Priming of human neutrophil superoxide generation by tumour necrosis factor-α is signalled by enhanced phosphatidylinositol 3,4,5-trisphosphate but not inositol 1,4,5-trisphosphate accumulation

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Abstract In human neutrophils, significant agonist-stimulated superoxide anion (O_2^-) release is observed only after exposure to a priming agent such as TNFα. We have investigated the potential for TNFα to modulate *N*-formyl-Met-Leu-Phe (fMLP)-triggered Ins(1,4,5)P_3 and PtdIns(3,4,5)P_3 accumulation. TNFα pretreatment did not affect basal or stimulated Ins(1,4,5)P_3 levels but greatly upregulated fMLP-stimulated PtdIns(3,4,5)P_3 accumulation, in a manner that matched, both temporally and in magnitude, the increase in O_2^- generation implying a possible role for PtdIns(3,4,5)P_3 in signalling primed O_2^- release.

Key words: Neutrophil; Tumor necrosis factor; Phosphoinositide 3-hydroxykinase

1. Introduction

Neutrophils are a vital component of the early non-specific immune response and hence patients who are neutropenic or who have defective leukocyte function are highly susceptible to bacterial infection [1]. However, neutrophils may also cause tissue damage when activated prematurely or to excess, and have been implicated in the pathogenesis of a wide variety of inflammatory diseases [2] including the adult respiratory distress syndrome [3].

The likelihood of neutrophil-mediated tissue damage is determined largely by the activation status of these cells, which can range from quiescent through primed to fully activated. Whereas incubation of an unprimed neutrophil with a secretagogue agonist induces only minimal cell activation, prior exposure to a priming agent (which on its own is unable to stimulate degranulation or O_2^- release) greatly amplifies the magnitude of the response to an activating agonist [4]. The wide variety of priming agents so far identified (e.g. lipopolysaccharide [4], tumour necrosis factor-α (TNFα) [5], granulocyte-macrophage colony-stimulating factor (GM-CSF) [6] and platelet-activating factor [7]) has made it difficult to dissect out the signal transduction mechanism(s) underlying priming. Activation of phospholipase A_2 (PLA_2) has been implicated in neutrophil priming [8] and activation [9], but while the release of arachidonic acid and O_2^- are both 'primable' events they display different sensitivities to calcium influx and inhibition of phosphoinositide 3-hydroxykinase (PI3K) [10], and when released under physiological conditions arachidonic acid appears unable to stimulate or modulate O_2^- release [11]. Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) have also been implicated in the priming process [12], but there have been no studies detailing the effects of priming agents on secretagogue-induced Ins(1,4,5)P_3 generation. To date, only single time point measurements of the effects of GM-CSF priming on fMLP-stimulated Ins(1,4,5)P_3 have been reported, which show either enhanced [13] or unaltered [14] Ins(1,4,5)P_3 accumulation.

In addition to activating phosphoinositidase C (PIC), many neutrophil secretagogues also stimulate PI3K, resulting in the formation of PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2 [15]. The ability of wortmannin, a specific and irreversible PI3K inhibitor, to abolish both fMLP- and phagocytosis-induced respiratory burst activity in the absence of any effect on agonist-induced $[Ca^{2+}]_i$ fluxes, phorbol ester-mediated NADPH oxidase activation or granule exocytosis [16–18] has provided evidence in support of a central second-messenger role for PtdIns(3,4,5)P_3 in O_2^- generation.

In view of the apparently independent but obligatory roles played by PtdIns(3,4,5)P_3 and Ins(1,4,5)P_3 in stimulating NADPH oxidase activity in the neutrophil [16], and the uncertainty regarding the mechanisms underlying the ability of priming agents to facilitate agonist-stimulated O_2^- generation, we have investigated the effects of the archetypal priming agent TNFα on fMLP-induced PIC and PI3K activation. We demonstrate that priming of human neutrophils by TNFα has no effect on fMLP-induced Ins(1,4,5)P_3 formation but results in a major enhancement of fMLP-stimulated PtdIns(3,4,5)P_3 accumulation, the degree and timing of which match precisely the priming of the O_2^- response. These data suggest that PtdIns(3,4,5)P_3 may be instrumental in signalling the primed release of O_2^-, with TNFα acting to enhance the efficacy of fMLP receptor coupling to PI3K.

