Tyrosine-Specific Protein Phosphorylation During Activation of Human Neutrophils

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The activation of human neutrophils by a variety of receptor-dependent and receptor-independent agonists induces the phosphorylation of a large number of proteins. Since we have previously shown that human neutrophils have at least two distinct tyrosine kinase activities, we examined protein tyrosine phosphorylation in human neutrophils stimulated with a variety of agonists. Using a monoclonal antibody specific for phosphorytrosine, the present study shows that the chemotactic peptides FMLP and leukotriene B4, the phorbol ester phorbol myristate acetate (PMA), and the calcium ionophore A23187 induce an increase in tyrosine phosphorylation of a number of neutrophil proteins. This increased protein tyrosine phosphorylation was dependent on the concentration of the agonist, as well as on the time of exposure to the agonist. Fractionation experiments showed that both a 150,000 g cytosolic and a particulate preparation showed increases in protein tyrosine phosphorylation with stimulation by FMLP or PMA, and showed that the pattern of protein tyrosine phosphorylation was slightly different in the FMLP- and PMA-stimulated cells. These data indicate that protein tyrosine phosphorylation is an early event in the activation of human neutrophils by a variety of receptor-dependent and receptor-independent agonists.

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METHODS

Materials used were as follows: PY-69 antiphosphotyrosine antibody and 125I-goat antimouse IgG from ICN (Cleveland, OH); dextran from Pharmacia (Piscataway, NJ); acrylamide and sodium dodecyl sulfate (SDS) from Bio-Rad (Richmond, CA); nitrocellulose from Gelman (Ann Arbor, MI); and phorbol myristate acetate (Consolidated Midland Corp, Brewster, NY). All other reagents were supplied by Sigma Chemicals, St Louis, MO.

Cell isolation. Neutrophils were isolated from fresh whole human blood, anticoagulated with 10% acid-citrate-dextrose, by dextran sedimentation, ficoll-hypaque density centrifugation, and hypotonic lysis of residual erythrocytes. The neutrophils were suspended at a density of 1 × 10^9/mL in phosphate-buffered saline (PBS), pH 7.4, containing 1 mmol/L CaCl2, 1 mmol/L MgSO4, and 5.5 mmol/L glucose. The cells were greater than 98% neutrophils and were greater than 99% viable by trypan blue exclusion.

Detection of neutrophil proteins phosphorylated on tyrosine residues. Neutrophils at 1 × 10^6/mL were prewarmed to 37°C for 5 minutes and then stimulated with the agonist indicated in the text and figure legends. The reaction was allowed to proceed for the specified time and terminated by the rapid addition of an equal volume of stop buffer. The samples were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the proteins were visualized by autoradiography.

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volume of "stop buffer," containing 20% trichloracetic acid (TCA), 1 mmol/L Vanadate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 7 μg/mL aprotinin and pepstatin, 2 mmol/L N-ethylemaleimide (NEM), 100 mmol/L NaF, and 5 mmol/L diisopropyl-fluorophosphate (DFP). After 60 minutes at 4°C, the protein pellet was washed three times with ice cold acetone, solubilized in SDS lysis buffer,17 and heated to 100°C for 5 minutes.

In experiments where neutrophil cytosol and particulate fractions were evaluated, the reaction was stopped with equal volumes of ice cold PBS, pH 7.4, containing 2 mmol/L NEM, 1 mmol/L Vanadate, 1 mmol/L PMSF, 10 mmol/L EDTA, 7 μg/mL of pepstatin and leupeptin, 100 mmol/L NaF, and 5 mmol/L DFP. After 10 minutes at 4°C, the cells were centrifuged at 500 g and sonicated in a buffer containing 20 mmol/L HEPES, pH 7.5, 100 μmol/L Vanadate (to inhibit phosphotyrosine phosphatase), 2 mmol/L PMSF, 2 mmol/L 2-mercaptoethanol, and 7 μg/mL of leupeptin and pepstatin. In some experiments, 2 mmol/L EDTA and 2 mmol/L NEM was added to fully prevent kinase activity during fractionation. The sonicate was centrifuged at 500 g for 5 minutes to remove unbroken cells and cell nuclei and then at 150,000 g for 60 minutes at 4°C. The 150,000 g supernatant (cytosol) was mixed with an equal volume of TCA stop buffer, and the 150,000 g pellet (particulate) was resuspended in sonication buffer and then in an equal volume of TCA stop buffer. The proteins were then handled as above. Separation of the neutrophil proteins is by SDS polyacrylamide gel electrophoresis (PAGE).17 The proteins were then transferred to nitrocellulose according to the method described by Burnette.18 The nitrocellulose filter was then incubated in Tris 10 mmol/L pH 7.3, NaCl 150 mmol/L, and sodium azide 0.01% (TNA) containing 3% bovine serum albumin (BSA) from 4 to 24 hours. The proteins were then probed with 2 μg/mL of a monoclonal antiphosphotyrosine antibody (PY-69) at 37°C for 2 hours. The filter was washed five times in TNA containing 0.1% NP-40 and then incubated with antimouse IgG labeled with 125I. The filter was again washed five times and dried, and autoradiography was done at −70°C for variable times using Kodak XAR film.

