Vol. 125, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1984 Pages 367-373

ACTIVATION OF NADPH OXIDASE OF HUMAN NEUTROPHILS. POTENTIATION OF CHEMOTACTIC PEPTIDE BY A DIACYLGLYCEROL

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Received October 24, 1984

Formyl-methionyl-leucyl-phenylalanine (fMLP) and 1-oleoyl-2-acetyl-glycerol (OAG) are synergistic stimuli of the respiratory burst of neutrophils. Simultaneous exposure to both agents greatly enhanced superoxide production, both in rate and extent. OAG potentiated the response to fMLP also in Ca^{++}-free medium. Pretreatment of the neutrophils with fMLP drastically shortened the lag of superoxide production in response to OAG. Our findings lead to the following conclusions: (i) Protein kinase C is likely to be involved in the activation of the NADPH oxidase by fMLP; (ii) OAG appears to be utilized as an intermediate in the activation process; (iii) prestimulation of the cells with fMLP facilitates the response to OAG.

The respiratory burst is a sudden increase in oxygen consumption that accompanies phagocytosis. It results from the activation of the NADPH oxidase which produces superoxide, an essential metabolite for the microbicidal function of phagocytes (1). The mechanism of activation of the oxidase is unknown. In neutrophils, the respiratory burst is initiated by phagocytosis and by three types of soluble stimuli, chemotaxins, e.g. formyl-methionyl-leucyl-phenylalanine (fMLP) and the anaphylatoxin C5a, calcium ionophores and protein kinase C ligands like phorbol myristate acetate (PMA). Protein kinase C is activated by diacylglycerols in combination with phosphatidylserine and calcium ions (2). Exogenous long-chain diacylglycerols do not stimulate intact cells. Their activity, however, is mimicked by 2-acetyl derivatives (3). In this paper, we present results showing that the activator of protein kinase C, 1-oleoyl-2-acetyl-glycerol

This study was supported by grant No. 3.353-0.82 from the Schweizerischer Nationalfonds.
Abbreviations: fMLP, formyl-methionyl-leucyl-phenylalanine; PMA, phorbol myristate acetate; OAG, 1-oleoyl-2-acetyl-glycerol

0006-291X/84 $1.50

(OAG) greatly enhances the activity of the chemotaxin fMLP as an inducer of the respiratory burst.

METHODS

Human neutrophils were prepared from buffy coats of donor blood stored overnight at 4° C (Swiss Red Cross Laboratory, Bern, Switzerland). Buffy coats of single donors, diluted 4-fold with PBS containing heparin (13 U/ml), were layered on Ficoll-Hypaque gradients (4). Following centrifugation, the neutrophils were isolated from the cell pellet according to Weening et al. (5).

Superoxide generation was measured spectrophotometrically at 37°C by incubating 3×10^6 neutrophils in a total volume of 1 ml PBS in the presence of 85 uM cytochrome c. The cells were preincubated at 37° C for 5 min in a 1 ml cuvette and the reaction was then started by addition of the appropriate stimulus. Cytochrome c reduction was recorded continuously at 550 nm (6). PBS had the following composition: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$. Where indicated (Ca^{++}-free conditions), PBS with no added $CaCl_2$ and $MgCl_2$ and containing 1 mM EGTA was used (7).

fMLP was obtained from Bachem AG, Bubendorf, Switzerland. OAG was synthetized as described by Mori et al. (8). The product was purified by chromatography on silica gel and the structure and purity of the preparation were confirmed by NMR and mass spectrometry. OAG was diluted in DMSO. The final DMSO concentration in the assays was 1 %. Superoxide dismutase from bovine blood was obtained from Sigma Chemical Co., St. Louis, MO.

