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MOLECULAR MECHANISMS OF PRIMING: EXUDATE HUMAN NEUTROPHILS ARE PRIMED TO fMET-LEU-PHE BUT HAVE NORMAL INTRACELLULAR CALCIUM AND cAMP RESPONSES

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ABSTRACT

Neutrophils were isolated both from peripheral blood (PB) and from skin-window inflammatory exudates (SW) of normal subjects. Comparison of metabolic and functional properties of the two cell populations showed that: 1) SW neutrophils had a two- to three-fold higher superoxide (O_2^-) production in response to the chemotactic peptide fMet-Leu-Phe as PB cells; 2) SW neutrophils had twice as many membrane fMet-Leu-Phe receptors than PB cells; 3) the adenosine 3'-5'-cyclic monophosphate (cAMP) levels and the intracellular free calcium concentration ($[Ca^{2+}]_i$) were similar in the two cell populations, both as basal level and as peak response to fMet-Leu-Phe. Therefore, the mechanisms underlying the priming of free radical production by SW neutrophils appear to be linked to receptor up-regulation rather than to the modification of cAMP or $[Ca^{2+}]_i$ responses.

INTRODUCTION

Neutrophils circulate in the bloodstream for only a few hours before migrating to extra-vascular tissues. Previous investigations on animal and human models have shown that exudate neutrophils are metabolically primed, being more responsive to various membrane

stimulants than blood neutrophils (1-7). Uncertainty still persists on the molecular nature of the priming phenomenon. Some authors have found that priming is associated with an increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) (8-10) and in the number of receptors for various agents including the chemotactic peptide fMet-Leu-Phe (2-4,10-12), while others have found no modifications in $[Ca^{2+}]_i$ (13,14) or in membrane receptors in primed cells (1,15,16). Modifications in other steps of the activation cascade such as protein kinase C or NADPH oxidase have also been described in primed cells (5,9,15,17,18), while other investigators observed no such changes (13,16,19). These differences suggest a variety of possible priming states, according to the experimental models utilized or, in-vivo, according to the various types of infection or inflammation.

In the present study, the biological events which are associated with priming of neutrophils were investigated using a human in-vivo model system. The oxidative metabolic responses (O_2^- production) of PB neutrophils were compared with those of neutrophils isolated from a skin experimental exudate obtained from the same subject. Specific cell resposes that are candidate for a role in cell priming, i.e., receptor expression and second messenger (cAMP and $[Ca^{2+}]_i$) changes, were evaluated in this model system.

METHODS

Cell isolation

Neutrophils were obtained from blood and from SW exudates of healthy human volunteers. Blood neutrophils were prepared from EDTA-anticoagulated blood by centrifugation over Percoll (Pharmacia, Uppsala, Sweden) gradients (20). The final cell preparation was suspended in Hank's balanced salt solution (Gibco, Paisley, Scotland) containing 5 mM glucose, 0.2% human serum albumin, 0.5 mM $CaCl_2$ and 1 mM $MgSO_4$ (medium H-GACM). Exudate neutrophils were isolated according to the skin-window technique proposed by Senn (21), using bell-shaped, sterile and disposable plastic skin chambers (FAR Italia, Verona, Italy). One ml of autologous serum was injected into the

chamber and twenty-four hours later the exudate was collected by aspiration. The exudate cells (>95% neutrophils) were then centrifuged at 1200 rpm, washed twice with phosphate buffered saline and finally suspended in medium H-GACM.

Superoxide anion production

Superoxide anion was measured by the reduction of ferricytochrome c with a microplate assay (22). The wells of fetal bovine serum-coated microplates (Linbro type, Flow) were supplemented with 25 µl of 0.6 mM cytochrome c (Boehringer, Mannheim, Germany) and with either 25 µl of fMet-Leu-Phe (Sigma, St.Louis, Mo) diluted in H-GACM at a concentration 4 times the final concentration in the assay (stimulated cells) or 25 µl of H-GACM (unstimulated cells). The plate was then brought to 37°C, and 50 µl of the neutrophil suspension (2 x 10^5 cells), pre-warmed at 37°C, were added to each well. The reduction of cytochrome c was measured with a microplate reader (Reader 400, SLT Labs Instruments) at 550 nm using 540 nm as reference wavelength (22).

