J. Biochem. Mol. Biol. Vol. 28, No. 3 pp. 210~215 May 31, 1995

Effects of Staurosporine and Genistein on Superoxide Generation and Degranulation in PMA- or C5a-Activated Neutrophils

Sung-Heon Ha and Chung-Soo Lee*

Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 156-756, Korea
(Received October 19, 1994)

Abstract: Effects of staurosporine, genistein and pertussis toxin on PMA-induced superoxide generation and degranulation in neutrophils were investigated. Their effects were also examined in C5a-stimulated superoxide generation. PMA-induced superoxide generation was inhibited by staurosporine but was not affected by pertussis toxin. Genistein enhanced the stimulatory effect of PMA in a dose dependent fashion. C5a-induced superoxide generation was inhibited by staurosporine, genistein and pertussis toxin. An NADPH oxidase system of resting neutrophils was activated by PMA, and the stimulatory effect of PMA was inhibited by staurosporine but was not affected genistein and pertussis toxin. The activity of NADPH oxidase in the membrane fraction of PMA-activated neutrophils was not affected by staurosporine and genistein. PMA-induced acid phosphatase release was inhibited by staurosporine and genistein, whereas the effect of pertussis toxin was not detected. These results suggest that the role of protein tyrosine kinase in neutrophil activation mediated by direct activation of protein kinase C may be different from receptor-mediated activation. The action of protein kinase C on the respiratory burst might be affected by the change of protein tyrosine kinase activity.
Key words: Staurosporine, Genistein, Superoxide generation, Degranulation, Neutrophils.

Neutrophils release reactive oxygen species and lysosomal enzyme including leukotrienes when stimulated by a variety of particulate and soluble stimuli (Fantone and Ward, 1982; Serhan *et al.*, 1982). Protein kinase C may play a crucial role in signal transduction and other cellular processes (Nishizuka, 1984). In neutrophils, activation of protein kinase C by phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) stimulates superoxide generation (Sha'afi *et al.*, 1983) and inhibits agonist-stimulated bivalent cation influx (McCarthy *et al.*, 1989). PMA has been shown to stimulate neutrophil responses, aggregation, superoxide generation, depolarization of the plasma membrane, phospholipid turnover and degranulation (Tauber, 1987). However, the inhibitors of protein kinase C including H-7 do not inhibit all neutrophil activation (Gerard *et al.*, 1986; Berkow *et al.*, 1987). Thus, it is suggested that the activation of other protein kinases may be involved in the biochemical signaling pathways in neutrophils. Protein kinase C-dependent and-independent activation of NADPH oxidase in neutrophils has been reported (Watson *et al.*, 1991). In addition, protein kinase C has been shown to be regulated by a cytosolic calcium- independent signal (O'Flaherty *et al.*, 1990). Thus, the role of protein kinase C in the activation process of neutrophils is still not clarified.

Neutrophils are considered to have distinct cytosolic and particulate tyrosine kinase activities (Berkow *et al.*, 1989). Neutrophils stimulated by fMet-Leu-Phe (fMLP) or PMA show an increase in protein tyrosine phosphorylation (Berkow and Dodson, 1990). Tyrosine kinase inhibitors inhibit superoxide generation in neutrophils stimulated by fMLP in the presence of granulocyte-macrophage colony stimulating factor (Tanimura *et al.*, 1992) and also inhibit prostaglandin production in platelet activating factor-activated macrophage-cell line (Glaser *et al.*, 1990).

These data suggest that protein tyrosine phosphorylation may also be involved in cellular activation. Effects of protein kinase inhibitors on agonist-induced superoxide generation in primed neutrophils are different from those on nonprimed neutrophils. It has been found that superoxide generation by protein kinase C activation without priming is stimulated by genistein and ST 638, inhibitors of protein tyrosine kinase (Tanimura *et al.*, 1992). Thus, it is suggested that protein tyrosine kinase possibly plays a regulatory role in protein kinase C-acti-

*To whom correspondence should be addressed.
Tel: 82-2-810-2501, Fax: 82-2-813-5387.

vated responses.

In this study, effects of protein kinase C inhibitor, protein tyrosine kinase inhibitor and G protein inhibitor on PMA-stimulated superoxide generation and degranulation in neutrophils were investigated. Their effects were also examined in complement component C5 a-stimulated superoxide generation.

Materials and Methods

Phorbol 12-myristate 13-acetate (PMA), C5a, staurosporine, pertussis toxin, DiC_8, phenylmethylsulfonyl fluoride (PMSF), ferricytochrome c, NADPH and a diagnostic kit for acid phosphatase were purchased from Sigma Chemical Co.. Genistein was obtained from Gibco BRL Life Tech., Inc.. Other chemicals were of analytical reagent grade.

