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### Research Article

The activation of human neutrophils by monosodium urate and calcium pyrophosphate dihydrate crystals is believed to play a critical role in the pathogenesis of arthritides such as acute gout and pseudogout, respectively. In this study, we investigated the potential involvement of tyrosine phosphorylation in microcrystal-mediated activation of human neutrophils. Immunoblot analysis with antiphosphotyrosine antibodies demonstrated that triclinic monosodium urate and calcium pyrophosphate dihydrate crystals stimulated a time- and concentration-dependent tyrosine phosphorylation of at least five proteins (pp130, 118, 80, 70, and 60). While phosphoprotein (pp) 118 and pp70 were the major phosphorylated substrates, pp70 was the dominant one in reactivity with antiphosphotyrosine antibodies. When the temporal patterns, as well as the levels of tyrosine phosphorylation for both types of crystals were compared, monosodium urate crystals were found to be more potent activators than calcium pyrophosphate dihydrate crystals. The tyrosine phosphorylation patterns induced by microcrystals differed from those stimulated by other soluble (FMLP, C5a, or leukotriene B4) or particulate (unopsonized latex beads or zymosan) agonists which stimulated preferentially the tyrosine phosphorylation of pp118. The ratio of the intensities of pp118 and pp70 were specific of the stimulation with microcrystals when compared to those observed with the other soluble or particulate agonists. Colchicine, a drug used specifically in the treatment of gout and pseudogout, inhibited microcrystal-induced tyrosine phosphorylation, while beta- and gamma-lumicolchicine [...]

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# Crystal-induced Neutrophil Activation

## III. Inflammatory Microcrystals Induce a Distinct Pattern of Tyrosine Phosphorylation in Human Neutrophils

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### Abstract

The activation of human neutrophils by monosodium urate and calcium pyrophosphate dihydrate crystals is believed to play a critical role in the pathogenesis of arthritides such as acute gout and pseudogout, respectively. In this study, we investigated the potential involvement of tyrosine phosphorylation in microcrystal-mediated activation of human neutrophils. Immunoblot analysis with antiphosphotyrosine antibodies demonstrated that triclinic monosodium urate and calcium pyrophosphate dihydrate crystals stimulated a time- and concentration-dependent tyrosine phosphorylation of at least five proteins (pp130, 118, 80, 70, and 60). While phosphoprotein (pp) 118 and pp70 were the major phosphorylated substrates, pp70 was the dominant one in reactivity with antiphosphotyrosine antibodies. When the temporal patterns, as well as the levels of tyrosine phosphorylation for both types of crystals were compared, monosodium urate crystals were found to be more potent activators than calcium pyrophosphate dihydrate crystals. The tyrosine phosphorylation patterns induced by microcrystals differed from those stimulated by other soluble (FMLP,  $C_{5a}$ , or leukotriene  $B_4$ ) or particulate (unopsonized latex beads or zymosan) agonists which stimulated preferentially the tyrosine phosphorylation of pp118. The ratio of the intensities of pp118 and pp70 were specific of the stimulation with microcrystals when compared to those observed with the other soluble or particulate agonists. Colchicine, a drug used specifically in the treatment of gout and pseudogout, inhibited microcrystal-induced tyrosine phosphorylation, while  $\beta$ - and  $\gamma$ -lumicolchicine were without effect. On the other hand, colchicine failed to inhibit FMLP-induced tyrosine phosphorylation. Furthermore, while colchicine inhibited the activation of the NADPH oxidase by microcrystals, it, on the other hand, enhanced the production of superoxide anions by FMLP. Taken together, these results (a) demonstrate that tyrosine phosphorylation is involved in the mechanism of activation of human neutrophils induced by microcrystals; and (b) suggest, on the basis of the characteristics of the observed patterns of tyrosine phosphorylation, that this response may be specific to the microcrystals and relevant to their phlogistic properties. (*J. Clin. Invest.*

1993. 91:1649–1655.) Key words: monosodium urate crystals • calcium pyrophosphate dihydrate crystals • colchicine • chemotactic factors • gout

### Introduction

Over the last 30 yr, several distinct microcrystals have been implicated in the pathogenesis of acute and chronic joint diseases. It is by now well established that the deposition of microcrystalline monosodium urate monohydrate (MSU)<sup>1</sup> and calcium pyrophosphate dihydrate (CPPD) in joints and their interactions with neutrophils play an important role in the development of gouty arthritis and joint chondrocalcinosis (pseudogout), respectively (1, 2). However, despite the fact that MSU and CPPD crystals are both involved in these inflammatory processes, they display differential phlogistic properties and metabolic responses, MSU crystals generally being more active than CPPD crystals (3–7).

The activation of neutrophils by MSU and CPPD crystals results in the release of inflammatory mediators that may be in part responsible for the local as well as the systemic inflammatory manifestations found in crystal-induced joint disorders. Interactions between crystals and neutrophils have been found to lead to the release of lysosomal enzymes (8, 9) and of a crystal-induced chemotactic factor (10, 11), to stimulate the generation of oxygen derived free radicals (12, 13), to induce phospholipase  $A_2$  enzyme activities and the synthesis of a phospholipase  $A_2$  (PLA<sub>2</sub>) activating protein in leukocytes (14), and to activate the synthesis of 5-lipoxygenase products (15). Furthermore, it has recently been demonstrated that human neutrophils, monocytes, and synoviocytes can synthesize and release various cytokines such as IL-1, IL-6, and IL-8 in response to MSU and/or CPPD crystals (16–18). However, little is known about the early intracellular signaling events that initiate and/or modulate these crystal-induced responses.