Intracellular free calcium

Intracellular free calcium concentration ($[Ca^{2+}]_i$ was measured fluorimetrically with Fura-2 as described (23). Neutrophils were loaded with Fura-2 acetoxymethylester (Calbiochem, La Jolla, Ca) by incubating the cell suspension in Hepes buffered saline (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, 0.5 mM Na_2HPO_4, 6 mM glucose, pH 7.4) with 1 mM Fura-2 for 45 minutes at room temperature. After loading, the cells were centrifuged for 5 minutes at 300 g and resuspended in the same buffer at 2 x 10^6 cells/ml. Aliquots (2 ml) of cell suspension were placed in a thermostated (37°C) quartz cuvette under magnetic stirring, and Fura-2 fluorescence (λex 340 nm, λem 500 nm) was measured in an F2000 Hitachi spectrophotometer. Calibration of $[Ca^{2+}]_i$ was performed as previously described (24). Fmin was obtained by the addition of 5 mM EGTA (final pH, 8.5) and by lysing the cells with 0.1% Triton X 100. Fmax was determined by addition of 5 mM $CaCl_2$ to the cell lysate.

cAMP concentration

cAMP was determined by the competitive protein binding method of Brown et al. (25), using cAMP assay kit from the Radiochemical Centre, Amersham. Neutrophils were suspended in H-GACM at 3 x 10^7 cells/ml and activated at 37°C with 10^{-7} M fMet-Leu-Phe. Just before (time zero) and at the indicated times after the addition, aliquots of 75 µl of the neutrophil suspension were withdrawn and added to 75 µl of potassium phosphate buffer (0.1 M, pH 5.5, containing 4 mM theophylline and 9 mM 2-mercaptoethanol) kept at 95-98°C in a thermostated Eppendorf microfuge tube holder. After incubation for a further 5 minutes at 95-98 °C, the samples were cooled in an icewater bath. Denaturated protein was removed by centrifugation at 10,000 g for 3 minutes and 50 µl of the supernatants were assayed in duplicate.

fMet-Leu-(^{3}H)Phe binding

Receptors for fMet-Leu-Phe were quantitated using radiolabeled f-Met-Leu-(^{3}H)Phe (NEN-Du Pont, Florence, Italy), exactly as described by Metcalf et al. (20). Binding was performed at 4°C for 30 minutes (saturation time) using the final concentration of 10^{-7} M radioactive ligand and 10^{-5} M nonradioactive ("cold") ligand when necessary to assess nonspecific and displaceable binding.

RESULTS

Peripheral blood and SW human neutrophils were compared for their O_2^--forming activity in a simultaneous assay. Table 1 shows that, in the absence of stimulants, both cell preparations produced very little O_2^-, indicating that even the exudate cells are metabolically in a resting state. Upon challenge with fMet-Leu-Phe, SW neutrophils exhibited a burst of superoxide production that was significantly higher than that of PB cells, the difference being invariably reproduced in 12 separate experiments performed on healthy subjects.

To identify possible mechanisms that might be correlated with the enhanced response

Table 1. O_2^- production by resting and fMet-Leu-Phe stimulated neutrophils from blood (PB) and from SW exudate. Mean values ±S.D. of samples from 12 subjects are reported. Means were compared by Student's t-test for paired data (values of blood cells versus values of SW cells for each subject).

	O_2^- nmoles/10 min/10^6 cells)		
Stimulant	PB	SW	t-test
None	0.43±0.45	0.57±0.36	P: 0.29
fMet-Leu-Phe (10^{-8} M)	4.23±1.8	15.9±7.1	P: 9.8×10^{-6}
fMet-Leu-Phe (10^{-7} M)	7.21±5.22	19.51±8.44	P: 1.7×10^{-5}

to fMet-Leu-Phe in SW neutrophils, we analysed some of the biological modifications that have been suggested play a role in cell priming, i.e., receptor expression, variations of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and cAMP. As shown in Table 2, the number of fMet-Leu-Phe receptors was increased by a factor of two in SW neutrophils compared to PB cells. The number of receptors detected on the membrane of blood neutrophils was in the range reported by Metcalf et al. (20). The increase in fMet-Leu-Phe binding in exudate cells was highly specific, and the bound peptide was almost totally displaceable by an excess of fMet-Leu-Phe. Our data are in agreement with others that suggested that one mechanism underlying the primed state is exposure of new cell surface receptors, possibly through limited degranulation of the specific granules (2-4,10,11).