RESULTS

As shown in Fig. 1, fMLP and OAG elicited a respiratory burst which was dependent on the stimulus concentration. The time course of the cellular responses, however, was completely different. fMLP induced a very rapid response which lasted for a short time only, while the effect of OAG was of slow onset and long duration. In Fig. 2 the actions of the single stimuli and their combination are compared. OAG applied slightly before, together with or slightly after fMLP greatly enhanced superoxide production. The maximum rate was higher than with fMLP alone and the response was much more extensive. With 0.02 uM fMLP and 300 uM OAG the maximum rate increased on average 3.2-fold over that observed with fMLP alone (Table I). A somewhat lower rate was obtained when OAG was supplied 60 or 120 sec after fMLP. As shown, potentiation of the fMLP response by OAG occurred well before significant superoxide production could be observed following

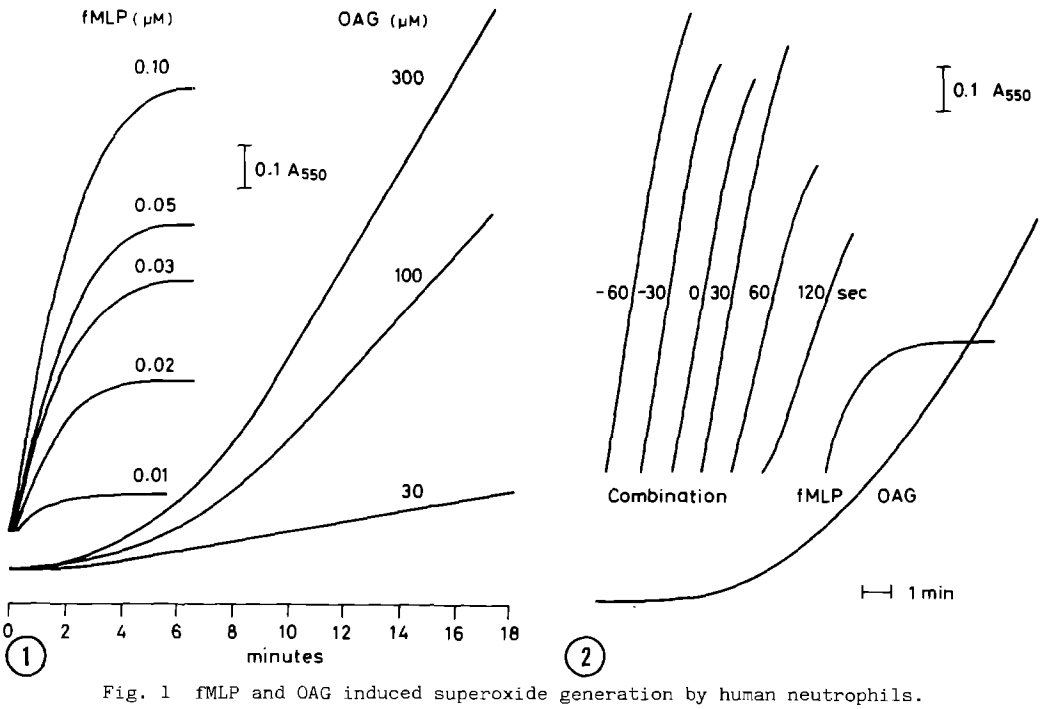

Fig. 1 fMLP and OAG induced superoxide generation by human neutrophils. Time course at various stimulus concentrations. Recording was started 15 - 20 sec after addition of the stimulus and mixing (time 0). Reproductions of the original tracings suitably arranged are shown in this and the following Figures.

Fig. 2 Synergistic effect of fMLP and OAG on superoxide production by neutrophils. fMLP (0.02 uM) and OAG (300 uM) were used either separately or in the following combinations: fMLP was added at time 0, and OAG was added either before or after fMLP at the indicated times or simultaneously with fMLP.