In some experiments phenylphosphate or phosphotyrosine was added to the PY-69 antiphosphotyrosine antibody to block the binding of the antibody to the neutrophil proteins.

**Neutrophil superoxide analysis.** The continuous release of neutrophil superoxide was determined by the superoxide dismutase inhibitable reduction of ferricytochrome C in a double beam spectrophotometer, as previously reported.19

**RESULTS**

When human neutrophils in suspension are exposed to the chemotactic peptide FMLP at 37°C, a respiratory burst is initiated within 10 to 15 seconds1 and continues for approximately 2 minutes before termination. To evaluate if, over this same time period, FMLP induces the phosphorylation of proteins on tyrosine residues, we used an antiphosphotyrosine monoclonal antibody, PY-69, (ICN Radiochemicals), produced using phosphotyrosine coupled to keyhole limpet hemocyanin as the antigen, and phosphotyrosine coupled to BSA as a boost antigen, as described by Glenney et al.20 As shown in Fig 1A, before the addition of any agonist (lane 0), several neutrophil proteins were recognized by the antiphosphotyrosine label. Immediately after this sample was obtained, 10−6 mol/L FMLP was added to the tube containing the neutrophils, and samples were obtained at the indicated time points (in seconds) after addition. By 10 to 20 seconds after addition of the FMLP, several of these proteins showed an increase in the labeling with the antiphosphotyrosine monoclonal antibody. Most notable were proteins between 110 and 120 Kd, between 70 and 85 Kd, and between 52 and 60 Kd (approximate molecular weight based on the midpoint of the band on repeat radiographs). A protein band between 42 and 46 Kd did not change significantly during this time course. The peak labeling was seen between 40 and 60 seconds after stimulation, with a decrease in the apparent label being noticed after the 120 second time point. In Fig 1B, the dose response for the FMLP-induced phosphorylation of proteins on tyrosine residues was evaluated. In this experiment, the neutrophils were incubated with the indicated concentration of FMLP for 45 seconds before termination of the reaction as in A. Approximate molecular weights are indicated on the left of each figure. These immunoblots are representative of at least five experiments.

![Fig 1](image-url)
the labeling of neutrophil proteins with the antiphosphotyrosine antibody. Figure 2A represents an autoradiograph of an immunoblot of proteins isolated from neutrophils stimulated with 50 nM PMA from 0 to 450 seconds. It can be seen that before the addition of PMA, proteins similar to those noted in Fig 1 were labeled by PY-69. Upon addition of the PMA, a protein between 110 and 115 Kd increased its label beginning at about 60 seconds, and continued to increase through the 450 seconds tested. A band between 70 and 85 Kd was labeled before stimulation (time 0) and increased its labeling slightly through 120 seconds. A band between 52 and 60 Kd remained at baseline levels through 120 seconds and then increased at 300 and 450 seconds. The band between 42 and 46 Kd increased its label for the PY-69 antiphosphotyrosine antibody through 60 seconds and then remained stable until 450 seconds. In the experiment shown, the 180 second time point was felt to be inadvertently underloaded. Figure 2B shows the dose response to PMA of labeling of these proteins with PY-69. An increase in label was noted at concentrations beginning at approximately 5 x 10^{-15} mol/L with an increase in label through 5 x 10^{-5} mol/L. The time of stimulation in this experiment was 180 seconds.