A reasonable target for amplification of the metabolic responses in primed cells would be the signal transduction pathways stimulated by fMet-Leu-Phe. Among the possible events of transmembrane signalling mechanisms, we explored calcium fluxes and cAMP intracellular levels. Fig. 1 gives the values of $[Ca^{2+}]_i$ in PB and SW neutrophils.

Table 2. Binding of fMet-Leu(^{3}H)Phe to neutrophils from blood (PB) and from SW exudate. Mean values±S.D. of triplicate determinations on cell samples from two subjects are reported

	fMet-Leu(^{3}H)Phe bound (fmoles/10^6 cells)	
	PB	SW
Subject n.1		
Total binding	49.2±0.7	95.2±10.0
Displaceable	42.1±0.7	82.2±10.0
Non displaceable	7.1±0.3	13.0±1.7
Subject n.2		
Total binding	41.1±1.1	79.7±6.8
Displaceable	36.7±1.2	73.7±6.9
Non displaceable	4.4±2.6	5.9±2.9

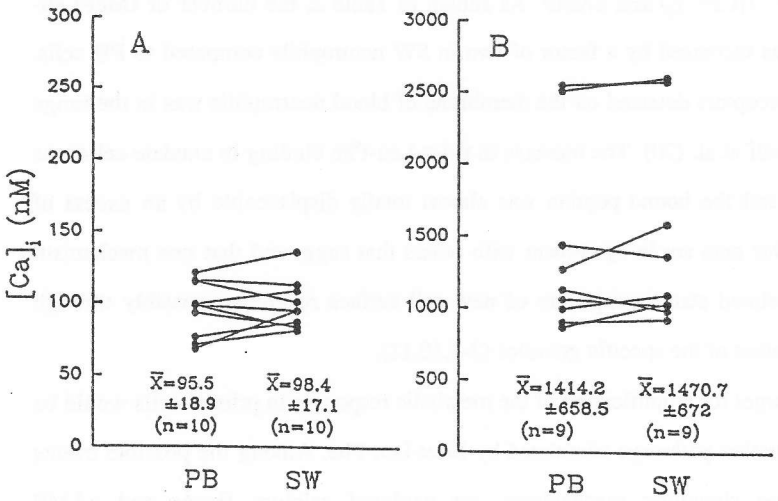

Fig. 1. Cytosolic free calcium in resting (A) and fMet-Leu-Phe-stimulated (10^{-8} M) (B) neutrophils from PB and from SW.

Neither the basal level nor the maximum fMet-Leu-Phe-stimulated increase showed any significant difference between the two cell populations. One experiment, representative of a total of six performed, involving a comparison between two complete dose-response curves, is shown in Fig. 2.

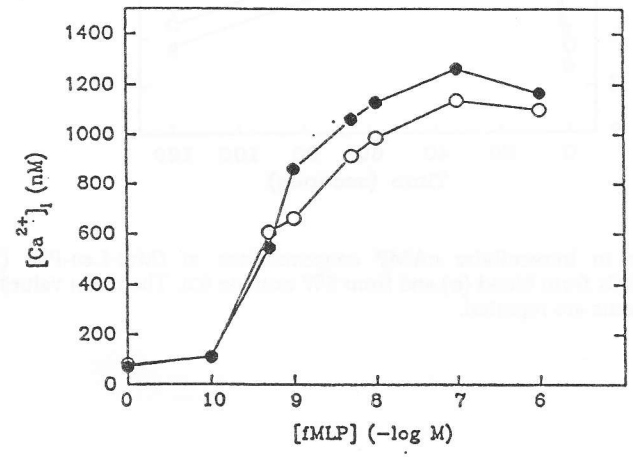

Fig. 2. Dose-dependency of stimulatory effect of fMet-Leu-Phe on cytosolic free calcium of neutrophils from blood (●) and from SW exudate (○). The values of a typical experiment, representative of 6, are shown.

Control experiments (not shown) demonstrated that: a) the cytosolic Fura 2 concentration (a parameter capable of affecting the sensitivity of the fluorimetric measurement) was similar in the two cell populations; and b) the differences in O_2^- response to fMet-Leu-Phe were maintained also in the Fura-2-loaded cells.