The addition of MSU and CPPD crystals to a suspension of human neutrophils leads to rapid increases in the cytoplasmic concentration of free calcium (15, 19). Moreover, MSU-induced formation of inositol 1,4,5 trisphosphate has been demonstrated in neutrophils (19). The mechanism of activation of phospholipase C (PLC) by microcrystals is presently unclear. In spite of evidence indicating that the interaction of MSU and CPPD with neutrophils stimulates a pertussis toxin (PT)-sensitive GTPase in purified membrane preparations (20), several of the effects induced by MSU and CPPD crystals in intact neutrophils (calcium mobilization, superoxide production,

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1. Abbreviations used in this paper: CPPD, calcium pyrophosphate dihydrate; ECL, enhanced chemiluminescence; MSU, monosodium urate; PLA<sub>2</sub>, phospholipase  $A_2$ ; pp, phosphoproteins; PT, pertussis toxin; TBS, Tris-buffered saline.

phagocytosis, and phosphatidylinositol 4,5-bisphosphate hydrolysis) are only weakly sensitive or insensitive to PT (15, 20, 21). These results indirectly suggest that the mechanisms of neutrophil activation by MSU and CPPD differ from those triggered in neutrophils by soluble chemotactic factors.

Recently, the presence of tyrosine-specific protein kinase activities has been demonstrated in neutrophils (22, 23). Stimulation of intact human neutrophils with GM-CSF (24), chemotactic peptides such as FMLP (22, 25–27), leukotriene B<sub>4</sub> (27), and platelet-activating factor (28) or with phorbol esters (26, 27) induces the rapid phosphorylation on tyrosine residues of several protein substrates. In addition, the results of recent studies have implicated the activation of tyrosine kinases in several neutrophil responses such as the oxidative burst (25), and adherence and chemotaxis (29).

The present study was thus undertaken to examine the potential involvement of tyrosine phosphorylation in the mediation of the activation of human neutrophils by inflammatory microcrystals. The results demonstrate that (a) microcrystals potently stimulate, in a colchicine-inhibitable manner, tyrosine phosphorylation in human neutrophils; (b) MSU crystals induce a more rapid and quantitatively more important tyrosine phosphorylation response than CPPD crystals; and (c) the pattern of tyrosine phosphorylation stimulated by microcrystals is characteristic in that it differs qualitatively from that observed in response to other neutrophil agonists (soluble or particulate).

## Methods

### Materials

N-FMLP, zymosan, latex beads, and cytochrome C were obtained from Sigma Chemical Co. (St. Louis, MO). Ficoll-Paque and Dextran T500 were obtained from Pharmacia (Dorval, Québec, Canada). Leukotriene B<sub>4</sub> and biosynthetic recombinant C<sub>5a</sub> were generous gifts from Dr. R. Young (Merck-Frosst, Dorval, Québec, Canada) and H. J. Shewell (Pfizer Central Research, Groton, CT), respectively. All stock solutions were made up in low endotoxin DMSO from Sigma Chemical Co. RPMI 1640 was from Gibco Laboratories (Grand Island, NY). The monoclonal antiphosphotyrosine antibody UB 05-321 was purchased from UBI (Lake Placid, NY). The enhanced chemiluminescence Western blotting system was obtained from Amersham Corp. (Arlington, IL).

### Methods

**Cell preparation.** Blood was obtained from the peripheral vein of healthy adults in preservative-free citrate/phosphate/adenine anticoagulant solution. The neutrophils were sterilely obtained by means of 6% dextran sedimentation followed by standard techniques of Ficoll-Paque gradients and hypotonic lysis of erythrocytes. The cells were resuspended in RPMI 1640 medium, pH 7.4, at a final concentration of  $15 \times 10^6$  cells/ml. The final cell preparations contained  $\geq 98\%$  neutrophils. Cell viability was measured by the release of lactate dehydrogenase activity and was  $\geq 98\%$  under all experimental conditions.

**Microcrystal preparation.** MSU and CPPD crystals were prepared under sterile pyrogen-free conditions by modifications of previously described methods (30, 31). Briefly, a boiling MSU solution (0.03 M, pH 7.5) was prepared by dissolution of equimolar quantities of uric acid and sodium hydroxide and filtered on an Acropor 3- $\mu$ m membrane filter (AN-3000; Gelman, Ann Arbor, MI). Sodium chloride (0.1 M final concentration) was added to speed up and improve the uniformity of the crystallization. CPPD was obtained by mixing a calcium nitrate solution (0.1 M final concentration) with an acidic solution of sodium pyrophosphate (final concentration 0.025 M in

Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub> and 0.03 M HNO<sub>3</sub>). The milky-white precipitate formed CPPD crystals after a 1-d incubation at 50–60°C.