In order to quantitate the changes observed in Figs 1 and 2, laser densitometry was done of the time course of tyrosine phosphorylation induced by FMLP (1 x 10^{-5} mol/L) and PMA (5 x 10^{-8} mol/L). The results are shown in Fig 3, which is representative of three densitometric tracings. In Fig 3A, which represents the results of a tracing of the radiograph represented in Fig 1A, the most dramatic increase in tyrosine phosphorylation is seen in the protein band between 110 and 120 Kd. The protein band at 42 to 46 Kd shows little to no change in phosphotyrosine labeling during this time course. When three separate experiments were
considered, we found that 40 seconds after stimulation, the bands at 110 to 120, 70 to 85, 52 to 60, and 42 to 46 Kd were 3.56 ± .82, 2.07 ± .36, 1.69 ± .26, and 1.37 ± .14-fold greater than the intensity of the band at time zero, respectively. Figure 3B shows the results of a representative densitometric tracing of labeling of phosphotyrosine of the radiograph represented in Fig 2A. The band at about 110 to 120 Kd increases in intensity within 30 to 60 seconds of stimulation and continues to increase up to the 450-second time point. The band between 70 and 85 Kd changed little early in the time course but increased after 120 seconds. The band in the 52 to 60 Kd range remained only minimally phosphorylated up to 90 seconds and then showed a marked increase in intensity through 450 seconds. The band between 42 and 46 Kd increased up to 60 seconds of stimulation and then remained constant (excluding the 180 seconds time point, which was inadvertently underloaded as stated). When three separate experiments were considered, we found that 120 seconds after stimulation the 110 to 120, 70 to 85, 52 to 60, and 42 to 46 Kd proteins were 1.43 ± .03, 1.11 ± .12, 1.23 ± .04, and 1.24 ± .07-fold greater, respectively, than the intensity of the band at time zero.

To determine the specificity of the PY-69 antibody for phosphotyrosine, we added either 40 mmol/L phenylphosphate, 20 mmol/L phosphotyrosine, or 20 mmol/L L-tyrosine to the antibody before immunoblotting identical neutrophil proteins. We found in multiple experiments that while phenylphosphate and phosphotyrosine both completely blocked the binding of the antibody to the neutrophil proteins, the L-tyrosine did not (data not shown).

We next evaluated the subcellular location of the proteins that were labeled with the antiphosphotyrosine antibody in unstimulated and stimulated neutrophils. The results from a representative radiogram are shown in Fig 4, and the results of a densitometric tracing of duplicate radiograms are shown in Figure 5. In neutrophils stimulated (Fig 4, S) with FMLP protein bands of approximately 120, 84, 57, and 44 Kd were seen in the cytosol that demonstrate an increased label with the antiphosphotyrosine antibody, compared with cytosolic proteins from neutrophils treated with control buffer (C) for 45 seconds. A band of approximately 42 Kd shows no change. In the particulate fraction from these same cells (Fig 4, lanes 3 and 4), a doublet is seen at 120 and 110 Kd. The 110 Kd protein showed increased labeling with the PY-69 antiphosphotyrosine antibody in the stimulated sample. Proteins of 89, 70, and 52 Kd are also noted to show an increased labeling with the PY-69 antiphosphotyrosine antibody in the particulate fraction of neutrophils stimulated for 45 seconds with FMLP compared with control cells. Quantitation of these changes are seen in Fig 5. In cells stimulated with 50 nmol/L PMA or control buffer for 180 seconds before fractionation, cytosolic proteins of approximately 110 to 115 and 42 Kd showed little change or a slight decrease (Fig 5) in the label with the PY-69 antiphosphotyrosine antibody upon stimulation. Cytosolic proteins of 84, 57, and 44 Kd showed increased labeling upon stimulation with the PMA. In the particulate fraction of neutrophils stimulated with PMA, proteins at 110 to 115 and 75 Kd showed enhancement in labeling with stimulation (1.5- and 1.4-fold increases over control, respectively, based on duplicate experiments). However, proteins at 89 and 52 Kd showed little or no change upon stimulation (see Fig 5 for quantitation). In some experiments, the fractionation buffer contained 2 mmol/L EDTA to prevent kinase activity (dependent on Mg²⁺ and Mn²⁺) during subcellular isolation, and the same results were seen.

In the experiments shown, the time course of phosphorylation on tyrosine residues of neutrophil proteins by FMLP and PMA was similar to the time course of O₂⁻ production. This could suggest that phosphorylation of tyrosine residues was a
result of \( \text{O}_2^- \) production. When neutrophils from a patient with X-linked chronic granulomatous disease, which are deficient in their ability to produce \( \text{O}_2^- \), were stimulated with either FMLP or PMA, the same changes in the stimulated patterns of labeling with PY-69 antiphosphotyrosine antibody were seen as were noted in simultaneously run normal neutrophils (data not shown). This indicates that the results seen above were not a result of \( \text{O}_2^- \) production.

