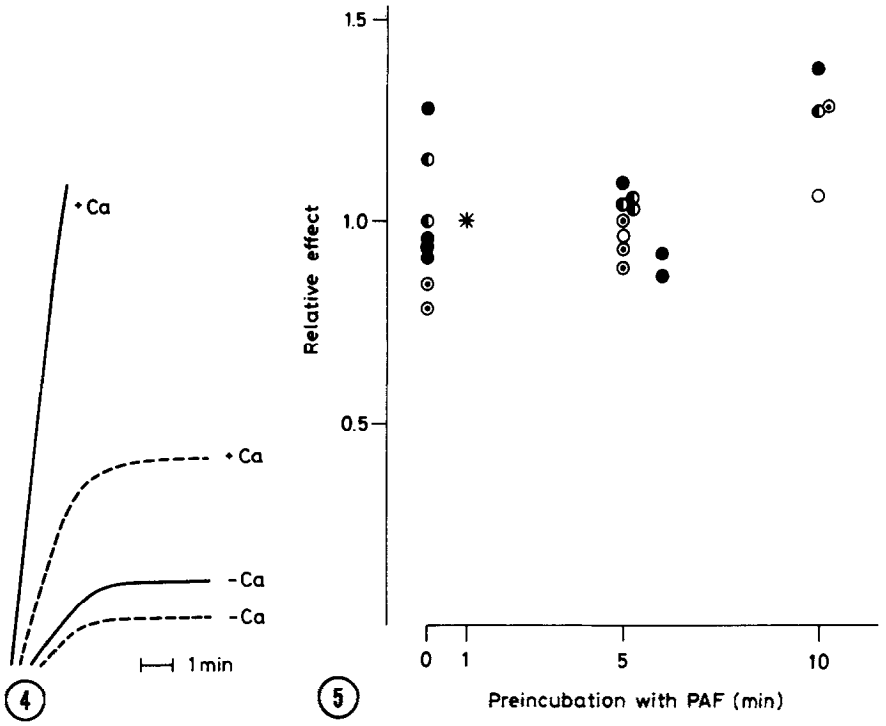

Fig. 4 Effect of extracellular calcium. The neutrophils were incubated in either Ca^{++}-containing or Ca^{++}-free medium. PAF (1.0 uM) was added 2 min before fMLP (0.1 uM). The responses to fMLP alone are represented by the broken lines.

Fig. 5 Effect of the time of pretreatment with PAF on fMLP-induced superoxide generation. PAF at various concentrations was added either simultaneously with fMLP (time 0) or at the indicated times before fMLP (0.02 uM). The extent of cytochrome c reduction during the first 5 min following addition of fMLP was determined and the relative increase over the value obtained without PAF was calculated. The data are presented relative to the value of 1 min pretreatment (*). The PAF concentrations used were 0.001 uM (O), 0.01 uM (◎), 0.1 uM (◐), 1.0 uM (●).

out by itself eliciting the burst, PAF greatly enhanced the rate and the extent of superoxide generation induced by fMLP. Priming with the chemotactic peptide, on the other hand, made the cells responsive to PAF. Enhancement of the fMLP-dependent respiratory burst was also obtained with LTB_4, although this effect was less pronounced. These results are in keeping with recent observations by other laboratories. In human neutrophils, PAF pretreatment was shown to increase fMLP-dependent oxygen consumption (12) and LTB_4 was reported to enhance fMLP responsiveness (14).

There are clear indications that the synergism between PAF and fMLP is of potential pathophysiological relevance. The effects were calcium-dependent and already significant at PAF concentrations as low as 0.0001 uM. Lyso-PAF was virtually inactive. The mechanism of the synergistic effects observed appears to differ depending on the sequence of stimulus addition: Priming with fMLP involves the activation of the superoxide-forming oxidase, while priming with PAF does not. Even with fMLP, however, the priming effect is fully preserved when the respiratory burst subsides. Both stimuli appear to induce the synthesis or activation of intermediates that are utilized in the transduction of the second stimulus.

On bacterial infection, the first signal to reach the circulating neutrophils is most likely that of chemotaxins, e.g. chemotactic peptides of bacterial origin or C5a (15). The neutrophils that migrate into the tissues are exposed to increasing concentrations of these agents. Under conditions of strong chemotactic stimulation, and in particular during phagocytosis, PAF and LTB_4 are liberated (6-10). The present results show that PAF can reinforce the oxygen-dependent microbicidal activity of the neutrophils. Such an upregulation through products of the responding cells appears to be a meaningful mechanism to maximize the targeted action of the neutrophils. In this respect, LTB_4 was less active than PAF. In view of its chemotactic and chemokinetic properties (16), LTB_4 may rather act as an amplifier of neutrophil recruitment. It is finally interesting to note that LTB_4 and PAF did not potentiate each other, thus precluding the (undesirable) possibility of autoactivation of the cells.

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