2. Materials and methods

2.1. Materials

Cytochrome *c*, fMLP, superoxide dismutase, and phosphate-buffered saline (PBS) were purchased from Sigma Chemical Company (Poole, Dorset, UK). Sterile phosphate-free HEPES-buffered Hanks solution was obtained from Gibco (Life Technologies Ltd., Renfrew-

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Abbreviations: TNFα, tumour necrosis factor-α; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; fMLP, *N*-formyl-Met-Leu-Phe; GM-CSF, granulocyte-macrophage colony-stimulating factor; PI3K, phosphoinositide 3-hydroxykinase; PIC, phosphoinositide-specific phospholipase C

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shire, UK). Human recombinant TNFα (Genzyme, Cambridge, MA) was stored at −70°C and diluted in PBS or phosphate-free buffer immediately prior to use. Percoll was obtained from Pharmacia (Uppsala, Sweden) and Ins(1,4,5)P_3 from Research Biochemicals International (St Albans, UK). [^{32}P]P_i (40 mCi/ml in HCl- and carrier-free aqueous solution), and [^{3}H]Ins(1,4,5)P_3 (17–20 Ci/mmol) were supplied by DuPont (UK) Ltd. (Stevenage, UK). Partisphere SAX HPLC columns were obtained from Whatman Chromatography (Maidstone, UK) and polyethyleneimine (PEI) TLC plates from CamLab (Cambridge, UK). All other reagents were purchased from BDH (Leicestershire, UK).

2.2. Preparation of human neutrophils

Human peripheral blood neutrophils were prepared using dextran sedimentation and centrifugation through discontinuous plasma-Percoll gradients as previously described [19]. Sterile, LPS-free reagents and plasticware were used throughout. Neutrophils were routinely >98% viable (trypan blue exclusion) and >95% pure, with <0.1% mononuclear cell contamination (cytospin and flow cytometry).

2.3. TNFα priming of superoxide anion (O_2^-) release

Neutrophils (10^6/100 μl) were incubated with TNFα (10–10 000 U/ml) in PBS containing divalent cations (2.3 mM $CaCl_2$, 0.48 mM $MgCl_2$) in a shaking water bath at 37°C for 30 min. Pre-warmed cytochrome *c* (final concentration 1.2 mg/ml in PBS) and fMLP (100 nM) or vehicle (final DMSO concentration <0.001%, v/v) were added and O_2^- release calculated from the superoxide dismutase-inhibitable reduction of cytochrome *c*. The kinetics of O_2^- release were also measured by lucigenin-dependent chemiluminescence (LDCL) using a ML 3000 microtitre plate luminometer (Dynatech Laboratories Ltd, Billinghurst, UK). Lucigenin (0.25 mM in PBS containing 1 mg/ml BSA) was added (100 μl) to each well and allowed to equilibrate at 37°C for 15 min prior to the addition of 80 μl (1×10^6) of TNFα primed (100 U/ml) or unprimed cells. Buffer (20 μl) or fMLP (100 nM) was added to each of the wells and chemiluminescence recorded continuously at 14-s intervals for 12 min. Data were recorded on-line (Cellular Chemiluminescence, Dynatech Laboratories Ltd) to produce mean LDCL values from triplicate wells.

2.4. Measurement of fMLP-stimulated Ins(1,4,5)P_3 accumulation

Neutrophils (225-μl aliquots of 32.2×10^6 cells/ml) in PBS containing divalent cations were incubated with TNFα (100 U/ml) or PBS for 30 min at 37°C prior to stimulation with fMLP (1 or 100 nM) or PBS. Reactions (300 μl) were stopped at 0–120 s by the addition of 60 μl ice-cold 3 M trichloroacetic acid (TCA). Neutralised extracts were prepared by the addition of 75 μl 10 mM EDTA (pH 7.0) and 300 μl 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine (1:1, v/v) to 300 μl of the TCA extracts. The pH of the upper phase was adjusted to 7.0 using 60 mM $NaHCO_3$ and Ins(1,4,5)P_3 mass determined by radioreceptor assay as previously detailed [20].