All the crystals used in this study were characterized by x-ray diffraction (Geigerflex D/max; Rigaku, Danvers, MA) and examination under phase and polarizing microscopy and scanning electron microscopy. The MSU and CPPD crystals showed triclinic morphologic characteristics. Their morphological properties, size, and specific area, determined by scanning microscopy and the BET method (32), are depicted in Table I.

**Tyrosine phosphorylation.** Freshly purified human neutrophils were incubated at  $15 \times 10^6$  cells/ml for 5 min at 37°C before stimulation for varying periods of time with either MSU or CPPD crystals. The reactions were terminated by adding 60  $\mu$ l of cell suspension to 60  $\mu$ l of boiling sample buffer (Tris HCl, pH 6.8, 2 mM Na orthovanadate, 10 mM nitrophosphate, 10 mM NaF, 10 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 20% SDS, 10% mercaptoethanol, 17.5% glycerol, and 0.1% bromophenol blue). The samples were denatured by boiling for 7 min at 100°C and loaded onto 7.5–20% SDS polyacrylamide gels. Electrophoretic transfer cells (Hoeffer Scientific Instruments, Canberra Packard, Canada, Mississauga, Ontario) were used to transfer proteins from the polyacrylamide gels to polyvinylidene difluoride membranes (Immobilon; Millipore Corporation, Bedford, MA). Nonspecific sites were blocked using 2% gelatin in Tris-buffered saline (TBS)-Tween 0.1% (25 mM Tris HCl, pH 8.0, 190 mM NaCl, and 0.1% vol/vol Tween 20) for 1 h at 37°C. The monoclonal antibody UB 05-321 was then incubated with the membranes for 1 h at 37°C at a final dilution of 1:4,000 in 2% gelatin in TBS-Tween 0.1%. The membranes were washed three times at room temperature in TBS-Tween 0.1% and further incubated with a horseradish peroxidase-labeled sheep anti-mouse IgG (Amersham Corp.) for 1 h at 37°C at a final dilution of 1:20,000 in 2% gelatin in TBS-Tween 0.1%. The membranes were washed three times and the phosphotyrosine bands were revealed using the enhanced chemiluminescence Western blotting detection system. The molecular weights of the phosphotyrosine bands were estimated using a log plot of the migration of molecular weight standards.

**Superoxide anion production.** Superoxide anion production was monitored as the (superoxide dismutase-sensitive) reduction of cytochrome C as previously described (21). Briefly, neutrophil suspen-

Table I. Morphological Characteristics of the Microcrystals\*

| Crystals        | Size                          | Specific area     |
|-----------------|-------------------------------|-------------------|
|                 | $\mu$ m                       | m <sup>2</sup> /g |
| CPPD            |                               |                   |
| 1               | 12 × 1.4 × 1.4–25 × 1.7 × 1.7 | 16.1              |
| 2               | 10 × 1.0 × 0.4–20 × 2.5 × .10 | 2.4               |
| 3               | 15 × 8.0 × 5.0–25 × 12 × 8.0  | 0.7               |
| 4               | 15 × 7.0 × 4.0                | 0.8               |
| MSU             |                               |                   |
| 1               | 10 × 1.0 × 1.0–25 × 1.5 × 1.5 | 6.7               |
| 2               | 10 × 0.7 × 0.7–30 × 1.3 × 1.3 | 7.3               |
| 3               | 1.5 × 0.3 × 0.3               | 12.1              |
| Potassium urate |                               |                   |
| 1               | 5.0 × 0.3 × 0.3               | 16.5              |
| 2               | 10 × 0.4 × 0.4                | 12.7              |
| 3               | 4.0 × 0.4 × 0.4               | 15.1              |

\* CPPD, MSU, and potassium urate crystals were prepared as described in Methods. The different crystals were characterized by x-ray diffraction and examination under phase and polarizing microscopy and by scanning electron microscopy. Specific area was determined by the BET method.

sions ( $1 \times 10^6$  cells/ml) were incubated under the desired experimental conditions in the presence of  $130 \mu\text{M}$  cytochrome C for 10 min at  $37^\circ\text{C}$ . After appropriate stimulation, the reactions were stopped by transferring the tubes to an ice-cold bath, followed by centrifugation. The optical density of the supernatants was read at 540 and 550 nm, and the amount of superoxide produced was calculated from the differences in optical densities at the two wavelengths using an extinction coefficient of 21.1.