Table I. Superoxide production induced by fMLP and OAG separately and in combination

Stimulus (uM)	Cytochrome c reduction		
	Maximum rate (a)	Total during first 3 min (b)	n
fMLP (0.02)	2.70 ± 0.78	5.77 ± 1.80	26
(0.1)	7.62 ± 1.29	19.53 ± 3.56	15
OAG (100)	1.25 ± 0.33	0.33 ± 0.16	7
(300)	1.51 ± 0.45	0.61 ± 0.23	17
OAG (300) + fMLP (0.02)	8.74 ± 0.51	18.61 ± 2.01	20

Superoxide-dependent cytochrome c reduction was measured as described in "Methods". Values are means ± SD obtained with neutrophil preparations from 4 to 7 individuals.
(a) nmol/min per 10^6 cells
(b) nmol per 10^6 cells during the first 3 min following stimulation

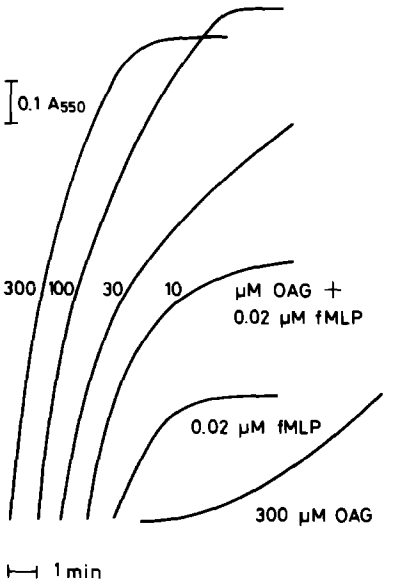

Fig. 3 Dependence of the potentiating effect of fMLP stimulation on the OAG concentration. OAG at the concentrations indicated was added 2 min before fMLP (0.02 uM).

stimulation with OAG alone. Fig. 3 shows the potentiation of the fMLP-induced response by increasing concentrations of OAG. Superoxide production approached a maximum on addition of 100 uM OAG. A substantial stimulation of superoxide production was obtained with OAG even at fMLP concentrations which by themselves induced barely detectable responses.

Since the cellular effects of fMLP are known to be influenced by the presence of extracellular calcium, we tested the response to both stimuli in normal and in Ca^{++}-free incubation media. Results from two experiments are presented in Fig. 4. Superoxide production induced by OAG alone was not influenced appreciably by extracellular Ca^{++} (Fig. 4a). Stimulation by fMLP, however, was drastically lowered when Ca^{++} was omitted. This is shown in Fig. 4b: No superoxide was produced in the presence of 0.02 uM fMLP, and the maximum rate induced by 0.1 uM fMLP dropped to 16 %. Fig. 4a also shows the influence of Ca^{++} on the effect of the combined stimuli. The yield of superoxide was very high when Ca^{++} was present. In the absence of added Ca^{++} the response was

