TNF-α Induces Phosphorylation of p47phox in Human Neutrophils: Partial Phosphorylation of p47phox Is a Common Event of Priming of Human Neutrophils by TNF-α and Granulocyte-Macrophage Colony-Stimulating Factor

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*J Immunol* 2003; 171:4392-4398; doi: 10.4049/jimmunol.171.8.4392
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TNF-α Induces Phosphorylation of p47\textit{phox} in Human Neutrophils: Partial Phosphorylation of p47\textit{phox} Is a Common Event of Priming of Human Neutrophils by TNF-α and Granulocyte-Macrophage Colony-Stimulating Factor

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Phosphorylation of p47\textit{phox} is a key event in NADPH oxidase activation. We examined the ability of proinflammatory cytokines such as TNFα, IL-1, and G-CSF to induce this process compared with GM-CSF. Only TNF-α and GM-CSF induced a clear p47\textit{phox} phosphorylation. This phosphorylation was time dependent and reached its maximum at 20 min. Two-dimensional phosphopeptide mapping of p47\textit{phox} phosphorylated in neutrophils primed with TNF-α revealed partial phosphorylation of p47\textit{phox} on the same peptide as for GM-CSF. Neutrophil incubation with TNF-α and subsequent addition of the chemotactic peptide fMLP resulted in more intense phosphorylation of p47\textit{phox} sites than with each reagent alone. A neutralizing Ab against the p55 TNF receptor, contrary to a neutralizing Ab against the p75 TNF receptor, inhibited TNF-α-induced p47\textit{phox} phosphorylation. Neutrophil treatment with both TNF-α and GM-CSF resulted in more intense phosphorylation of the same p47\textit{phox} peptide observed with each cytokine alone, suggesting that they engaged pathways converging on common serines. This additive effect was also obtained on the priming of NADPH oxidase activity. The use of protein kinase inhibitors pointed to the involvement of a protein tyrosine kinase, but not protein kinase C. These findings show that TNF-α, via its p55 receptor, induces a protein tyrosine kinase-dependent selective phosphorylation of p47\textit{phox} on specific serines. The ability of TNF-α and GM-CSF, two different cytokines with different receptors to induce this specific p47\textit{phox} phosphorylation, suggests that this event could be a common element of the priming of neutrophils by TNF-α and GM-CSF. The Journal of Immunology, 2003, 171: 4392–4398.

Humans polymorphonuclear cells are the first line of host defense against invading pathogens. In response to stimulating agents such as the bacterial fMLP peptide, neutrophils release a large amount of superoxide anions and other reactive oxygen species in a phenomenon called the respiratory burst (1). The functions of these cells are enhanced by a variety of mediators, including proinflammatory cytokines (2, 3), such as TNF-α, GM-CSF, G-CSF, and IL-1. These agents prime in particular the respiratory burst in nonadherent neutrophils (4–6), but the underlying molecular mechanisms are poorly documented.

NADPH oxidase, the enzyme responsible for superoxide anion production in phagocytes, is a multicomponent enzyme consisting of a membrane-associated cytochrome b558 (composed of two subunits: gp91\textit{phox} and p22\textit{phox}) and the cytosolic components: p47\textit{phox}, p67\textit{phox}, p40\textit{phox}, and a small GTPase rac2 (7, 8). Neutrophil activation with fMLP or PMA leads to phosphorylation of cytosolic components, especially p47\textit{phox}, a protein that has an important role in translocation of the complex to cytochrome b558, and in the assembly and activation of NADPH oxidase (9–12). p47\textit{phox} is phosphorylated on several serines located in the carboxyl-terminal portion of the protein (11, 12). The multiplicity of these sites suggests that p47\textit{phox} could be targeted by multiple phosphorylation pathways, such as protein kinase C (PKC), protein kinase A, and mitogen-activated protein kinase (12–14).

We (15) and others (16) have recently shown that priming agents such as GM-CSF and bacterial LPS induce partial or limited p47\textit{phox} phosphorylation in primed neutrophils. This partial phosphorylation of p47\textit{phox} could be one mechanism by which GM-CSF primes neutrophil respiratory burst. Other candidate-priming mechanisms include increased membrane expression of cytochrome b558 (16, 17), increased expression of triggering receptors such as fMLP receptors, activation of heterotrimeric G proteins (18), and other mechanisms (2, 3).