Preparation of human neutrophils

Neutrophils were prepared from fresh whole human blood, anticoagulated with 10% acid-citrate-dextrose, by dextran sedimentation, hypotonic lysis of erythrocytes and Ficoll-Hypaque density centrifugation (Markert *et al.*, 1984). The neutrophils were suspended in Dulbecco's phosphate-buffered saline at a concentration of 1×10^7/ml. Final suspensions of neutrophils were comprised of about 97% neutrophils as judged from by Wright-Giemsa stain and viability was more than 98% as judged from trypan blue dye exclusion.

Preparation of membrane and cytosolic fractions from neutrophils

Membrane and cytosolic fractions were prepared from neutrophils by the method of Verhoeven *et al.* (1993). Resting neutrophils or activated neutrophils (0.1 μg/ml PMA/10^7 cells at 37℃ for 5 min) were suspended (1×10^8 cells/ml) in cold sonication buffer (10 mM HEPES, 1 mM EGTA, 0.15 M sucrose and 0.5 mM PMSF in phosphate-buffered saline, pH 7.2) for 10 min on ice. The neutrophil suspensions were sonicated for three 15 sec intervals at 40 watts power with a Branson sonifier cell disruptor (Mod. W 185D). The suspensions were centrifuged at 6,000 g for 10 min to remove unbroken cells and nuclei. Two ml of supernatants were layered on a discontinuous sucrose gradient consisting of 2 ml of 40% (W/V) sucrose and 2 ml of 20% (W/V) sucrose and were centrifuged at 100,000 g for 1 h in a Beckman L5-50B ultracentrifuge. The supernatant 75% (the cytosolic fraction) and the interface of the sucrose layers (membrane fraction) were taken, respectively and were centrifuged at 100,000 g for 45 min. The membrane fraction was suspended in 0.1 M mannitol, 10 mM sodium phosphate and 20% ethylene glycol, pH 7.4. The membrane suspension and the cytosolic fraction were stored at −70℃. Protein concentration was determined by the method of Lowry *et al.* (1951).

Assay of superoxide generation

Superoxide generation was determined by superoxide dismutase- inhibitable cytochrome c reduction with a UV max kinetic microplate reader (Molecular Devices) (Markert *et al.*, 1984). Assay mixtures (0.2 ml) consisted of 5×10^5/ml neutrophils, 75 μM ferricytochrome c, 10 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1% bovine serum albumin in Dulbecco's phosphate-buffered saline. The reference well contained 60 μg/ml SOD. Before measuring superoxide generation, neutrophils were preincubated with cytochalasin B (5 μg/10^7 cells) for 5 min at 37℃. The reactions were started by adding stimuli to each well, and the change in absorbance at 550 nm was record. The amount of reduced cytochrome c was calculated by using an extinction coefficient of 2.1×10^4 $M^{-1}cm^{-1}$ at 550 nm (Cohen and Chovaniec, 1978).

For cell priming, neutrophils were incubated with 10 μM DiC_8 in the absence of cytochalasin B for 5 min at 37℃ and then superoxide generation was initiated immediately by adding the stimulating agents.

Assay of NADPH oxidase activity

The activity of NADPH oxidase was measured as the reduction of ferricytochrome c by superoxide anion produced from oxidation of NADPH by oxidase (Markert *et al.*, 1984). The reaction mixtures consisted of 0.1 mg protein of membrane fraction, 0.2 mg protein of cytosol fraction, 100 μM NADPH, 10 μM flavine adenine dinucleotide, 75 μM ferricytochrome c, 1.23 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM HEPES-tris, pH 7.4 in a total volume of 1.0 ml. The reaction mixtures were preincubated for 5 min at 37℃ and the reaction was initiated by adding NADPH and the stimulating agents. The reduction rate of ferricytochrome c was measured at 550 nm.

Assay of acid phosphatase activity

The amount of acid phosphatase released from activated neutrophils was measured using a Sigma diagnostic kit. The reaction mixtures contained 2×10^6 neutrophils, 0.1 μg/ml PMA, 20 mM HEPES-tris and HBSS buffer, at pH 7.4 in a total volume of 0.5 ml. After 15 min of incubation at 37℃, the reaction mixtures were centrifuged at 830 g for 10 min and the supernatants were taken. Aliquots (0.2 ml) were mixed with 0.5 ml of 4 mg/ml p-nitrophenyl phosphate disodium and 0.5 ml of 90 mM citrate buffer solution, pH 4.8.