2.5. Measurement of fMLP-stimulated [^{32}P]PtdIns(3,4,5)P3 accumulation

Neutrophils were washed twice in sterile, phosphate-free HEPES-buffered Hanks solution (110 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 1.5 mM $CaCl_2$, 30 mM HEPES, pH 7.4) and resuspended (10^8 cells in 2.25 ml buffer) with 5 mCi [^{32}P]P_i. The cells were incubated at 37°C in a shaking water bath for 70 min, washed twice and resuspended in 1.2 ml phosphate-free buffer. Aliquots (100 μl) were incubated for 30 min with TNFα (100 U/ml) or buffer, and stimulated with fMLP (100 nM) or vehicle for 10 or 60 s. Reactions were stopped with 675 μl methanol/chloroform (2:1, v/v). Samples were acidified by the addition of 158 μl of 2.4 M HCl/5 mM tetrabutyl-ammonium sulphate and partitioned using 675 μl chloroform. The lower phase was washed with 658 μl synthetic upper phase (methanol:HCl:chloroform, 48:47:3, v/v/v), dried under vacuum and the lipids deacylated with monomethylamine reagent. Deacylated lipids were extracted in the presence of 200 μl phytate hydrolysate (12 mg phosphorus per sample) with 240 μl *n*-butanol/light petroleum/ethylformate (20:4:1, v/v/v) and the lower phase dried. The [^{32}P]glycerophosphoesters were dissolved in 2 ml H_2O and resolved by anion-exchange HPLC on a Partisphere SAX column. In experiments where resolution of [^{32}P]glycerophosphoester isomers was not required, samples were redissolved in 2 ml 20 mM HCl, 1 mM K_2PO_4 chromatographed on polyethyleneimine-cellulose plates and developed in 0.48 M HCl. The radioactivity in the [^{32}P]GroPInsP$_3$ spots was quantified using a phosphoimager (Molecular Dynamics).

3. Results

3.1. TNFα priming of O_2^- generation in human neutrophils

Unprimed neutrophils released minimal quantities of O_2^- when challenged with a maximal concentration of fMLP (Fig. 1A,B). Priming with TNFα for 30 min resulted in a concentration-dependent increase in fMLP-stimulated O_2^- generation (Fig. 1A). Since TNFα at 100 U/ml amplified fMLP-induced O_2^- release by 4.5-fold and had no effect on