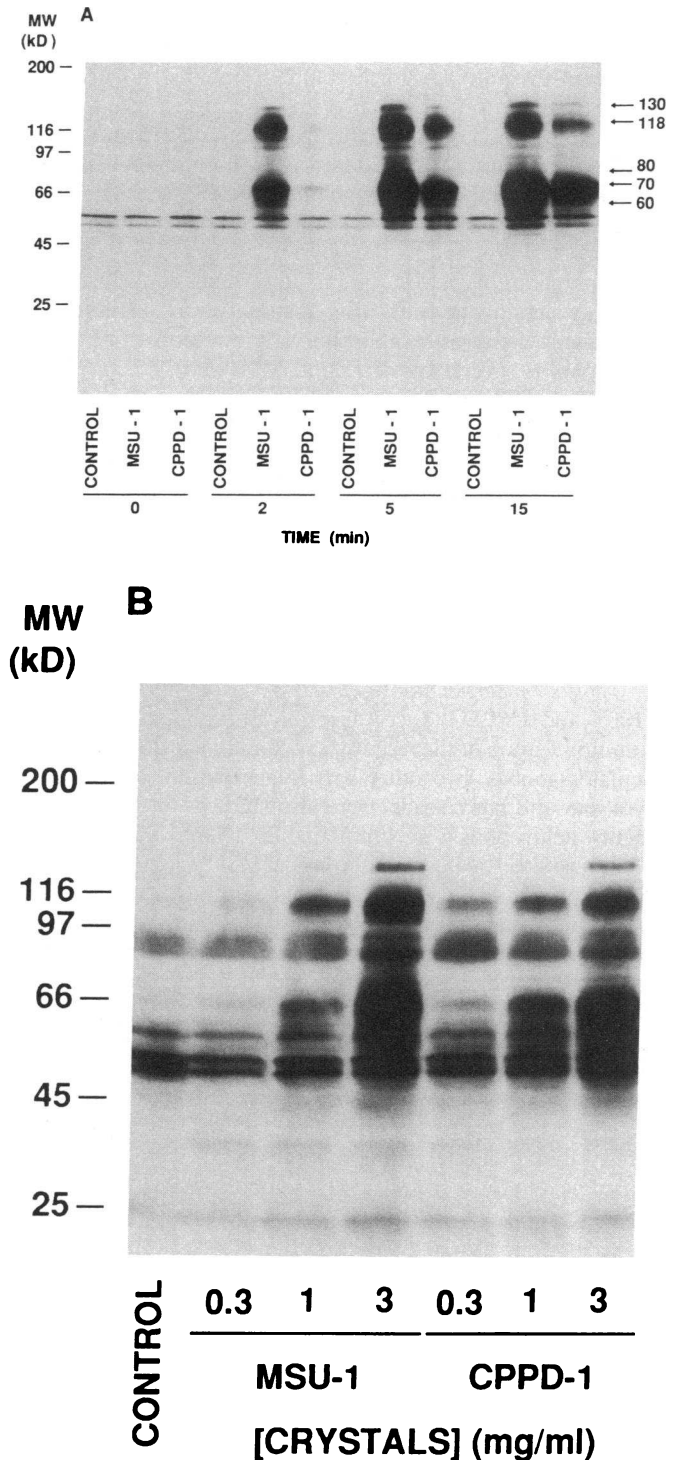
**Statistical analysis.** Results are expressed as the mean  $\pm$  SEM. Statistical analysis was performed by Student's paired *t* test (two tailed), and significance was considered attained when *P* was  $< 0.05$ .

## Results

**MSU and CPPD crystals stimulate tyrosine phosphorylation in human neutrophils.** Only low levels of tyrosine phosphorylation were observed after the addition of RPMI 1640 alone. A doublet of molecular mass  $\sim 55$  kD was always found constitutively tyrosine phosphorylated, and its level of tyrosine phosphorylation did not change under any of the experimental conditions tested in this study (Figs. 1–7, control lanes). The addition of MSU or CPPD crystals (3 mg/ml) induced the tyrosine phosphorylation of at least five proteins whose approximate molecular masses were 130, 118, 80, 70, and 60 kD (Fig. 1A). The phosphoproteins (pp) of 118 and 70 kD consistently showed the greatest increase in labeling, pp70 being the dominant one in reactivity with the antiphosphotyrosine antibody. Although other tyrosine phosphorylated proteins were also occasionally detected, including in particular a relatively diffuse band at  $\sim 90$ – $100$  kD, these were not observed as consistently as the five bands just identified. Similar patterns of tyrosine phosphorylation were obtained when the antiphosphotyrosine antibody PY 20 (ICN Radiochemicals, Montréal, Canada) was tested (data not shown).

While similar profiles were detected in neutrophils stimulated with MSU or CPPD crystals, the temporal patterns and the levels of protein tyrosine phosphorylation did differ. MSU-induced tyrosine phosphorylation of all five substrates was detected within 2 min of stimulation, reached a maximum at 5 min and was maintained over a period of 15 min (Fig. 1A). A decline in the phosphorylation level of most of the substrates was observed 30 min after MSU treatment. A densitometric analysis of pp118 and pp70 showed a significant decrease in intensity of  $81 \pm 6\%$  ( $P < 0.05$ ,  $n = 5$ ) and  $52 \pm 4\%$  ( $P < 0.01$ ,  $n = 5$ ), between the 15- and 30-min time points, respectively. A significant decrease was also observed for the other tyrosine phosphorylated bands (data not shown). The decreases in tyrosine phosphorylation observed at later times were not caused by a cytotoxic effect of the crystals, since incubation of neutrophils with crystals for 60 min did not promote significant lactic dehydrogenase release from the cells (data not shown). In comparison, CPPD-induced tyrosine phosphorylation only reached maximal levels after 15 min of stimulation (Fig. 1A) and remained constant for the next 45 min. A comparative densitometric analysis of the maximal levels of CPPD-induced tyrosine phosphorylation showed that the intensities of tyrosine phosphorylation of pp118 and pp70 were only  $44 \pm 15\%$  ( $P < 0.05$ ,  $n = 5$ ) and  $44 \pm 13\%$  ( $P < 0.01$ ,  $n = 5$ ) of those induced by MSU microcrystals.