The cytosolic cAMP concentration (Fig. 3) was found to be similar in two cell populations, both in the basal level and in the fMLP-induced transient peak.

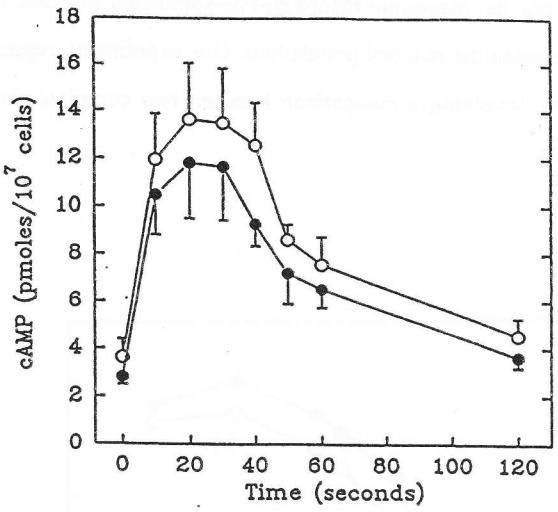

Fig. 3. Variations in intracellular cAMP concentrations in fMet-Leu-Phe (10^{-7} M) stimulated neutrophils from blood (●) and from SW exudate (○). The mean values ±S.E. of 8 separate experiments are reported.

DISCUSSION

Neutrophils isolated from an inflammatory exudate can be metabolically primed, as demonstrated by an enhanced O_2^- production when stimulated. Increased production of toxic oxygen derivatives is beneficial to antimicrobial defenses, but can also be a potential threat to the host. The purpose of this study was to shed some light on the mechanisms of the priming phenomenon.

A large number of publications have indicated that Ca^{2+} plays a central role in regulating neutrophil function, the current view being that an increase in $[Ca^{2+}]_i$ is important in chemotaxis and in priming, while it is itself insufficient to cause stimulation of NADPH oxidase activity (26). Other experimental systems seem to exclude even that enhanced levels of $[Ca^{2+}]_i$ are essential in priming of oxidase activity (14,27,28). In the experiments reported here, comparing exudate and blood neutrophils, no significant differences in basal $[Ca^{2+}]_i$ levels and in $[Ca^{2+}]_i$ response to fMet-Leu-Phe were found.

Molecular mechanisms of priming

Since the methods of $[Ca^{2+}]_i$ measurements utilized in different laboratories are essentially based on the same principle, i.e., the use of fluorimetric indicators, the different results regarding $[Ca^{2+}]_i$ changes in primed cells are not due to methodological reasons but, more conceivably, to differences in the experimental models of priming utilized. Therefore, we may conclude that the enhanced production of O_2^- in this priming model involves a pathway that does not require increased intracellular Ca^{2+} in primed cells versus normal cells. However, our data do not exclude the possibility that a Ca^{2+} increase may be necessary during priming itself. It is possible that the 24-hour exudate neutrophils used in these studies have completed their priming, and that a burst of $[Ca^{2+}]_i$ could occur during extravasation and migration into exudate, this burst being instrumental for receptor expression or for other biological modifications related to priming.

The chemotactic factor fMet-Leu-Phe induces a transient elevation in intracellular levels of cAMP. It has been suggested that, instead of serving as an initial signal, changes in cAMP levels may function as an inhibitory feedback mechanism (29) and that cytokines affect neutrophil functions through alterations in the level of cAMP (30). These observations prompted us to investigate whether the enhanced fMet-Leu-Phe-dependent O_2^- production in SW cells might be associated to a diminished cAMP response and therefore to a delayed termination of the respiratory burst. However, no significant differences were found either in the basal cAMP content of the two cell populations or in the kinetics of the cAMP response to fMet-Leu-Phe. Our results are in accord with others showing that exudate neutrophils have an oxidase inactivation rate similar to blood neutrophils (7).

Thus, the present study provides evidence that in this in vivo model of aseptic inflammation the priming effect observed on the superoxide formation seems not dependent on significant changes of either $[Ca^{2+}]_i$ and cAMP basal levels and responses. Whether priming of the metabolic response to fMet-Leu-Phe might be accounted for by the increase of receptors for this peptide or whether it requires other changes of specific

transduction pathways (phospholipid metabolism, protein phosphorylation, G-proteins, etc.) that have not been considered here, is a matter of investigation.

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