In this study, we analyzed the effect of TNF-α, G-CSF, and IL-1 compared with GM-CSF on p47\textit{phox} phosphorylation, together with the mechanisms underlying the effect of TNF-α.

Materials and Methods

Reagents

fMLP, protease, and phosphatase inhibitors were from Sigma-Aldrich (St. Louis, MO). [32\textit{P}]Orthophosphoric acid was from NEN Life Science Products (Boston, MA). Kinase inhibitors were from Calbiochem (San Diego, CA). Injection-grade water and 0.9% NaCl were endotoxin free (<0.4 pg/ml) in the limulus test (Charles River, Charleston, SC). Endotoxin-free buffers and salt solutions were from Life Technologies (Cergy, France). Human rTNF-α (rTNF-α), rhGM-CSF, rhIL-1, rhG-CSF, and neutralizing Abs against p55 and p75 TNF receptor (TNF-R55 and TNF-R75) were from R&D Systems (Minneapolis, MN). Rabbit polyclonal Ab against p47\textit{phox} (from B. M. Babior, The Scripps Research Institute, La Jolla, CA) was raised against the 10 COOH-terminal residues of p47\textit{phox} and purified, as described by Babior’s laboratory (19, 20).

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3 Abbreviations used in this paper: PKC, protein kinase C; rh, recombinant human.
Neutrophil preparation

Human neutrophils were obtained in LPS-free conditions by means of dextran sedimentation and Ficoll centrifugation, as described previously (15).

Superoxide anion production assay

Neutrophils were treated with various cytokines at 37°C for 20 min in sterile HBSS. Thereafter, superoxide production was measured in response to 0.1 μM fMLP in the superoxide dismutase-inhibitable ferricytochrome c reduction assay (13, 15).

$^{32}$P labeling, stimulation, and fractionation of neutrophils

Neutrophils were incubated in phosphate-free buffer (20 mM HEPES, pH 7.4, 140 mM NaCl, 5.7 mM KCl, 0.8 mM MgCl$_2$, and 0.025% BSA) (15) containing 0.5 μCi of $[^{32}$P]$^3$orthophosphoric acid/10$^6$ cells/ml for 60 min at 30°C. Neutrophils were then incubated at 37°C for 5 min before treatment with cytokines for 20 min. In experiments with inhibitors, neutrophils were incubated for 15 min with inhibitors before treatment with TNF-α. The reaction was stopped by adding ice-cold buffer and centrifugation at 400 × g for 7 min at 4°C. The cells were lysed by resuspending them in lysis buffer, as previously described (12, 15). The suspension was sonicated on ice for 3 × 15 s. The lysate was centrifuged at 100,000 × g for 20 min at 4°C in a TL100 Ultracentrifuge (Beckman, Fullerton, CA).

Immunoprecipitation of p47$^{phox}$

The cleared supernatant was incubated overnight with anti-p47$^{phox}$ (1/200) Ab; protein was then immunoprecipitated using γ-bind G-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) and washed four times, as previously described (15).

Electrophoresis and blotting

The samples were subjected to SDS-PAGE in 10% polyacrylamide gels, using standard techniques (21). The separated proteins were transferred to nitrocellulose following Towbin's procedure (22). After blocking in 5% milk, the membrane was probed with the anti-p47$^{phox}$ Ab (1/5000 dilution), then an alkaline phosphatase-labeled goat anti-rabbit Ab, and developed using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate staining. The intensity of p47$^{phox}$ band was quantified by densitometry using the Scion Image analysis program from National Institutes of Health. For $^{32}$P radioactivity quantification (cpm) of p47$^{phox}$, nitrocellulose membranes were analyzed in an Instant Imager apparatus (Packard, Meriden, CT) equipped with Instant Imager software. Radioactivity counts were corrected for the actual amounts of p47$^{phox}$ present on the membrane.

