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TUMOR NECROSIS FACTOR-α/CACHECTIN ACTIVATES THE O_2^--GENERATING SYSTEM OF HUMAN NEUTROPHILS INDEPENDENTLY OF THE HYDROLYSIS OF PHOSPHOINOSITIDES AND THE RELEASE OF ARACHIDONIC ACID

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SUMMARY. We have investigated the mechanisms of transmembrane signalling implicated in the activation of the respiratory burst of adherent neutrophils by tumor necrosis factor-α/cachectin (TNF). The activation of the respiratory burst by TNF is insensitive to pertussis toxin and weakly sensitive to protein kinase C inhibitors Cytochalasin B and dibutyryl cyclic AMP have an inhibitory effect. The activation of the respiratory burst by TNF takes place in the absence of formation of ^{3}H-inositol phosphates, ^{32}P-phosphatidic acid, and ^{3}H-arachidonic acid. These results demonstrate that the activation of the respiratory burst by an endogenous, physiologic stimulus can be independent of the formation of messengers derived from hydrolysis of phosphoinositides.

Tumor necrosis factor-α (TNF; also referred to as cachectin) is a cytokine able to modulate the functions of cells involved in inflammatory reactions, host defences and repair of injured tissues (1). TNF is a neutrophil chemoattractant (2-4), promotes neutrophils adhesion to endothelial surfaces (5), and enhances the neutrophils capability to exert effector functions such as antibody-mediated cell cytotoxicity (6), ingestion of zymosan (7), and production of toxic oxygen molecules in response to different stimuli (7-10). Assays with suspensions of cells indicated that TNF was a weak agonist for the stimulation of toxic oxygen molecules production (7,9-13). Studies by Nathan (14), confirmed by subsequent observations (15,16), demonstrated that when neutrophils adhere to surfaces coated with serum or extracellular matrix proteins, they produce elevated amounts of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) in response to TNF.

The nature of the signals involved in the activation by TNF of NADPH oxidase, the enzymatic system responsible for the generation of toxic oxygen molecules, is not known. Several studies (reviewed in 17) have shown that ligand-receptor interactions which activate NADPH oxidase are accompanied by hydrolysis of

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phosphoinositides. However, studies performed in our laboratory (18-20), have shown that receptor-mediated stimuli can activate the NADPH oxidase independently of stimulation of phosphoinositide turnover in calcium-depleted neutrophils.

We report in this paper that stimulation of the respiratory burst by an endogenous physiologic stimulus like TNF is accompanied neither by the hydrolysis of phosphoinositides nor by the release of arachidonic acid.

MATERIALS AND METHODS

Neutrophils preparation. Neutrophils were isolated and purified as previously described (20). Cells were suspended in Hank's balanced salt solution (pH 7.4) containing 20 mM Hepes, 5.5 mM glucose and 0.25 mM $CaCl_2$ (HBSS). All assays were performed with adherent neutrophils. Six-wells culture plates were precoated for 2 hours at room temperature with foetal calf serum (FCS) and washed with Phosphate buffered saline (PBS) immediately before use. Cells ($1-1.5\times10^7$/well in 1 ml of HBSS) were settled for 5 min in 5% CO_2 at 37°C, and then TNF (kindly donated by Bachem. Inc.) or concanavalin A (Con A) (Sigma) were added. All the incubations were performed at 37°C in 5% CO_2 .

Superoxide anion generation. Neutrophils were incubated as described above in HBSS containing 160 µM cytochrome c and 2 mM NaN_3 and with or without 50 µg/ml superoxide dismutase. After different times of incubation the reduced cytochrome c was measured in the supernatants by reading the absorbance at 550 minus 468 nm and using a molar extinction coefficent of 24.5 (21).