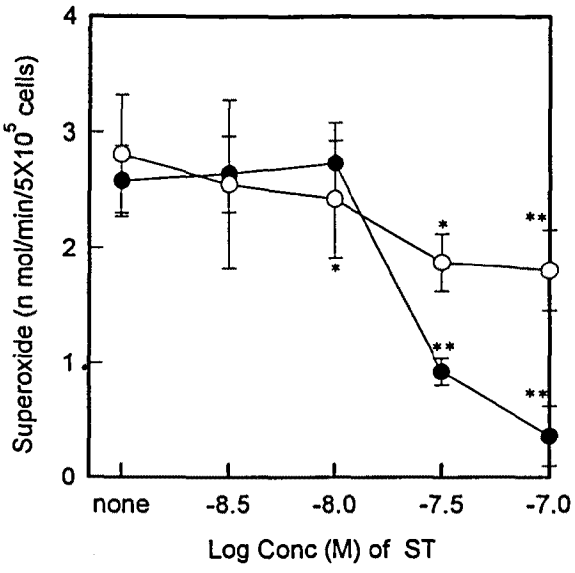

Fig. 1. Inhibitory effect of staurosporine on superoxide generation in activated neutrophils. Neutrophils (5×10^5 cells/0.2 ml) were stimulated with 0.1 μg/ml PMA (or 11.6 nM C5a) in the presence of varying concentration of staurosporine (ST). Values are means± SD, n=4~6. ●–●, PMA; ○–○, C5a. **p<0.01, *p<0.05 by Student's t-test.

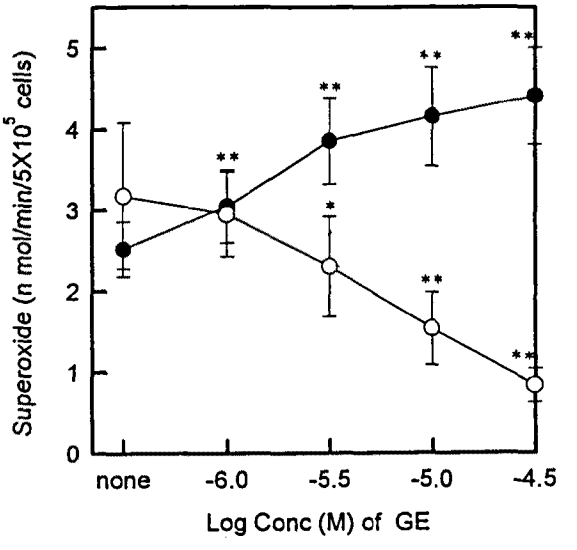

Fig. 2. Effect of genistein on superoxide generation in activated neutrophils. Neutrophils (5×10^5 cells/0.2 ml) were stimulated with 0.1 μg/ml PMA (or 11.6 nM C5a) in the presence of varying concentration of genistein (GE). Values are means± SD, n=6~10. ●–●, PMA; ○–○, C5a. **p<0.01, *p<0.05 by Student's t-test.

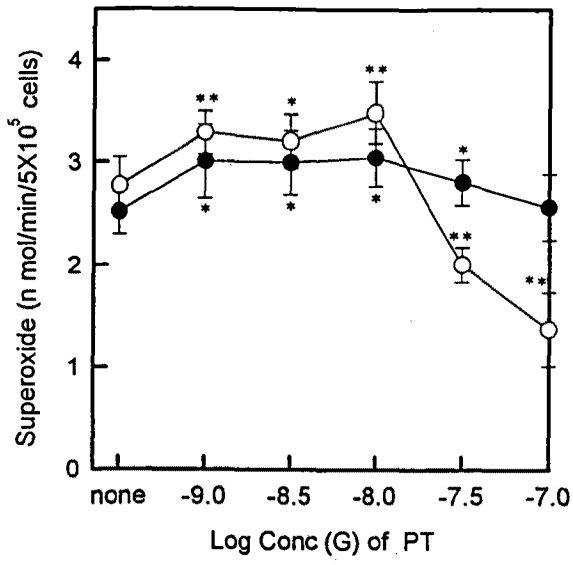

Fig. 3. Effect of pertussis toxin on superoxide generation in activated neutrophils. Neutrophils (5×10^5 cells/0.2 ml) were stimulated with 0.1 μg/ml PMA (or 11.6 nM C5a) in the presence of pertussis toxin (PT). Values are means± SD, n=3~6. ●–●, PMA; ○–○, C5a. **p<0.01, *p<0.05 by Student's t-test.