To determine if other activators of neutrophils could induce increased phosphorylation of proteins on tyrosine residues, we tested the effect of the calcium ionophore A23187, as well as leukotriene \( \text{B}_4 \), on these events. Figure 6 shows that A23187, which activates neutrophils through an influx of \( \text{Ca}^{2+} \) ions, induced a rapid increase in the PY-69 labeling of neutrophil proteins similar to that seen with PMA. This was evident by 30 seconds and increased through 120 seconds, with a subsequent dephosphorylation of the phosphotyrosine labeled proteins at about 110 to 120, 75 to 85, and 52 to 60 Kd. This latter protein showed a slightly more delayed increase in PY-69 label than that noted for the other prominent proteins. The 42 to 46 Kd protein did not significantly change its level of phosphotyrosine labeling upon stimulation.

Figure 7 represents the results when LTB\(_4\), which, like FMLP, binds to specific receptors and activates polymorphonuclear neutrophil leukocytes (PMN) by a pertussis toxin sensitive pathway,\(^21\) was used to activate neutrophils before determining the labeling of proteins with PY-69 antiphospho-

**Fig 6. Autoradiograph showing immunoblotting with PY-69 antiphosphotyrosine antibody of proteins from neutrophils stimulated with the calcium ionophore A23187. Neutrophils warmed to 37°C were stimulated with 5 \( \mu \)mol/L A23187 for 0 to 600 seconds before stopping the reactions with TCA stop buffer. Proteins were analyzed by 7.5% SDS-PAGE and transferred for immunoblotting. Each lane represents the time in seconds after addition of A23187. Equal protein samples were analyzed.**
tyrosine. It was apparent that the same bands as noted for A23187 rapidly increase their label after addition of the LTB₄ with the 110 to 120 Kd band showing peak intensity at 40 to 60 seconds. The 75 to 85 Kd band showed a peak of intensity earlier, at approximately 10 to 20 seconds, and the 52 to 60 Kd band showed an increase at 10 and 20 seconds with a subsequent decrease beyond 40 seconds.

**DISCUSSION**

Phosphotyrosine-containing proteins usually constitute less than 1% of total cellular phosphoproteins. Because of this, phosphorylation of proteins on tyrosine residues by tyrosine kinase is an attractive mechanism for regulation of cellular events. Tyrosine phosphorylation has been suggested as the mechanism of action of a variety of growth hormone receptors that are involved in the regulation of cell growth and proliferation. Similarly, the protein products of several viral oncogenes are tyrosine kinases, and phosphorylation of proteins on tyrosine residues is thought to be involved in malignant transformation events. Tyrosine kinase activities have also been found in a number of nonproliferative hematopoietic cells, including mature lymphocytes, erythrocytes, platelets, and neutrophils. As lymphocytes, platelets, and neutrophils respond to stimuli with physiologic responses, it is possible that tyrosine kinases are involved in stimulus-response coupling. This has been shown in platelets where stimulation with thrombin increases the total cellular content of phosphotyrosine and induces the phosphorylation of specific proteins on tyrosine residues as shown using antiphosphotyrosine antibodies. Additional studies suggest a role for the platelet integrin, glycoprotein IIb-IIIa, in these events.

In the present study, we have shown that stimulation of human neutrophils with the chemotactic peptide FMLP or the phorbol ester PMA induces an increase in the labeling of several neutrophil proteins with a monoclonal antibody directed against phosphotyrosine. The increase in tyrosine phosphorylation upon stimulation was both time- and concentration-dependent. The time course and concentration dependency of the tyrosine phosphorylation is similar to that noted for the induction of neutrophil superoxide production by these agonists. Studies using neutrophils from a patient with chronic granulomatous disease, which do not produce O₂⁻ when stimulated, showed the same stimulated patterns of labeling with the antiphosphotyrosine antibody as was seen in normal control neutrophils, indicating that the results seen are an effect of the stimulation and not of the O₂⁻ produced.