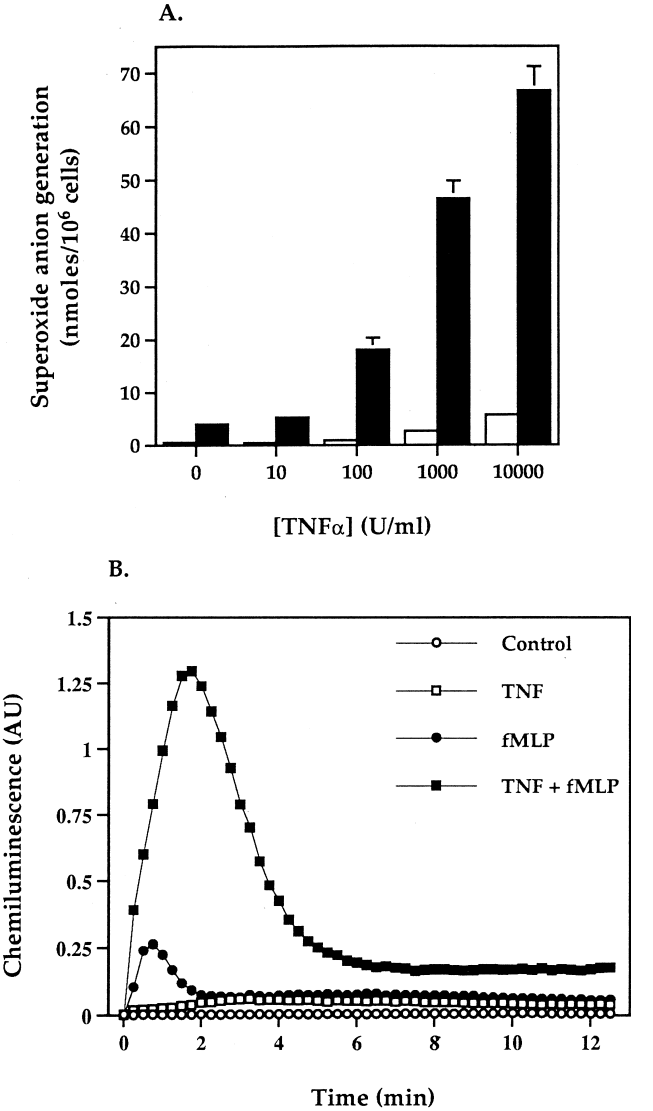

Fig. 1. TNFα priming of fMLP-stimulated O_2^- generation in human neutrophils: concentration response and time course. Human neutrophils were incubated with 0–10 000 U/ml TNFα for 30 min prior to stimulation with buffer (open bars) or 100 nM fMLP (closed bars). Quantification of O_2^- release (A) during a 10 min incubation with fMLP by superoxide dismutase-inhibitable reduction of cytochrome *c* (data points represent mean ± S.E.M. of three separate experiments each performed in triplicate) and (B) with lucigenin-dependent chemiluminescence (LDCL), monitored at 14-s cycle intervals in: control unprimed cells (○), TNFα-primed (100 U/ml, 30 min) unstimulated cells (□), unprimed fMLP (100 nM)-stimulated cells (●) and TNFα-primed, fMLP-stimulated cells (■). Data are from a single experiment, representative of four.

spontaneous O_2^- production, this concentration was used in subsequent experiments. Analysis of the kinetics of O_2^- release following fMLP stimulation (Fig. 1B) revealed that this response was rapid (onset within 14 s, peak at 60–90 s) and both augmented and prolonged following TNFα priming.

3.2. *Ins(1,4,5)P_3 generation in TNFα primed and unprimed neutrophils*

Stimulation of unprimed neutrophils with 100 nM fMLP (a maximal stimulus for Ins(1,4,5)P_3 and O_2^- generation, data not shown) caused a rapid and transient elevation of Ins(1,4,5)P_3 reaching a value 4-fold higher than resting levels at 10 s and thereafter declining to control levels by 60 s (Fig. 2A). Incubation with TNFα (100 U/ml) for 30 min (conditions that induced a major upregulation in the O_2^- response) altered neither basal nor fMLP-stimulated Ins(1,4,5)P_3 levels (Fig. 2A). TNFα also failed to influence the time course of Ins(1,4,5)P_3 accumulation following stimulation with a submaximal (EC_{40}) concentration of fMLP (1 nM) (Fig. 2B). Ins(1,4,5)P_3 mass was also determined at 5 and 10 min following fMLP stimulation and again, no significant differences were observed between primed and unprimed cells (data not shown).

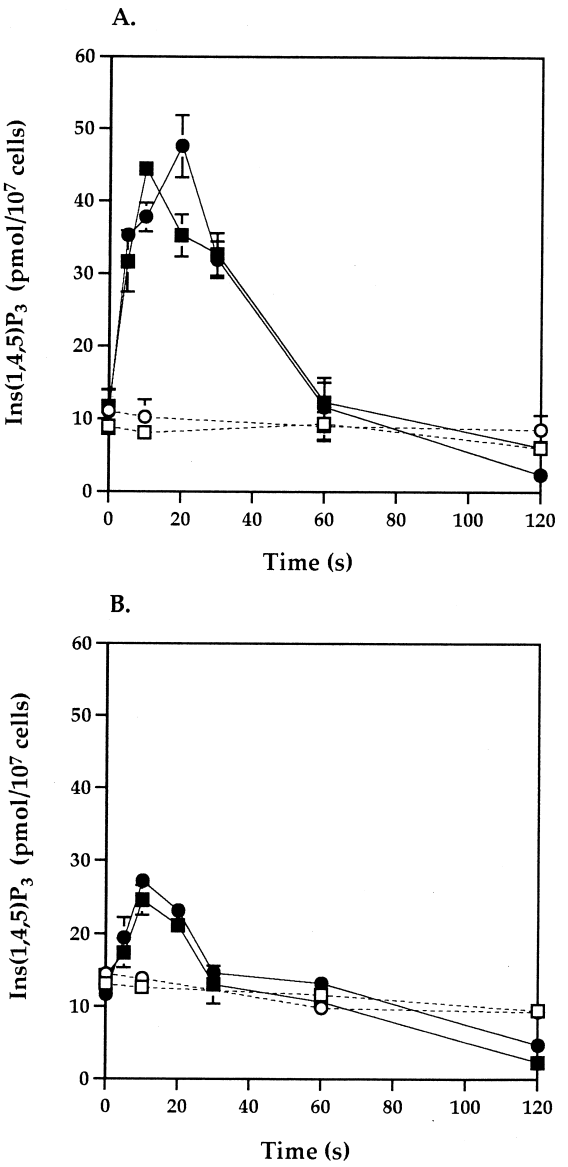

Fig. 2. Effect of TNFα priming on fMLP-stimulated Ins(1,4,5)P_3 mass in human neutrophils. Human neutrophils were incubated with TNFα 100 U/ml (circles) or PBS (squares) at 37°C for 30 min and stimulated with fMLP (closed symbols) or PBS (open symbols). The concentration of fMLP used was 100 nM in A and 1 nM (EC_{40}) in B. Ins(1,4,5)P_3 mass was determined in neutralised TCA extracts by radioreceptor assay. Data represent mean ± S.E.M. for three separate experiments each performed in duplicate.