Two-dimensional tryptic phosphopeptide mapping

Tryptic digestion of p47$^{phox}$ on nitrocellulose, thin-layer electrophoresis, and thin-layer chromatography were performed, as previously described (12, 15). Briefly, the nitrocellulose area containing $^{32}$P-labeled p47$^{phox}$ was incubated for 30 min at 37°C with polyvinylpyrrolidone, washed, then incubated overnight with trypsin (50 μg/ml) in carbonate buffer. Released peptides were washed three times in a Speed-Vac, redissolved in electrophoresis buffer (17 vol of water/3 vol of 88% formic acid), and applied to one corner of a cellulose thin-layer plate (Merck, West Point, PA). After electrophoresis (1000 V for 20 min), ascending chromatography was performed in isobutric acid buffer (12). The plates were autoradiographed for 1 wk at −75°C.

Statistical analysis

All results are expressed as means ± SEM. Significant differences were identified using Student’s t test (significance for p < 0.05).

Results

Comparison of p47$^{phox}$ phosphorylation and priming in human neutrophils induced by various cytokines

We have recently shown that GM-CSF induces phosphorylation of the cytosolic NADPH oxidase component p47$^{phox}$ in human neutrophils (15). Because p47$^{phox}$ phosphorylation is an essential event in NADPH oxidase activation and priming, in this study we compared the potency of various cytokines to induce p47$^{phox}$ phosphorylation in nonadherent neutrophils.

$^{32}$P-labeled neutrophils were treated with the following proinflammatory cytokines: TNF-α (250 U/ml), G-CSF (500 U/ml), IL-1β (50 ng/ml) compared with GM-CSF (12 ng/ml) for 20 min, then lysed; p47$^{phox}$ was immunoprecipitated, as described above in Materials and Methods; proteins were submitted to SDS-PAGE and revealed by autoradiography. As shown in Fig. 1A (upper panel), only TNF-α and GM-CSF induced a clear p47$^{phox}$ phosphorylation. The degree of TNF-α-induced p47$^{phox}$ phosphorylation was close to that obtained with GM-CSF. G-CSF induced a moderate phosphorylation, but IL-1β did not induce any p47$^{phox}$ phosphorylation. Western blot analysis showed that the same amount of p47$^{phox}$ was immunoprecipitated from each sample. Different concentrations of G-CSF and IL-1β and different incubation times yielded the same results (data not shown). Phosphorylated p47$^{phox}$ from different experiments was quantified by phosphor imager analysis and corrected for the amount of p47$^{phox}$,

![Image](http://www.jimmunol.org/)

**FIGURE 1.** Comparative potency of proinflammatory cytokines to induce p47$^{phox}$ phosphorylation and priming of fMLP-induced superoxide production in human neutrophils. A. Upper panel, $^{32}$P-labeled neutrophils were incubated with the following proinflammatory cytokines: TNF-α (250 U/ml), GM-CSF (12 ng/ml), G-CSF (500 U/ml), and IL-1β (50 ng/ml) for 20 min. p47$^{phox}$ was then immunoprecipitated with a specific Ab, subjected to SDS-PAGE, blotted on nitrocellulose, and detected by autoradiography or with anti-p47$^{phox}$ Ab. A. Lower panel, Phosphorylated p47$^{phox}$ from five experiments was quantified by phosphor imager analysis and corrected for the amount of p47$^{phox}$, which was quantified by densitometry, as described in Materials and Methods (mean ± SEM, n = 5). B. Neutrophils were incubated with the cytokines, then stimulated with fMLP (10$^{-7}$ M). Superoxide anion production was measured using the cytochrome c reduction assay at 550 nm. Control superoxide anion production (100%) is 3.9 nmol/min/10$^6$ cells for fMLP (10$^{-7}$ M) (mean ± SEM, n = 5).
which was quantified by densitometry, as described in Materials and Methods. The mean of these experiments (Fig. 1A, lower panel) shows that the order of potency of these cytokines to phosphorylate the neutrophil NADPH oxidase component p47\textsubscript{phox} was GM-CSF > TNF-\(\alpha\) > G-CSF > IL-1\(\beta\). These results show that, apart from GM-CSF, TNF-\(\alpha\) was the only cytokine tested that could induce a clear reproducible p47\textsubscript{phox} phosphorylation.