The concentration-dependence of the tyrosine phosphorylation induced by microcrystals was tested next after a 5- and 15-min incubation with MSU or CPPD crystals, respectively. Microcrystals stimulated detectable tyrosine phosphorylation



**Figure 1.** Time dependence and dose response of MSU- and CPPD-induced tyrosine phosphorylation. Neutrophils ( $15 \times 10^6$ /ml) were incubated with diluent (RPMI 1640) as control, 3 mg/ml MSU and CPPD for various periods of time (A), or with different concentrations of MSU and CPPD crystals, for 5 and 15 min, respectively (B). Blotting and revelation of the phosphotyrosine bands were carried out as described under Methods.

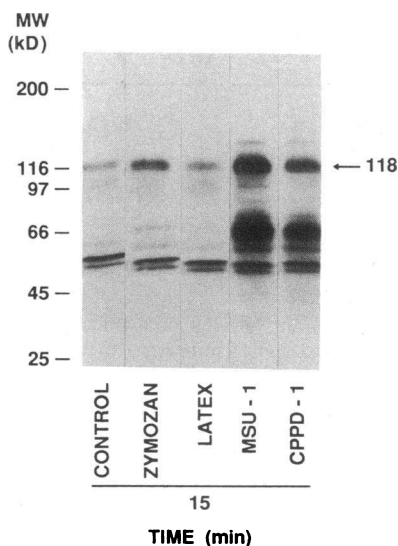
in human neutrophils at concentrations  $\geq 0.3$  mg/ml (Fig. 1B). A concentration-dependent increase in the labeling of all substrates was observed between 0.3 and 3 mg/ml.

The amino acid specificity of the crystal-stimulated phosphorylation as detected by immunoblot was tested by determining the ability of phosphoaminoacids to compete with the binding of the antibody to the blotted proteins. Whereas 50 mM phosphotyrosine eliminated the binding of the antiphosphotyrosine antibody to proteins from both control and crystal-stimulated cells, neither 50 mM phosphoserine nor 50 mM phosphothreonine had any significant effects (data not shown).

The variability among the physical characteristics of individual preparations of MSU and CPPD crystals could affect the responsiveness of the cells. This possibility was examined by determining the tyrosine phosphorylation response of human neutrophils to several lots of triclinic MSU and CPPD crystals, significantly differing in size and specific area. The various crystal preparations were found to elicit essentially identical responses. However, the quantitative differences between the responses to MSU and CPPD crystals described above were consistently observed, whatever the size or the morphology of the crystals tested. In addition, potassium urate crystals were found to induce a similar pattern of tyrosine phosphorylation as MSU crystals. Finally, it was also observed that the addition of uric acid did not stimulate tyrosine phosphorylation (data not shown).

**Effects of particulate agonists.** To examine whether the MSU- and CPPD-induced tyrosine phosphorylation was a common feature of the responses of human neutrophils to particulate agonists, two other particulate stimuli, unopsonized zymosan and latex beads, were also studied. In these experiments, neutrophils were stimulated for 15 min with 3 mg/ml of unopsonized zymosan, latex beads, MSU, or CPPD crystals. As can be seen in Fig. 2, unopsonized latex beads and zymosan induced only a slight increase in the tyrosine phosphorylation of pp118, while the inflammatory MSU and CPPD microcrystals, on the other hand, induced their characteristic responses (high pp70/pp118 ratio). Hydroxy-apatite crystals (3 mg/ml) elicited a weak response that resembled that to latex beads or zymosan; i.e., a predominance of the 118-kD band (results not shown).

**Effects of soluble agonists.** The patterns of tyrosine phosphorylation induced by inflammatory microcrystals were compared next to those stimulated by soluble agonists. Neutrophils



**Figure 2.** Effects of particulate agonists on protein tyrosine phosphorylation. Neutrophils ( $15 \times 10^6$ /ml) were incubated with 3 mg/ml of unopsonized zymosan, latex beads, MSU, or CPPD crystals for 15 min. Blotting and revelation of the phosphotyrosine bands were carried out as described under Methods.

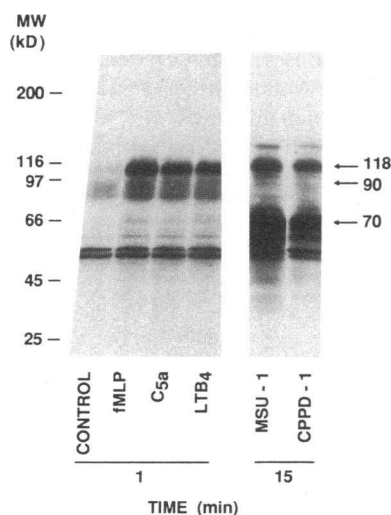
were stimulated for 1 min with  $10^{-7}$  M FMLP,  $\text{LTB}_4$ , or  $\text{C}_{5a}$ , a time at which it had previously been shown that maximal tyrosine phosphorylation in response to these agonists occurred. As demonstrated in Fig. 3, the patterns of tyrosine phosphorylation induced by the soluble agonists differed from those obtained with MSU and CPPD crystals. Exposure of neutrophils to  $10^{-7}$  M FMLP,  $\text{C}_{5a}$ , or  $\text{LTB}_4$  stimulated principally the tyrosine phosphorylation of a major protein with a molecular mass  $\sim 118$  kD, and to a minor extent that of the 130, 90, 60, and 70 kD proteins (Fig. 3). While the addition of the microcrystals also resulted in the tyrosine phosphorylation of pp118, they, as previously shown, stimulated to a much greater extent that of pp70.