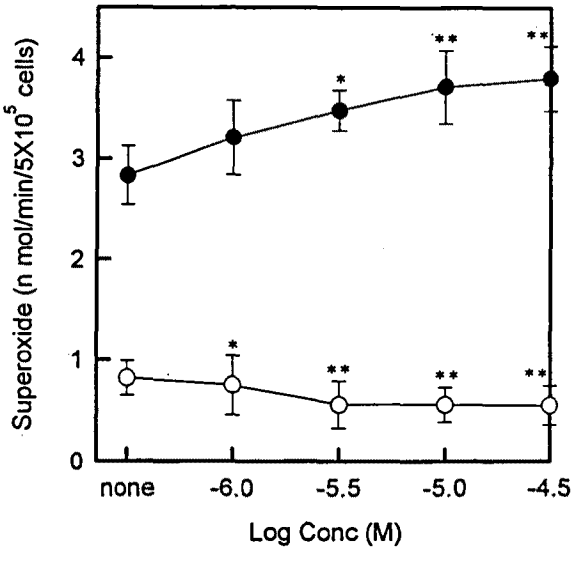

Fig. 4. Enhancement of PMA-stimulated superoxide generation in DiC_8-primed neutrophils by genistein. Ten μM DiC_8-primed neutrophils (5×10^5 cells/0.2 ml) were stimulated with 0.1 μg/ml PMA in the presence of varying concentration of genistein (●–●). Effect of genistein on DiC_8-induced superoxide generation was examined (○–○). Values are means± SD, n=6~10. **p<0.01, *p<0.05 by Student's t-test.

After 30 min of incubation at 37℃, the incubation was stopped by adding 5 ml of 0.1 N NaOH. The absorbance was read spectrophotometrically at 405 nm. Activity of acid phosphatase was estimated from the standard curve using p-nitrophenol standard solution and is expressed as the mUnit/2×10^6 cells.

Results

Effects of staurosporine, genistein and pertussis toxin on neutrophil superoxide generation

Staurosporine, an inhibitor of protein kinase C, inhibited superoxide generation in PMA-or C5a-activated

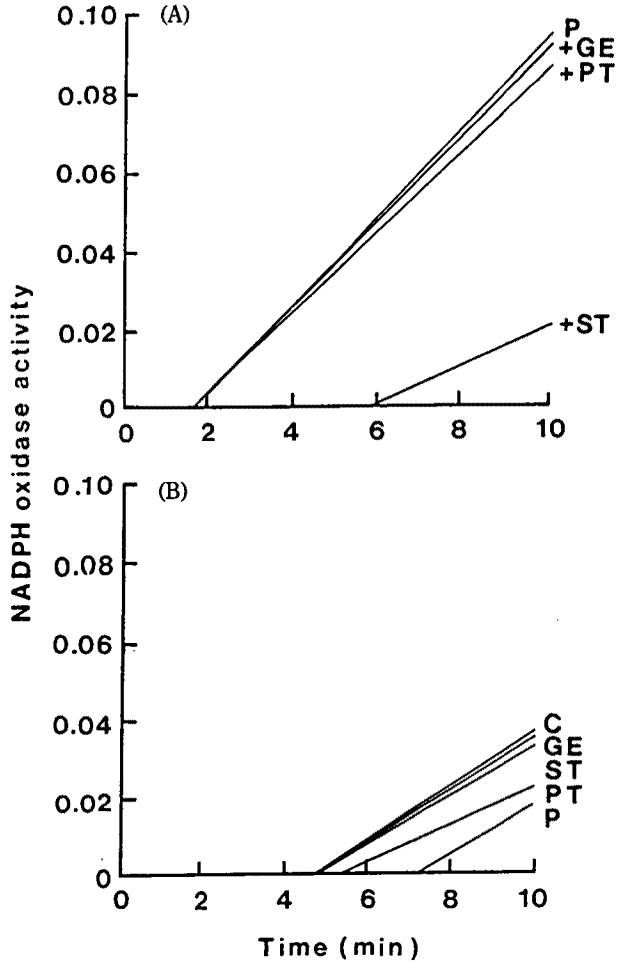

Fig. 5. Effects of staurosporine and genistein on NADPH oxidase activity. Reaction mixtures contained 0.1 mg/ml membrane and 0.2 mg/ml cytosolic fraction from resting neutrophils (A) and 0.1 mg/ml membrane fraction from PMA-activated neutrophils (B). The response was initiated by adding NADPH and PMA. C, no addition of PMA; ST, 100 nM staurosporine; GE, 10 μM genistein; PT, 0.1 μg/ml pertussis toxin; P, 0.1 μg/ml PMA.