To ensure that the chosen cytokines exerted biological effects on human neutrophils, we tested their effects on the fMLP-induced respiratory burst. None of the cytokines tested showed any significant direct activation of the respiratory burst (data not shown); however, they increased fMLP-induced respiratory burst at different degrees (Fig. 1B). The priming effect of the cytokines on fMLP-stimulated superoxide production (as a percentage of the mean fMLP-stimulated superoxide production (as a percentage of the phosphor imager analysis corrected for the amount of p47\textsubscript{phox}) was 527.5 ± 106.5% (\(n = 8\)) with TNF-\(\alpha\) (250 U/ml), 523.6 ± 108.8% (\(n = 8\)) with GM-CSF (12 ng/ml), 145.3 ± 29.1% (\(n = 8\)) with G-CSF (50 ng/ml), and 182 ± 48.4% (\(n = 5\)) with IL-1\(\beta\) (50 ng/ml). As shown from these results, TNF-\(\alpha\) and GM-CSF induced equal intense priming of the respiratory burst (>5 times increase), while G-CSF and IL-1\(\beta\) exerted a weak priming effect parallelizing the effect of these cytokines on p47\textsubscript{phox} phosphorylation.

**Kinetics of p47\textsubscript{phox} phosphorylation in neutrophils exposed to TNF-\(\alpha\) and to GM-CSF**

To compare the kinetics of p47\textsubscript{phox} phosphorylation in neutrophils treated with TNF-\(\alpha\) and GM-CSF, cells were incubated with each cytokine for 10, 20, 30, and 40 min before lysis and immunoprecipitation. As shown in Fig. 2, increased p47\textsubscript{phox} phosphorylation was detected at 10 min of incubation with TNF-\(\alpha\) or GM-CSF. The same p47\textsubscript{phox} amount was present in the gels, as determined by Western blotting (data not shown). Phosphorylated p47\textsubscript{phox} from different experiments was quantified by phosphor imager analysis and corrected for the amount of p47\textsubscript{phox}, which was quantified by densitometry, as described in Materials and Methods. The mean of these experiments (Fig. 2, lower panel) shows that TNF-induced-p47\textsubscript{phox} phosphorylation reached its maximum at 20 min and remained constant, then weakly decreased at 40 min. However, GM-CSF-induced-p47\textsubscript{phox} phosphorylation increased gradually up to 40-min incubation. TNF-\(\alpha\)-induced phosphorylation was also concentration dependent in parallel with the priming effect on fMLP-induced superoxide production (data not shown).

**TNF-\(\alpha\) enhances fMLP-induced p47\textsubscript{phox} phosphorylation and induces the phosphorylation of one p47\textsubscript{phox}-selective peptide through p55 TNF receptor (TNF-R55)**

As mentioned above, TNF-\(\alpha\) has a priming effect on NADPH oxidase activation by fMLP (4–6). The priming effect of TNF-\(\alpha\) was tested on fMLP-induced p47\textsubscript{phox} phosphorylation in human neutrophils. Neutrophils were preincubated with 250 U/ml TNF-\(\alpha\) for 20 min and stimulated with 0.1 \(\mu\)M fMLP for 3 min. The results (Fig. 3, lower and upper panels) showed that p47\textsubscript{phox} phosphorylation with TNF-\(\alpha\) potentiated the phosphorylation induced by fMLP, in keeping with its effect on the respiratory burst. Western blot analysis showed that the same amount of p47\textsubscript{phox} was immunoprecipitated from each sample.

Two-dimensional phosphopeptide mapping analysis shows (Fig. 4) that TNF-\(\alpha\) induced the phosphorylation of a major p47\textsubscript{phox} peptide (shown by an arrow), whereas fMLP induced phosphorylation of several peptides. TNF-\(\alpha\) treatment of the cells resulted in the increase of the intensity of all phosphorylated peptides induced by fMLP. The new peptides that seem to appear in the fMLP + TNF map are actually weakly phosphorylated in fMLP alone; as we can see on the original film, TNF enhances their degree of phosphorylation.

These results suggest that selective p47\textsubscript{phox} phosphorylation by TNF-\(\alpha\) may facilitate the induction of p47\textsubscript{phox} phosphorylation on the other sites targeted by the pathways induced by the chemotactic agent such as fMLP.