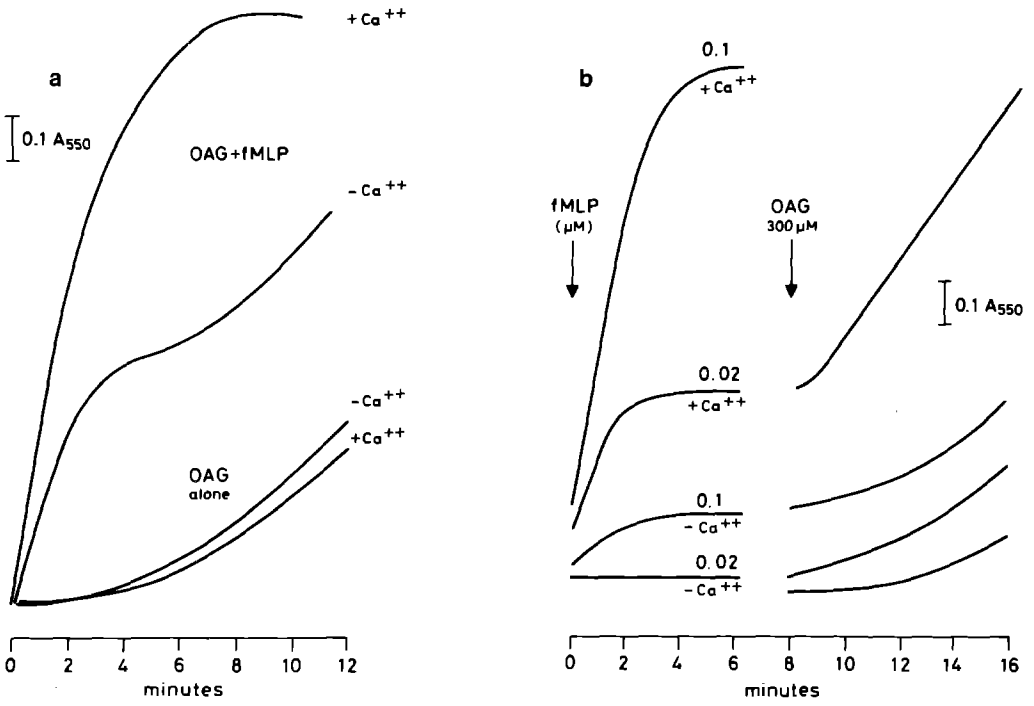

Fig. 4 Effect of extracellular calcium. The neutrophils were incubated in either Ca^{++}-containing or Ca^{++}-free PBS as defined in "Methods". a. OAG (300 uM) and fMLP (0.02 uM) were added simultaneously. b. fMLP was added at the indicated concentration at time 0, and OAG (300 uM) was added 8 min later at a time when superoxide production induced by fMLP had subsided.

lower, both in rate and extent. Superoxide production was biphasic. An initial rise to a plateau, which was similar to that induced in the presence of Ca^{++} by an intermediate concentration of fMLP (see Fig. 1), was followed by a delayed increase of the type obtained with OAG alone. Quite clearly, the response to fMLP was strongly potentiated by OAG even under Ca^{++}-free conditions. This is evident when the effect of the combined stimuli is compared with that of 0.02 uM fMLP alone (Fig. 4b). In addition, Fig. 4b shows another aspect of the synergism between fMLP and OAG. When OAG was added to cells which had already responded to fMLP (in the presence of Ca^{++}), superoxide production resumed almost immediately without the lag observed after OAG alone. On the other hand, in Ca^{++}-free medium the lag typical for the OAG response was apparent again.

The tracings shown in Figs. 1 - 4 are the results of single experiments. They are, however, representative since closely comparable responses were obtained in repeated assays with the same or different neutrophil preparations. The reproducibility of the effects is shown in Table I which presents average data.

DISCUSSION

This study shows that the chemotactic peptide fMLP and the diacylglycerol OAG act synergistically in eliciting the respiratory burst of human neutrophils.

Two findings are worth discussing, which could shed some light on the mechanism of activation of the respiratory burst oxidase. The first is that superoxide production induced by fMLP is faster and more extensive when OAG is present. This obviously suggests that OAG may be utilized as an intermediate in the activation process. The fact that the rate and extent of superoxide production increase with increasing amounts of OAG (Fig. 3) and that OAG is more effective when supplied before rather than after fMLP support this possibility. OAG stimulates protein kinase C (3) and, like PMA, could activate the NADPH oxidase by this mechanism (9). The synergism between fMLP and OAG observed in our experiments suggests that the activation of the burst by chemotactic factors may also be mediated by protein kinase C.

The second finding to discuss is that pretreatment of the neutrophils with fMLP even at very low concentrations drastically shortens the lag time of the response to the subsequent addition of OAG. This suggests that a change induced by fMLP persists for several minutes and facilitates the response to OAG. This change could be related to a redistribution of calcium. fMLP rises cytosolic Ca^{++} by enhancing influx and the release from intracellular stores (10). A priming through Ca^{++} is also suggested by recent results of Robinson et al. (11) indicating synergism between

A 23187 and PMA and by our own observations (data not shown) that pretreatment with A 23187 shortens the lag and increases the rate of superoxide production induced by OAG.

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