**Effect of colchicine on crystal-induced tyrosine phosphorylation.** The effects of colchicine on the tyrosine phosphorylation induced by microcrystals were tested next. Neutrophils were preincubated for 1 h with  $10 \mu\text{M}$  colchicine or with its inactive analogs  $\beta$ - or  $\gamma$ -lumicolchicine. The cells were further stimulated with 3 mg/ml MSU or CPPD crystals and a densitometric analysis of the levels of four of the major phosphotyrosine bands were performed (Table II) for colchicine-treated cells, while a representative immunoblot illustrates the effects of the three alkaloids on MSU-induced tyrosine phosphorylation (Fig. 4). It is noteworthy that the 1 h preincubation at  $37^\circ\text{C}$  resulted in a shift in the optimal stimulation time from 30 to 45 min (as compared to 5 and 15 min without preincubation) for MSU and CPPD crystals, respectively. Colchicine by itself, increased to a small extent the levels of phosphorylation of pp60, pp70, and pp118 in control cells, but inhibited the tyrosine phosphorylation induced by both MSU and CPPD microcrystals. Densitometric analysis indicated that the stimulation of tyrosine phosphorylation of pp118 and of pp70 was inhibited by  $> 60\%$  by colchicine for both MSU and CPPD crystals and that of pp130 and pp60 by  $> 55\%$  for MSU and  $> 30\%$  for CPPD (Table II). Although inhibited, the densitometric analysis of pp80 was not performed because of its proximity to pp70. The specificity of the effects of colchicine on the crystal-induced tyrosine phosphorylation was confirmed by the failure of the inactive analogs of colchicine,  $\beta$ - and  $\gamma$ -lumicolchicine, to inhibit MSU- (Fig. 4) or CPPD- (data not shown) induced tyrosine phosphorylation. The stimulus-specific effect of colchicine was confirmed by its inability to inhibit the FMLP-induced tyrosine phosphorylation (Fig. 5). Pretreatment of neutrophils with colchicine did, however, increase the tyrosine phosphorylation of the pp60 and pp70 in response to FMLP. A densitometric analysis of the FMLP-induced tyrosine phosphorylation showed a 1.9- and 3.3-fold increase for the pp60 and pp70 proteins, respectively (Table II).

**Effect of colchicine on microcrystal- and FMLP-induced superoxide anion production.** Neutrophils were preincubated for 1 h with  $10 \mu\text{M}$  colchicine or its diluent (DMSO). The cells were then further stimulated either with 3 mg/ml MSU or CPPD crystals for 15 min or with  $10^{-7}$  M FMLP for 5 min. The amounts of superoxide anion produced during this time in untreated or colchicine-treated neutrophils were then measured. While pretreatment of neutrophils with colchicine inhibited crystal-induced superoxide production, it, on the other hand, enhanced that induced by FMLP (Fig. 6).

## Discussion

We have observed that inflammatory microcrystals stimulate tyrosine phosphorylation in human neutrophils. In addition to



**Figure 3.** Effects of soluble agonists on protein tyrosine phosphorylation. Neutrophils ( $15 \times 10^6/\text{ml}$ ) were stimulated for 1 min with  $10^{-7}$  M FMLP,  $C_{5a}$ , or  $LTB_4$  and for 15 min with 3 mg/ml MSU or CPPD crystals. Blotting and revelation of the phosphotyrosine bands were carried out as described under Methods.

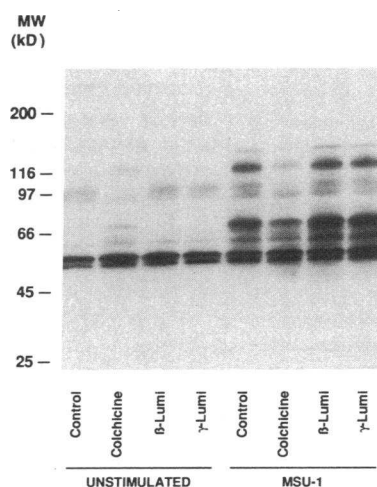
demonstrating for the first time the stimulation of this signaling pathway by MSU and CPPD crystals, these results also provide evidence that this response is characteristic of these stimuli, and that it can be distinguished from that induced by other particulate or soluble neutrophil agonists.