TNF-\(\alpha\) exerts its cellular effects by binding to two types of receptors, the p55 TNF receptor (TNF-R55) and the p75 TNF receptor (TNF-R75) (23). To determine through which receptor TNF-\(\alpha\) induced p47\textsubscript{phox} phosphorylation, we used specific neutralizing mAbs (24). The phosphorylation results illustrated in Fig. 5.
show that when neutrophils were preincubated with anti-TNF-R55 Ab before adding TNF-α/H9251, p47phox phosphorylation was inhibited; in contrast, anti-TNF-R75 had no significant effect. Radioactive p47phox was quantified by Instant Imager software and corrected for the amount of p47phox, which was quantified by densitometry, as described in Materials and Methods. The results are expressed in this work as percentage of the phosphorylated p47phox control in the presence of TNF alone (100%); in the presence of anti-TNF-R55/H11001 TNF p47phox phosphorylation degree was 47.5 ± 10.2% of the control, and in the presence of anti-TNF-R75/H11001 TNF the phosphorylation was 98.6 ± 11.6% of control (mean ± SEM (n = 3) (p < 0.05 for anti-TNF-R55 + TNF)). These data indicated a major role of p55-TNF-R in the induction of p47phox phosphorylation by TNF-α.

TNF-α and GM-CSF have additive effects on the common p47phox-phosphorylated site and on fMLP-induced NADPH oxidase activation: evidence for a common priming pathway. TNF-α and GM-CSF are two different cytokines with two different receptors that prime the respiratory burst. To answer the question as to whether they induce the phosphorylation of the same peptide on p47phox, we analyzed the effect of TNF-α and GM-CSF and

FIGURE 3. Priming effect of TNF-α on fMLP-induced p47phox phosphorylation. [32P]-labeled neutrophils were incubated with or without TNF-α (250 U/ml) for 20 min. Then neutrophils were stimulated with fMLP (0.1 μM) for 3 min. p47phox was immunoprecipitated and revealed by autoradiography and Western blotting (upper panel). Phosphorylated p47phox from five experiments was quantified by phosphor imager analysis and corrected for the amount of p47phox, which was quantified by densitometry, as described in Materials and Methods (mean ± SEM, n = 5) (lower panel).

FIGURE 4. Tryptic phosphopeptide mapping of phosphorylated p47phox from TNF-α-primed and fMLP-activated neutrophils. [32P]-labeled neutrophils were incubated with or without TNF-α (250 U/ml) for 20 min. Then neutrophils were stimulated with fMLP (0.1 μM) for 3 min. p47phox was immunoprecipitated and revealed by autoradiography and Western blotting. [32P]-p47phox was recovered from nitrocellulose sheets and submitted to trypsin digestion. Peptides were subjected to first-dimension electrophoresis and second-dimension liquid chromatography, as described in Materials and Methods. Phosphorylated peptides were detected by autoradiography. +, Represents the sample application point. Data are representative of three experiments.

FIGURE 5. Effect of anti-TNF receptor Abs on TNF-induced p47phox phosphorylation. [32P]-labeled neutrophils were preincubated with anti-p55-neutralizing Ab (TNF-R55, 10 μg/ml) or anti-p75-neutralizing Ab (TNF-R75, 10 μg/ml) for 30 min before the addition of TNF-α (250 U/ml) for 20 min. p47phox was immunoprecipitated with a specific Ab, subjected to SDS-PAGE, blotted on nitrocellulose, and detected by autoradiography. Data are representative of three experiments.