Most tyrosine kinases described are either integral membrane proteins or associated with the cytoplasmic side of the plasma membrane. We have previously shown that neutrophils have tyrosine kinase activity in both a cytosolic fraction and a detergent extractable particulate fraction. Therefore, it was of interest to determine the location of tyrosine phosphorylated proteins from neutrophils. From the data presented in Figs 4 and 5, it was apparent that stimulation of neutrophils in vivo with either FMLP or PMA induces the tyrosine phosphorylation of proteins from both the cytosolic as well as the particulate fraction of the cell. Cytosolic proteins of 120, 84, 52, and 44 Kd and particulate proteins of 110, 89, 70, and 52 Kd showed increases in phosphotyrosine after stimulation with FMLP. When PMA was the stimulant, cytosolic proteins of 84, 57, and 44 Kd and particulate proteins of 110 to 120 and 75 Kd demonstrated consistent increases in labeling for phosphotyrosine. It was surprising that although phosphotyrosine-containing proteins in all of these molecular mass ranges were seen in the whole cell lysates, several were more prominent in the fractionated samples as compared with the whole cell samples. To rule out the possibility that this was due to continued kinase activation during the fractionation, these same experiments were...
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done with EDTA in the fractionation buffer to inhibit kinase activity. No change in the results was evident. Finally, differences between the whole cell lysates and fractionated experiments might be the result of the improved resolution obtained by fractionating the cell and removing cell nuclei. In addition, there are subtle but distinct differences seen in the pattern of phosphotyrosine-containing proteins between cells stimulated with either PMA or FMLP, indicating that activation of the cell by differing pathways may slightly alter the substrates for the neutrophil tyrosine kinases.

The findings presented using leukotriene B4 and A23187 to stimulate neutrophils suggest that phosphorylation of proteins on tyrosine residues is associated with activation of the neutrophils by a wide variety of receptor-dependent (FMLP and LTB4) receptor-independent (A23187) and PKC-dependent (PMA) mechanisms. However, it is not possible from the present studies to determine which of the neutrophil tyrosine kinase activities are responsible for phosphorylation of specific proteins, the identity of those proteins, the function of tyrosine phosphorylated proteins, or whether the phosphorylation of proteins on tyrosine is the result of stimulated changes in phosphotyrosine phosphatase activity, as we have previously reported.

Tyrosine phosphorylation of neutrophil proteins has previously been reported by Huang et al. In their study, the most prominent phosphotyrosine-containing proteins were of 125 Kd and about 60 Kd, similar to proteins that we have seen. In more recent data presented by these authors, a wide variety of stimuli, including PMA, was seen to increase tyrosine phosphorylation of a number of proteins from rabbit peritoneal neutrophils.

Tyrosine phosphorylation of human neutrophil proteins has also been seen in electrophoremeobilized neutrophils stimulated with guanine nucleotides in the presence of magnesium and adenosine triphosphate. Several proteins with tyrosine phosphorylation, similar in molecular weight to the proteins seen in the present study, were seen. This suggests that the FMLP- and LTB4-induced tyrosine phosphorylation seen in the present study may be a result of the interaction of the receptor-ligand complex with guanine nucleotide-binding proteins and subsequent activation of tyrosine phosphorylation. Other mechanisms for tyrosine phosphorylation must be involved to explain the tyrosine phosphorylation occurring in response to PMA or A21387, ie, mechanisms that bypass receptor-ligand interaction with G proteins.

The finding of stimulus-induced tyrosine phosphorylation in this study must also be interpreted in light of our previous observations that the tyrosine kinase inhibitor, ST638, inhibits O2- production in neutrophils stimulated by FMLP, but not in neutrophils stimulated with PMA or A21387. Several explanations exist for these observations. It is possible that (1) the phosphorylation of neutrophil proteins on tyrosine by PMA or A21387 is unrelated to their activation of the oxidative burst; (2) the particulate-associated tyrosine kinase (inhibited by ST638 in vitro) is involved in O2- production by agonists that interact with G proteins but not by agonists, such as PMA or A21387, that bypass this interaction; or (3) the ability of ST638 to inhibit O2- production in vivo in neutrophils is not related to its in vitro inhibition of the particulate-associated neutrophil tyrosine kinase.

In summary, we report that intact human neutrophils increase the labeling with antiphosphotyrosine monoclonal antibody of a number of cytosolic and particulate proteins when the cells are stimulated in vivo with both receptor-dependent and receptor-independent agonists. Further studies will be aimed at identification of specific proteins that are phosphorylated on tyrosine.

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