3.3. *[^{32}P]PtdIns(3,4,5)P_3 generation in TNFα-primed and unprimed neutrophils*

Use of sterile, endotoxin- and phosphate-free HEPES-buffered Hanks solution and a 70 min incubation with 2 mCi/ml of [^{32}P]P_i permitted sufficient [^{32}P]PtdIns(4,5)P_2 labelling to quantify changes in [^{32}P]PtdIns(3,4,5)P_3 levels yet did not abrogate the O_2^- priming response or induce spontaneous O_2^- generation (Fig. 3). Basal and fMLP-stimulated [^{32}P]PtdIns(3,4,5)P_3 accumulation was quantified at both 10 s (to correspond with the time of peak Ins(1,4,5)P_3 and [^{32}P]PtdIns(3,4,5)P_3 accumulation in unprimed cells; Fig. 2 and [15]) and 60 s (to correspond with the peak primed O_2^- response; Fig. 1B) following fMLP addition, with O_2^- assayed in parallel. While TNFα alone (100 U/ml, 30 min) did not affect basal [^{32}P]PtdIns(3,4,5)P_3 levels, it enhanced the fMLP-stimulated [^{32}P]PtdIns(3,4,5)P_3 signal by 1.4-fold at 10 s (Table 1), and more dramatically by 6.2-fold at 60 s (Table 1 and Fig. 3). As shown in Table 1 there was a substantial (> 100-fold) increase in [^{32}P]PtdIns(3,4,5)P_3 accumulation in the TNFα primed, fMLP-stimulated cells compared to levels in control unstimulated cells. This ability of TNFα to augment fMLP-stimulated [^{32}P]PtdIns(3,4,5)P_3 matched precisely the observed increases in O_2^- generation when measured under identical conditions (Fig. 3).

3.4. *Effects of TNFα and fMLP on [^{32}P]phosphoinositide metabolism*

Incubation of [^{32}P]P_i-labelled neutrophils with 100 U/ml TNFα for 30 min resulted in a small but significant increase in [^{32}P]PtdIns4P levels but no consistent effect on [^{32}P]PtdIns(4,5)P_2, the immediate precursor of PtdIns(3,4,5)P_3 (Table 1). The changes in [^{32}P]PtdIns(3,4,5)P_3 were paralleled very closely by changes in its immediate metabolite [^{32}P]PtdIns(3,4)P_2, indicating that the ability of TNFα to augment fMLP-stimulated [^{32}P]PtdIns(3,4,5)P_3 accumulation does not reflect inhibition of its metabolism. Following 10 s fMLP stimulation, very similar changes in [^{32}P]PtdIns(3,4)P_2 and [^{32}P]PtdIns3P levels were observed in both primed and unprimed cells whilst at 60 s post fMLP stimulation the accumulation of both phosphoinositides was much greater in primed cells (Table 1) and mirrored the changes observed in [^{32}P]PtdIns(3,4,5)P_3 levels. At 60 s following fMLP stimulation, depletion of [^{32}P]PtdIns(4,5)P_2 was also much greater in the primed cells.

4. Discussion

We have demonstrated that priming of human neutrophils by TNFα results in a considerable enhancement of fMLP-induced [^{32}P]PtdIns(3,4,5)P_3 accumulation. The time course and magnitude of this response match precisely the effect of TNFα on fMLP-stimulated O_2^- generation suggesting that