FIGURE 6. Effects of cytokine combination on p47phox phosphorylation. [32P]-labeled neutrophils were incubated with GM-CSF (12 ng/ml) alone, TNF-α (250 U/ml) alone, or in combination for 20 min. p47phox was immunoprecipitated from lysates and revealed by autoradiography or with Western blotting with a specific Ab. Phosphorylated p47phox from three experiments was quantified by phosphor imager analysis and corrected for the amount of p47phox, which was quantified by densitometry, as described in Materials and Methods (mean ± SEM, n = 3) (lower panel).
their combination on p47phox phosphorylation. As shown in Fig. 6 (upper and lower panels), neutrophil incubation with both cytokines resulted in a more intense p47phox phosphorylation than with TNF-α or GM-CSF alone. Two-dimensional phosphopeptide mapping was used to determine whether the phosphorylation induced by the combination of TNF-α and GM-CSF targeted the same peptide. As shown in Fig. 7, when each cytokine was added alone, the same major peptide was phosphorylated, as previously observed; GM-CSF was more potent than TNF-α. Incubation with both cytokines resulted in more intense phosphorylation of the same major peptide, and phosphorylation of some new minor peptides. In keeping with this result, the priming effect of TNF-α and GM-CSF on fMLP-induced superoxide production was additive (Fig. 8). These results suggest that the kinases activated by TNF-α and GM-CSF converge on the same target serines of p47phox and could participate in the priming process of the respiratory burst.

To further bring evidence that TNF-α and GM-CSF activate a common priming pathway that targets selective p47phox phosphorylation site, we tested the effect of protein kinase inhibitors on TNF-α-induced p47phox phosphorylation. As illustrated in Fig. 9, the TNF-α-induced p47phox phosphorylation was markedly reduced by neutrophil preincubation with the protein tyrosine kinase inhibitor genistein (100 μM). Phosphorylated p47phox from three experiments was quantified and corrected for the actual amount of p47phox present; the phosphorylation of p47phox in the presence of genistein was 27.4 ± 9.4% of control TNF alone (mean ± SEM, n = 3 (p < 0.05)). The PKC inhibitor GF109203X (5 μM) had moderate effect (phosphorylation degree of p47phox in the presence of GF109203X was 79.5 ± 7.9% of control TNF alone (mean ± SEM, n = 3). Viability of neutrophils was not affected in the presence of genistein or GF109203X, as assessed by trypan blue exclusion test. These results suggest that a protein tyrosine kinase controls TNF-α-induced p47phox phosphorylation. It is noteworthy that in a previous study we showed that GM-CSF-induced p47phox phosphorylation is inhibited by genistein, but not by GF109203X (15). This suggests that although TNF-α and GM-CSF have different receptors, the two cytokines share a common pathway to target the same peptide on p47phox.

Discussion
Proinflammatory cytokines such as TNF-α and GM-CSF prime the neutrophil respiratory burst and NADPH oxidase activation (4–6). A direct link between this effect and oxidase components is not well documented. In this study, we demonstrate that TNF-α induces partial phosphorylation of the cytosolic oxidase component p47phox, suggesting a role of this event in the priming effect of TNF-α on NADPH oxidase activation. It is noteworthy that IL-1β and G-CSF, which have a moderate priming effect, did not induce or induce a moderate p47phox phosphorylation. IL-1β and G-CSF stimulate many signals in neutrophils through their specific receptors. P47phox phosphorylation might not be coupled to the transduction pathways triggered by these cytokines; nevertheless, these cytokines could use distinct mechanisms to prime the respiratory burst. Other mechanisms of NADPH oxidase priming have been reported, such as increased cytochrome b558 translocation (16, 17, 25) and increased G protein activation (18). G-CSF induced translocation of cytochrome b558 by gelatinase-granule release (25). It is clear from our results that the potent priming cytokines (TNF-α and GM-CSF) induce p47phox phosphorylation, while weakly priming cytokines (IL-1β and G-CSF) do not. TNF-α exerts its cellular effects by binding to its two receptors, p55 and p75 TNF-α receptors. Neutrophils express both receptor

FIGURE 7. Two-dimensional phosphopeptide mapping of p47phox from TNF-α- and GM-CSF-treated neutrophils. 32P-labeled neutrophils were incubated with GM-CSF (12 ng/ml) alone, TNF-α (250 U/ml) alone, or in combination for 20 min. p47phox was immunoprecipitated from lysates and revealed by autoradiography. Phosphorylated p47phox was subjected to two-dimensional phosphopeptide mapping, as described in Materials and Methods. Data are representative of three experiments.