The addition of MSU and CPPD crystals to human neutrophils caused a time- and concentration-dependent increase in the tyrosine phosphorylation level of at least five major proteins, pp130, pp118, pp80, pp70, and pp60 (Fig. 1, A and B). The pp118 and pp70 consistently showed the greatest increases, the pp70 being the major phosphorylated substrate in response to the crystals (Fig. 1 A). The tyrosine phosphorylation response to MSU crystals was more rapid and more pronounced than that induced by CPPD crystals. Thus, the relative abilities of the microcrystals to stimulate tyrosine phosphorylation in human neutrophils parallel their phlogistic

**Table II.** Effect of Colchicine on Microcrystal-induced Tyrosine Phosphorylation\*

| Conditions      | Level of tyrosine phosphorylation (arbitrary scanning units) |                     |                     |                  |
|-----------------|--|---------------------|---------------------|------------------|
|                 | pp60   | pp70                | pp118               | pp130            |
| Control         | 28±5   | 4±1                 | 5±1                 | 1±1              |
| Colchicine      | 36±5 <sup>‡</sup>  | 10±1 <sup>‡</sup>   | 7±1*                | 2±1              |
| MSU             | 155±18   | 314±38              | 70±14               | 15±3             |
| MSU/colchicine  | 69±9 <sup>‡</sup>  | 122±33 <sup>‡</sup> | 21±4 <sup>‡</sup>   | 5±1 <sup>‡</sup> |
| CPPD            | 58±9   | 145±31              | 38±8                | 6±1              |
| CPPD/colchicine | 54±10 <sup>‡</sup>   | 53±9 <sup>‡</sup>   | 14±4 <sup>‡</sup>   | 4±1 <sup>‡</sup> |
| FMLP            | 74±16 <sup>‡</sup>   | 35±14*              | 350±33 <sup>‡</sup> | 23±9*            |
| FMLP/colchicine | 151±36 <sup>‡</sup>  | 136±32 <sup>‡</sup> | 396±41              | 26±6             |

\* Neutrophils were treated with either diluent or with colchicine ( $10^{-5}$  M) for 1 h at 37°C and further incubated with MSU or CPPD crystals for 30 or 45 min, respectively, or with  $10^{-7}$  M FMLP for 1 min. The autoradiographs from 7 to 12 experiments were analyzed using a densitometer (Research Analysis Systems; Amersham Canada, Oakville, Ont. Canada), the values were corrected for background and are expressed in arbitrary scanning units (mean±SEM). The values that differ significantly from the appropriate (no colchicine) controls are indicated as follows: \*  $P < 0.05$ , <sup>‡</sup>  $P < 0.01$ .

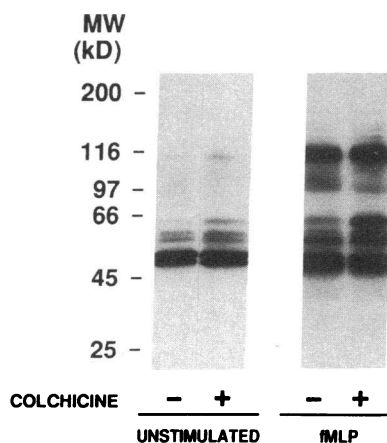


**Figure 4.** Effects of colchicine,  $\beta$ - and  $\gamma$ -lumicolchicine on MSU-induced tyrosine phosphorylation. Neutrophils ( $15 \times 10^6/\text{ml}$ ) were incubated for 1 h with 10  $\mu\text{M}$  colchicine,  $\beta$ - or  $\gamma$ -lumicolchicine, and then stimulated with 3 mg/ml MSU crystals for 30 min. Blotting and revelation of the phosphotyrosine bands were carried out as described under Methods.

potential (3–7, 18), suggesting that these two properties may be causally related.

It has been pointed out that the inflammatory potential of microcrystals might be related in part to their size, “small” crystals, such as MSU crystals, are found to be more inflammatory than “large” crystals, such as CPPD crystals (33). On the other hand, Antommatei et al. (34) found no correlation between crystal size or shape and severity of inflammation. These conflicting findings prompted us to test different batches of MSU and CPPD crystals with varying physical characteristics. The results of these investigations demonstrated that all of the microcrystals tested stimulated a similar pattern of tyrosine phosphorylation. Furthermore, no correlation between the physical characteristics of the crystals (size, surface area, or density) and their ability to stimulate tyrosine phosphorylation was found.

The experiments performed in the course of these studies allow several conclusions to be drawn about the interactions of microcrystals with human neutrophils. Firstly, the requirement for crystallization was demonstrated by the lack of effect of uric acid. Secondly, the nature of the cation mattered little as potassium urate crystals were nearly as potent as MSU crystals. Thirdly, the tyrosine phosphorylation pattern elicited by microcrystals is not a generalized response to the phagocytic process, since the responses to other particulate stimuli, such as unopsonized zymosan and latex beads (Fig. 2), were both



**Figure 5.** Effect of colchicine on FMLP-induced tyrosine phosphorylation. Neutrophils ( $15 \times 10^6/\text{ml}$ ) were incubated for 1 h with 10  $\mu\text{M}$  colchicine and then stimulated for 1 min with  $10^{-7}$  M FMLP. Blotting and revelation of the phosphotyrosine bands were carried out as described under Methods.