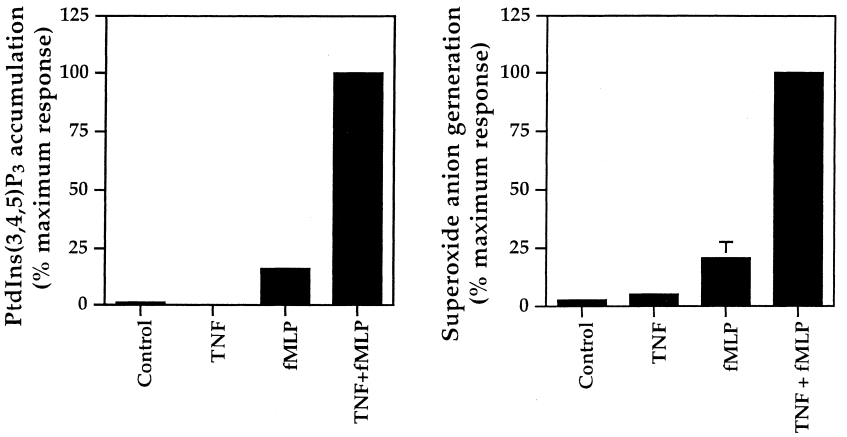

Fig. 3. Effect of TNFα priming on fMLP-stimulated [^{32}P]PtdIns(3,4,5)P_3 accumulation and superoxide generation in human neutrophils. Left panel: Human neutrophils were suspended in phosphate-free buffer and labelled in bulk with 5 mCi [^{32}P]P_i for 70 min at 37°C. Cells were subsequently washed and incubated for 30 min with TNFα (100 U/ml) or buffer. Following 60 s stimulation with fMLP (100 nM) the reactions were quenched and [^{32}P]PtdIns(3,4,5)P_3 levels determined by TLC as detailed. Data represent mean of % maximum responses for three separate experiments each performed in triplicate. S.E.M.s were all $<5\%$ of individual values. Right panel: Human neutrophils derived from the same cell preparations as used above and incubated and primed in an identical manner were stimulated with buffer or fMLP (100 nM) for 10 min in the presence of cytochrome *c* to allow measurement of O_2^- release. Data (expressed as % of maximum response) represent mean ± S.E.M. from three separate experiments. Where not shown, S.E.M.s were $<5\%$ of individual values.

PtdIns(3,4,5)P_3 may play a vital role in signalling the primed release of O_2^-. In contrast, TNFα did not influence fMLP-stimulated Ins(1,4,5)P_3 accumulation.

The mechanism whereby TNFα enhances fMLP-induced PtdIns(3,4,5)P_3 accumulation in neutrophils, and the downstream effectors of PtdIns(3,4,5)P_3 that result in O_2^- generation, are at present, unknown. Under the conditions employed in this study we have previously shown that the priming effect of TNFα is mediated exclusively by stimulation of the p55 TNFα receptor (TNF-R1) [21] and is not mimicked by ceramide [22]. Current knowledge of TNF-R1 signalling protein interactions provides little insight into how this receptor may interact with phospholipid signalling pathways. Since there was no apparent increase in PtdIns(4,5)P_2 substrate availability or obvious inhibition of PtdIns(3,4,5)P_3-5 phosphatase activity (as judged from the similar increases in [^{32}P]PtdIns(3,4)P_2 levels), it is likely that TNFα acts directly at the level of the PI3K, or at a more proximal transduction step, and does not relate to the reported ability of TNFα to enhance agonist-stimulated phospholipase D activity [23]. Furthermore, the fact that fMLP-stimulated Ins(1,4,5)P_3 accumulation was not increased by TNFα strongly suggests that the enhanced [^{32}P]PtdIns(3,4,5)P_3 response does not reflect a change in plasma membrane fMLP receptor or $G_{i2\alpha}$ expression or enhanced $G_{\beta\gamma}$ release [24]. The possibility that TNFα induces the recruitment of a tyrosine kinase-regulated PI3K to augment, or work in parallel with, the $G_{\beta\gamma}$-regulated PI3K is supported by the recent demonstration [25] that fMLP and insulin exert a synergistic effect on PtdIns(3,4,5)P_3 formation in THP1 cells, suggesting that there may be cross-talk between the tyrosine phosphorylation-dependent and the G-protein-dependent PI3K pathways.