FIGURE 8. Effects of cytokine combination on fMLP-induced superoxide production. Neutrophils were incubated with GM-CSF (12 ng/ml) alone, TNF-α (250 U/ml) alone, or in combination for 20 min, then stimulated with fMLP (10^{-7} M). Superoxide anion production was measured using the cytochrome c reduction assay at 550 nm. Control superoxide anion production (100%) is 3.5 nmol/min/10^6 cells for fMLP (10^{-7} M) (mean ± SEM, n = 8; * p < 0.05).

FIGURE 9. Effect of protein kinase inhibitors on TNF-α-induced p47phox phosphorylation. 32P-labeled neutrophils were incubated in the presence or absence of different kinase inhibitors for 15 min: a PKC inhibitor GF109203X (5 μM) and tyrosine kinase inhibitor genistein (100 μM) before treatment with TNF-α (250 U/ml) for 20 min. p47phox was immunoprecipitated from lysates and revealed by autoradiography (upper panel) and blotting (lower panel). Data are representative of three experiments.
types (23), which are linked to different signaling pathways (26). We used neutralizing mAbs (24) to investigate the role of TNF receptors in p47phox phosphorylation. The results showed that p47phox phosphorylation induced by TNF-α in neutrophils is predominantly mediated by TNF-α-R55. The lack of effect of anti-TNF-α-R75 on p47phox phosphorylation cannot be ascribed to a lack of binding, because the Abs used in our study are equally effective in blocking L929 cell lysis (24). This result is in keeping with the findings of Della Bianca et al. (27), showing that only the TNF-R55 is involved in the signaling process during phagocytosis in human neutrophils. Other studies (28) have shown that when neutrophils are adherent to fibrinogen, TNF-α-R55 and R75 cooperate to induce the respiratory burst, but TNF-α-R55 has been described as the main mediator of intracellular signaling. Our results strongly support a primary role of TNF-α-R55 in p47phox phosphorylation by TNF-α in neutrophils in suspension.

Although p47phox can be phosphorylated on several serines, TNF-α used in priming conditions induced only limited p47phox phosphorylation. This was also the case with GM-CSF and LPS (15, 16). This partial p47phox phosphorylation could accelerate phosphorylation of other sites by chemoattractants present in inflammatory sites, such as fMLP, leukotriene B4, platelet-activating factor, and C5a. The phosphopeptide map of p47phox (12) suggests that serines 345 to 370 are candidate targets for priming-induced phosphorylation. It has been reported that phosphorylation of serines 359 and 370 precedes the phosphorylation of other p47phox serines (29). These two serines may be phosphorylated in priming conditions. Research is underway in our laboratory to identify the serines phosphorylated when neutrophils are primed with TNF and GM-CSF.

TNF-α receptors and GM-CSF receptor are completely different, but p47phox phosphorylation induced by these two cytokines showed the same sensitivity to protein tyrosine kinase inhibitors, suggesting that TNF-α and GM-CSF priming-induced p47phox phosphorylation is controlled by the same pathway. This is further supported by our observation that GM-CSF- and TNF-α-induced p47phox phosphorylation occurs on the same peptide, and that the two cytokines have additive effects on phosphorylation of this peptide and on the priming of fMLP-induced respiratory burst. As no phosphotyrosine was present in the phosphorylated p47phox (15) (data not shown from this study), this suggests that TNF-α and GM-CSF activate a protein tyrosine kinase upstream of a ser/thr-protein kinase, which phosphorylates directly p47phox. The ability of TNF-α of GM-CSF, two different cytokines with two different receptors to induce this p47phox phosphorylation, suggests that this event could be a shared pathway of the priming of neutrophils by TNF-α and GM-CSF. TNF-α induces a variety of signals in human neutrophils, stimulating the activation and phosphorylation of phospholipase A2 (30, 31), extracellular signal-regulated kinase 1/2, and p38 mitogen-activated protein kinase (32, 33), and several protein tyrosine kinases (34, 35). The involvement of these pathways in TNF-induced p47phox phosphorylation is under investigation, together with the target protein kinase.

In conclusion, TNF-α induces partial p47phox phosphorylation in human neutrophils via its p55 receptor, thereby facilitating subsequent fMLP-induced phosphorylation. This effect of TNF-α, which is shared by GM-CSF, could be one common mechanism underlying human neutrophil respiratory burst priming.

References