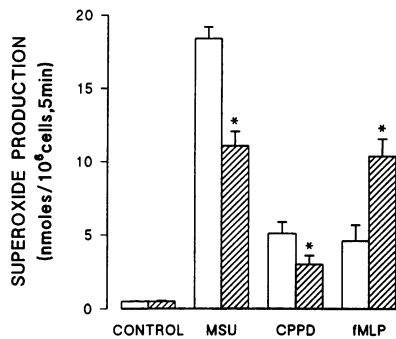


Figure 6. Effect of colchicine on microcrystal- and FMLP-induced superoxide anion production. Neutrophils ( $15 \times 10^6/\text{ml}$ ) were incubated for 1 h with  $10 \mu\text{M}$  colchicine and then stimulated for 5 min with  $10^{-7}$  M FMLP or for 15 min with 3 mg/ml MSU or CPPD crystals. Superoxide anion

production was monitored as described in Methods. Statistical significance: \* $P < 0.05$ , Student's two-tailed, paired  $t$  test. □, Control; ▨, colchicine.

weaker and qualitatively different. Thus, the response monitored in the present study is a sensitive and characteristic feature of the stimulation of human neutrophils by microcrystals.

An additional level of specificity of the tyrosine phosphorylation response to microcrystals was revealed by comparing it to those induced by soluble agonists. Neutrophil activation by chemotactic factors such as FMLP (22, 25, 26, 29), platelet-activating factor (28), leukotriene  $B_4$  (27), and cytokines (24) has previously been shown to be associated with increased tyrosine phosphorylation. These results suggest that tyrosine phosphorylation may play important roles in the activation of neutrophils, a suggestion strengthened by the ability of tyrosine kinase inhibitors to inhibit the chemotactic factor-stimulated locomotory and adherence responses of the cells (29), generation of superoxide anions (25), and activation of phospholipase D (34a). Previous reports have demonstrated that MSU and CPPD crystals activate the excitation-coupling sequence of human neutrophils (increase in intracytoplasmic calcium (15, 20, 21) and leukotriene synthesis (15) among others) in a manner suggesting the involvement of certain common transductional pathways between microcrystals and soluble or particulate agonists. On the other hand, the interactions between microcrystals and human neutrophils have been found to be significantly less sensitive to inhibition by pertussis toxin than those initiated by chemotactic factors (20, 21). The distinctive patterns of tyrosine phosphorylation induced by microcrystals and soluble agonists observed in the present study further indicate that qualitative differences exist between the mechanism of activation of human neutrophils by these two classes of stimuli. The present results are both more direct and of significant additional relevance to the functional status of the cells than those based on inhibitor studies.

The relevance of the microcrystal-induced tyrosine phosphorylation to the mechanism of neutrophil activation is underlined by the effects of colchicine on crystal and FMLP-induced tyrosine phosphorylation and production of superoxide anions. Colchicine is unique among therapeutic agents used in the treatment of acute gout and pseudogout, in that its usefulness is generally felt to be relatively limited to these rheumatic diseases (35). The ability of colchicine (but not of its inactive analogs) to specifically inhibit microcrystal-induced tyrosine phosphorylation and the parallel effects of colchicine on tyrosine phosphorylation and on superoxide anion generation (Figs. 4–6) provide additional support for the hypothesis that the latter response is linked to the phlogistic properties of microcrystals. It is important, however, to point out that a caus-

ative link between these two responses is only suggested but not established by the data at hand. Although the precise mechanism of action of colchicine has remained elusive (14, 36), the present results suggest a novel putative site of action by demonstrating that the alkaloid interferes either with one or more elements of the activation pathways used by microcrystals or directly with a specific subset of tyrosine kinases. The elucidation of the biochemical mechanisms by which colchicine exerts its inhibitory effect on crystal-induced tyrosine phosphorylation and to a larger extent on crystal-induced neutrophil activation while of significant basic and clinical interest, is, however, not directly relevant to the aim of the present study.

While the present study provides strong evidence for a role of tyrosine phosphorylation in crystal-induced neutrophil activation, the key to understanding the details of signal transduction pathways is likely to lie in the identification of the phosphotyrosine substrates detected in neutrophils stimulated by MSU and CPPD crystals. The nature of the predominant phosphotyrosine band, pp70, remains unknown. Among the potential substrates, ribosomal S6 kinase (70 kD) is an attractive candidate, since it has been shown to be one, among others, of the PDGF-induced phosphorylated substrate (37). In addition, Raf-1, a 74-kD protein, has been reported to be implicated as an intermediate in signal transduction mechanisms (38). While five major substrates were reported in this study, a colchicine-sensitive band of an apparent molecular mass of  $\sim 100$  kD was found phosphorylated under most of the microcrystals experimental conditions tested. Recent reports have shown that MSU crystals stimulate  $\text{PLA}_2$  activity and induce the activity of  $\text{PLA}_2$ -activating protein (14), two crystal-induced responses found to be inhibited by colchicine. Since a  $\text{PLA}_2$  of an apparent molecular weight of 100 kD has been recently cloned in the human monoblast U937 cells (39), a colchicine-sensitive  $\text{PLA}_2$  could be an attractive substrate for the pp100 described in this study.

In summary, the results of the present investigation have uncovered a biochemical response, the specificity and characteristics of which make it likely that it is of significant relevance to the interactions between microcrystals and human neutrophils. Furthering our understanding of the stimulation of tyrosine phosphorylation by microcrystals (nature and mechanism of activation of the kinases and/or phosphatases involved, as well as the identification of the substrates and their physiological functions) will lead to a significantly improved understanding of neutrophil physiology and potentially to more efficient strategies to control the associated inflammatory reactions.

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