Phosphoinositides turnover. Phosphoinositides turnover was investigated by measuring accumulation of ^{3}H-inositol phosphates according to Berridge (22). Neutrophils were suspended at 5×10^7/ml in HBSS containing 0.025% BSA and 30 µCi/ml myo-^{3}H-inositol (10-20 Ci/mmol; Amersham) and incubated for 120 min at 37°C. After 110 min, 10 mM LiCl was added to minimize inositol phosphates hydrolysis. Labelled neutrophils were washed and resuspended at 2×10^7 in HBSS containing 10 mM LiCl; assays were performed with $1,5\times10^7$ cells/well in adhesion, as described above. The reaction was stopped at different time points with 10% cold TCA and samples kept on ice for 30 min. Different species of inositol phosphates (InsP, $InsP_2$, $InsP_3$) were separated on AG anion exchange resin as previously described (19,22). For phosphatidic acid formation, neutrophils, suspended in HBSS at 5×10^7/ml, were incubated at 37 °C for 90 min with 100 µCi/ml $^{32}P_i$ (3000 Ci/mmol; Amersham) and then washed and resuspended at 2×10^7/ml in HBSS. Assays were performed on 1.5×10^7 cells/well in adhesion, as described above. The reaction was stopped at appropriate times by adding a 2:1 methanol/chloroform mixture;lipids were separated and phosophatidic acid formation detected as previously described (19).

Arachidonic acid release. Neutrophils at 1.5×10^7/ml in HBSS were labelled for 60 min at 37 °C with 2 µCi/ml of [^{3}H] arachidonic acid, washed and resuspended in the same buffer containing 0.1% BSA free fatty acid. Assays were performed with 10^7 cells/well in adesion as described above. At appropriate times, 0.5 ml were withdrawn, diluted with 0.5 ml cold 154 mM NaCl containing 2 mM EDTA, rapidly (60 sec) centrifuged in a microfuge (13,000xg) and aliquots (0.8 ml) of the supernatant were counted (19).

RESULTS

Stimulation of O_2^- generation by TNF in adherent neutrophils.

TNF stimulated O_2^- generation by neutrophils adherent to tissue culture plastic coated with FCS (see figures 1-3). As previously reported (14), O_2^- generation started after a lag time of 15-30 minutes and was maximal at 60-90 minutes. The effect of TNF was dose-dependent, and optimal at 20 ng/ml (1.2 nM). This concentration was used in the experiments described below.

Effect of different inhibitors on the stimulation of O_2^- generation by TNF in adherent neutrophils.

Table I reports the effect of different agents on the response of adherent neutrophils to TNF. In accord with previous observations (11,14,15) cytochalasin B inhibited the response to TNF . As judged by microscopic observation, cytochalasin B, inhibited neutrophils adhesion. As previously reported (15), pertussis toxin, at doses which inhibited by 90% the response to FMLP in parallel experiments (not shown), had no effect on the response to TNF. Dibutyryl cyclic AMP, in the presence of theophylline, almost completely inhibited the response to TNF and Con A. As observed with cytochalasin B , also the cyclic AMP analogue inhibited the spontaneous and the TNF-induced neutrophils adherence. H-7 and

TABLE I. Effects of different inhibitors on the O_2^- generation by adherent neutrophils stimulated with TNF

	% of the control	
STIMULUS	TNF	Con A
INHIBITORS		
CB (5µg/ml)	20	126
Pertuxis toxin (0.3µg/ml)	92	97
dibutyryl cyclic AMP (0.1 mM) plus theophylline (1 mM)	7	4
H-7 (75 µM)	65	78
Staurosporine (10 nM)	68	102

O_2^- generation was assayed after 60 min from the addition of 20 ng/ml TNF or 50 µg/ml Can A as described in Materials and Methods. Results are expressed as per cent of the activity in the absence of the inhibitor. The results of one of three similar experiment are reported. All the inhibitors tested, a part Pertussis toxin, were added to the wells where the assay was performed 2-4 min before the cells. To test the effect of Pertussis toxin, neutrophils (at $5x10^6$/ml) were incubated at 37 °C for 90 min with 0.3 µg/ml of the toxin, washed and then assayed. In assays performed in parallel, H-7 and staurosporine inhibited the response to PMA by 75 and 85 per cent respectively.