Whilst PtdIns(3,4,5)P_3 generation has been implicated in a variety of cellular processes [26], the precise role(s) of PtdIns(3,4,5)P_3 and its immediate product PtdIns(3,4)P_2 remains to be elucidated. Studies in a variety of cell lines, utilising receptor and enzyme mutants and PI3K inhibitors, have suggested that PI3K may lie upstream of several protein kinase cascades, including p70 S6 kinase [27], the MAPK pathway [28] and the serine-threonine kinase Akt-1/PKB [29]; however, the relevance of these observations to O_2^- generation in neutrophils is at present unclear. Activation of Ca^{2+}-inde-

Table 1
Effects of TNFα on fMLP-stimulated changes in human neutrophil [^{32}P]phosphoinositide levels

		PtdIns4P	PtdIns(4,5)P_2	PtdIns3P	PtdIns(3,4)P_2	PtdIns(3,4,5)P_3
Control	10 s	39 ± 3	100	1.3 ± 0.1	0.07 ± 0.01	0.23 ± 0.39
	60 s	40 ± 3	100	1.0 ± 0.2	0.10 ± 0.02	0.13 ± 0.01
TNF	10 s	58 ± 1	119 ± 6	1.9 ± 0.1	0.12 ± 0.01	0.29 ± 0.09
	60 s	55 ± 3	95 ± 9	1.6 ± 0.1	0.16 ± 0.02	0.12 ± 0.01
fMLP	10 s	37 ± 2	80 ± 4	1.4 ± 0.1	0.97 ± 0.07	4.10 ± 0.24
	60 s	45 ± 1	91 ± 1	1.3 ± 0.1	1.28 ± 0.12	2.22 ± 0.15
TNF+fMLP	10 s	54 ± 2	100 ± 5	20 ± 0.2	1.35 ± 0.10	6.49 ± 0.26
	60 s	49 ± 2	65 ± 1	2.6 ± 0.2	9.76 ± 0.85	13.78 ± 1.47

[^{32}P]P_i-labelled human neutrophils were incubated in the presence or absence of 100 U/ml TNFα for 30 min prior to stimulation with buffer or 100 nM fMLP. Reactions were quenched at 10 s or 60 s and the lipids extracted and deacylated as described. The [^{32}P]glycerophosphoesters were resolved by HPLC using a Partisphere SAX column. To permit a clearer understanding of the relative amounts of ^{32}P-labelled 3-phosphoinositides in neutrophils, and the changes observed under the defined conditions, data are expressed as per cent of control [^{32}P]PtdIns(4,5)P_2 dpm values (41 027 ± 2232 dpm, experiment quenched at 10 s; 24 062 ± 830 dpm, experiment quenched at 60 s). The results shown are mean ± S.E.M. of triplicate determinations from a single representative experiment.

pendent isoforms of PKC by PtdIns(3,4,5)P_3 has also been demonstrated [30]; since $p47^{phox}$ phosphorylation represents a key step in NADPH oxidase assembly and can be induced by PKC activation, this represents a potential mechanism by which PI3K could influence O_2^- generation. However, activation of PKC by alternative routes (e.g. PIC-mediated increases in $[Ca^{2+}]_i$ and diacylglycerol levels) also occurs during fMLP stimulation, hence PKC is unlikely to be the sole PtdIns(3,4,5)P_3 effector pathway involved. The small G-protein $p21^{rac}$, whose translocation to the neutrophil membrane is essential for O_2^- generation [31], is also a downstream target of PtdIns(3,4,5)P_3 [32], and increased translocation of $p21^{rac}$ may lead to an enhanced O_2^- response [33].

Forehand and colleagues have previously demonstrated a small enhancement of fMLP-stimulated $[Ca^{2+}]_i$ responses in lipopolysaccharide-primed neutrophils [12]. Our results indicate that such differences are unlikely to result from priming-induced changes in Ins(1,4,5)P_3 formation or metabolism. Calcium studies performed at a single cell level, however, indicate that priming probably results from the recruitment of only a proportion of cells into a more highly agonist-responsive pool [34]. Hence in view of the marked heterogeneity that may exist in the density of Ins(1,4,5)P_3-sensitive Ca^{2+} channels within intracellular Ca^{2+} stores [35], small or localised changes in Ins(1,4,5)P_3 could conceivably still contribute to signalling the primed state, with the current mass assay not sufficiently sensitive to detect such changes.

In summary, we have demonstrated that fMLP-stimulated PtdIns(3,4,5)P_3, but not Ins(1,4,5)P_3, accumulation is augmented in TNFα-primed human neutrophils and that this effect mirrors very closely the ability of TNFα to enhance fMLP-mediated O_2^- generation. These data, together with the documented ability of wortmannin to abolish respiratory burst activity in these cells, suggest that enhanced PtdIns(3,4,5)P_3 accumulation may represent a key mechanism underlying the ability of priming agents to modulate secretagogue-stimulated O_2^- generation.

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