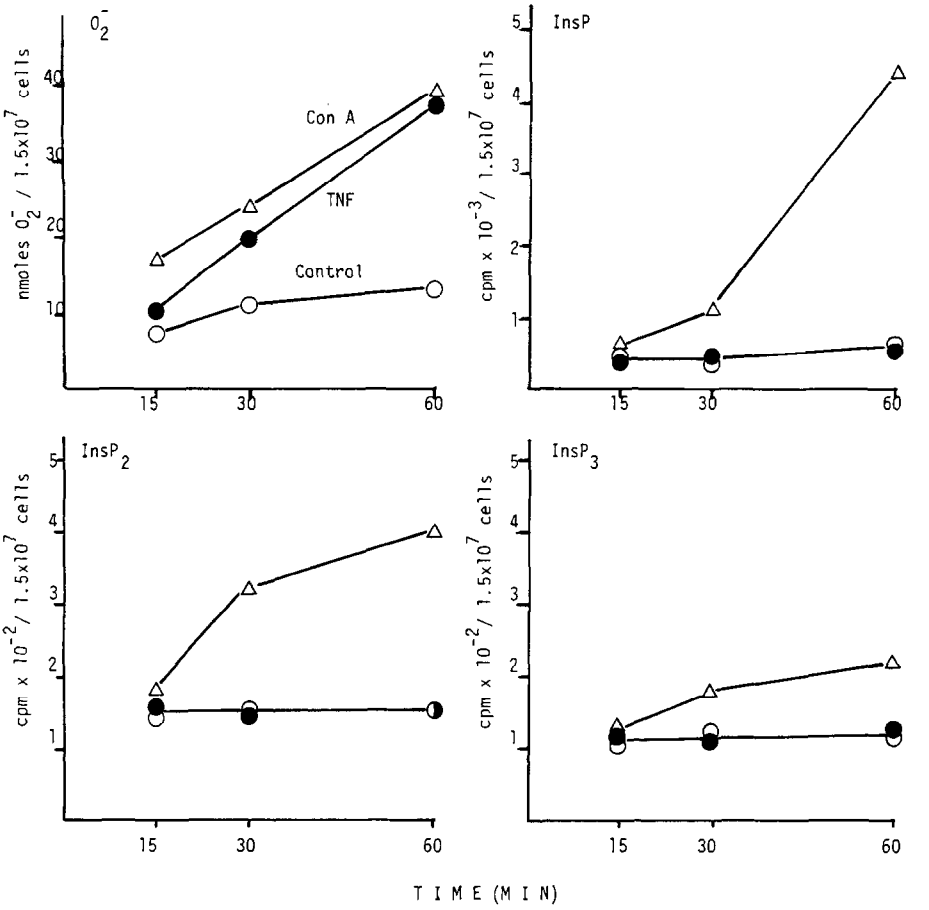

Figure 1. Inositol phosphates formation by adherent neutrophils in response to TNF. Formation of inositol phosphate (InsP), inositol biphosphate ($InsP_2$) and inositol trisphosphate ($InsP_3$) was assayed as described in Materials and Methods in untreated (○——○) TNF-stimulated (20 ng/ml;●——●) or Con A-stimulated (30 µg/ml;△——△) neutrophils. Mean results of duplicate assays of one representative of four similar experiments are reported. O_2^- generation in control (○——○), or TNF-(●——●) and Con A-stimulated (△——△) neutrophils in the same experiment is reported in the upper left panel.

staurosporine, at doses which inhibited the response to PMA by more than 80% in parallel experiments were weak inhibitors of the TNF-induced O_2^- generation by adherent neutrophils. Studies with inhibitors indicate that the activation of the respiratory burst by TNF does not likely involve a pertussis toxin-sensitive GTP-binding protein, is weakly sensitive to protein kinase C inhibitors and, as previously suggested (14), requires that neutrophils are adherent.

Stimulation of phosphoinositides turnover and release of arachidonic acid by TNF.

Figure 1 shows O_2^- generation and the associated formation of InsP, $InsP_2$ and $InsP_3$ by adherent neutrophils stimulated with Con A

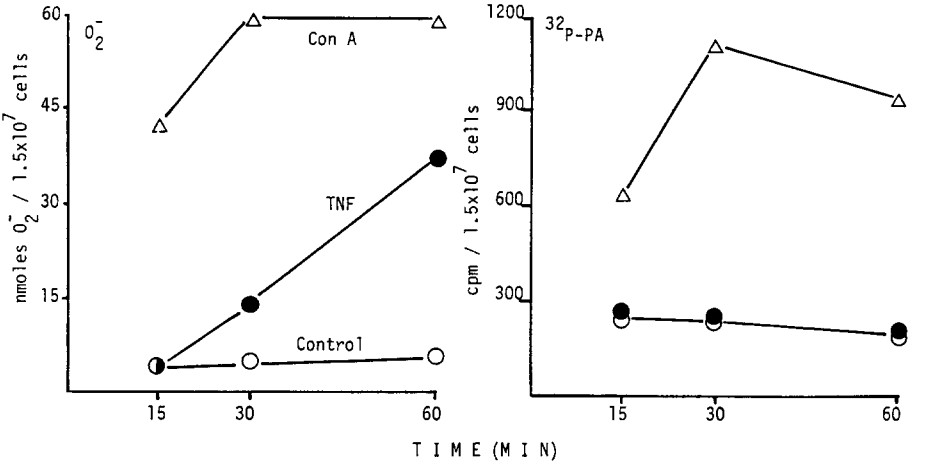

Figure 2. Phosphatidic acid formation by adherent neutrophils in response to TNF. Phosphatidic acid was assayed as described in Materials and Methods in untreated (○——○), TNF-stimulated (20 ng/ml;●——●) or Con A-stimulated (50 μg/ml;△——△) neutrophils. Mean results of duplicate assays of one representative of three experiments are reported. The left panel shows O_2^- generation in response to TNF and Con A in the same experiment.

and TNF. The Con A-stimulated formation of inositol phosphates was time-dependent and paralleled O_2^- generation. On the contrary, TNF did not increase the formation of inositol phosphates above the background up to 60 minutes of incubation. These data indicate that TNF stimulates the respiratory burst without activating phosphoinositides turnover.

Further support to this conclusion derived from studies on ^{32}P-phosphatidic acid (^{32}P-PA) formation. As shown in figure 2, Con A stimulated ^{32}P-PA formation while TNF had no effect. Incubation of neutrophils with $^{32}P_i$ for 90 min is known to cause the labelling mainly of phosphoinositides(23). In these conditions, the agonist-induced formation of ^{32}P-PA reflects the hydrolysis of phosphoinositides via phospholipase D or of phosphoinositides and other phospholipids via phospholipase C with the production of diacylglycerol (DAG) and its phosphorylation to ^{32}P-PA with ^{32}P-ATP by a DAG kinase. The lack of formation of ^{32}P-PA in adherent neutrophils stimulated with TNF does indicate that neither a phospholipase D nor a phospholipase C active on phosphoinositides are activated by TNF.

Figure 3 reports experiments concerning the release of arachidonic acid. Con A and the calcium ionophore A23187 stimulated the release of arachidonic acid and the generation of O_2^- in parallel. In response to A23187 arachidonic acid release was substantial already at 15 minutes and in response to Con A

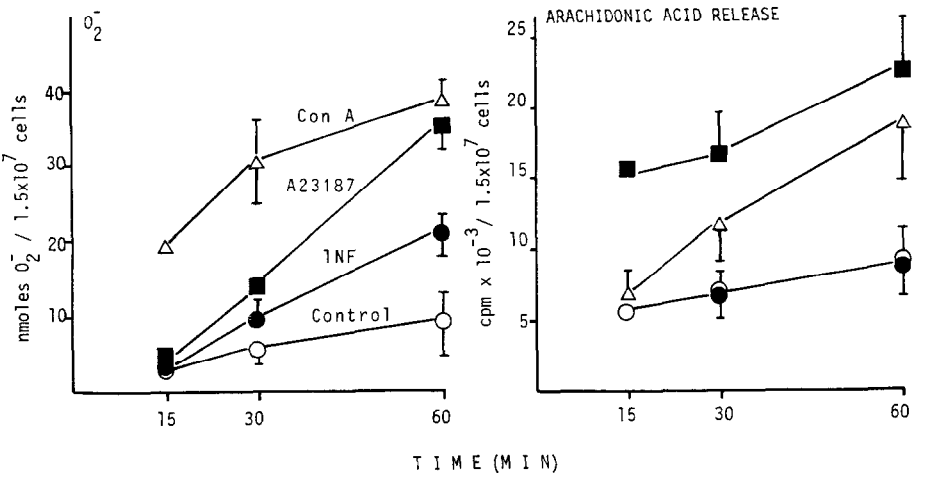

Figure 3. Arachidonic acid release by adherent neutrophils in response to TNF. Arachidonoc acid release was assayed as described in Materials and Methods in untreated (○——○), TNF-stimulated (20 ng/ml;●——●), A23187-stimulated (8 μM;■——■) or Con A-stimulated (50 μg/ml;△——△) neutrophils. Mean results ± SD of two experiments are reported. The left panel shows O_2^- generation in response to TNF, A23187 and Con A in the same experiments.

increased with time up to 60 min. TNF did not trigger arachidonic acid release above background at all the time intervals tested.

DISCUSSION

Many agonists able to trigger a respiratory burst in neutrophils also activate phosphoinositides turnover generating messengers responsible for increase in $[Ca]_i$ and activation of protein kinase C (reviewed in 17). This evidence does not however prove that the two events are causally related. Studies performed in our laboratory showed that buffering of $[Ca]_i$ to about 10-20 nM with Quin-2 and EGTA prevents the stimulation either of the respiratory burst and of phosphoinositides turnover by different agonists . However, calcium-depleted neutrophils can undergo a respiratory burst when stimulated with two soluble agonists, such as Con A and FMLP (18) , or yeast particles coated with Con A (19) and IgG or C3b (20). The results presented in this paper are the first to show that activation of the respiratory burst by an endogenous, physiologic stimulus like TNF, and in conditions likely occurring in vivo, is independent of activation of phosphoinositides turnover.

It may be relevant that activation of the respiratory burst in calcium-depleted neutrophils requires the ligation of more than one surface receptor. Calcium-depleted neutrophils do not respond to

Con A or FMLP when added alone, but become responsive when challenged with the two stimuli added in sequence or at the same time (18). Yeast particles coated with Con A (19), IgG or C3b (20) are recognized by receptors for the opsonin but likely also for glucose residues of the glucan yeast surface (24). Since it has been shown that TNF enhances the expression of the CD11/CD18 complex of adhesion receptors in neutrophils and, at the same time, "activates" adhesion receptors to mediate adhesion (25) the experimental system we used could involve a double interaction beween TNF and adhesion molecules with their specific receptors.

The results presented in this paper show that the stimulation of the respiratory burst by TNF takes place independently of formation of inositolphosphates, the products of the hydrolysis of phosphoinositides by phospholipase C, and of phosphatidic acid, the product of hydrolysis of phosphoinositides by phospholipase D, and of phosphorylation of diacylglycerol generated by phospholipase C active also on phospholipids different from phosphoinositides. These results do not exclude that other modifications of phospholipids are induced by TNF. Studies are in progress in our laboratory on hydrolysis of phosphatydylcholine by phospholipases D and C (26). However it is worth pointing out that the weak sensitivity to H-7 and staurosporin could indicate that the activation of NADPH oxidase by TNF occurs independently of messengers able to stimulate the activity of protein kinase C.

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