neutrophils (Fig. 1). The stimulatory effects of 0.1 μg/ml PMA and 11.6 nM C5a were inhibited 87% and 39%, respectively by 100 nM staurosporine. The inhibitory effect of staurosporine on PMA-induced superoxide generation was greater than its effect on C5a. On the contrary, superoxide generation in activated neutrophils was differently affected by genistein, a protein tyrosine kinase inhibitor. Fig. 2 shows that genistein further stimulated PMA- induced superoxide generation in a dose-dependent fashion but inhibited C5a-induced superoxide generation. The stimulatory effect of PMA was enhanced 65% by 10 μM genistein. Pertussis toxin, a G protein inhibitor, did not affect PMA-induced superoxide generation but inhibited the stimulatory effect of C5a by 50% at the concentration of 0.1 μg/ml (Fig. 3).

Effect of genistein on PMA-induced superoxide generation in synthetic diacylglycerol DiC_8-primed neutrophils was examined. The amount of superoxide in 10 μM DiC_8-primed neutrophils was 0.82± 0.17 nmol/min/5×10^5 cells (n=10). As shown in Fig. 4, genistein inhibited superoxide generation in DiC_8-primed neutrophils, whereas it enhanced PMA-induced superoxide generation in primed neutrophils and at 10 μM genistein, a stimulation of 31% was observed.

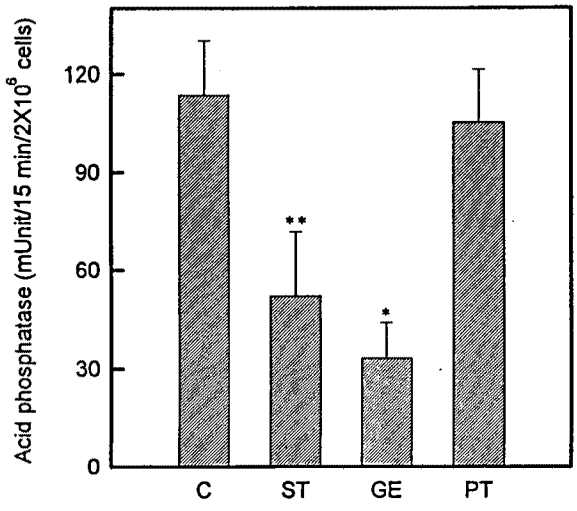

Fig. 6. Effects of staurosporine, genistein and pertussis toxin on acid phosphatase release. Neutrophils (2×10^6 cells/0.5 ml) were stimulated with 0.1 μg/ml PMA (C) in the presence of 100 nM staurosporine (ST), 10 μM genistein (GE) and 0.1 μg/ml pertussis toxin (PT), respectively. Acid phosphatase activity were expressed as mUnit/2×10^6 cell. Values are means± SD, n=3. **p<0.01, *p<0.05 by Student's t-test.

Effects of staurosporine and genistein on NADPH oxidase activity

Influences of inhibitors of protein kinase C and protein tyrosine kinase on superoxide forming NADPH oxidase in a cell-free system were investigated. NADPH oxidase, which consists of a mixture of the membrane and cytosolic fractions of resting neutrophils, was activated by 0.1 μg/ml PMA (Fig. 5A). The PMA-activated NADPH oxidase was inhibited by 100 nM staurosporine but was not affected by 10 μM genistein and 0.1 μg/ml pertussis toxin. The NADPH oxidase, which is obtained from PMA-activated neutrophils, was inhibited by 0.1 μg/ml PMA and 0.1 μg/ml pertussis toxin, whereas 100 nM staurosporine and 10 μM genistein did not affect it (Fig. 5B).

Effects of staurosporine and genistein on acid phosphatase release

After neutrophils were preincubated for 5 min with the inhibitors, acid phosphatase release was initiated by the addition of PMA. As shown in Fig. 6, 0.1 μg/ml PMA-induced acid phosphatase release was inhibited

by 100 nM staurosporine and 10 μM genistein but was not affected by 0.1 μg/ml pertussis toxin.

Discussion

Activation of neutrophils by chemoattractants, such as the platelet-activating factor and C5a leads to ionic and molecular changes in the plasma membrane or intracellular components followed by an alteration of neutrophil response (Ingraham *et al.*, 1982; Becker *et al.*, 1985). Receptor binding induces phosphoinositide hydrolysis by phospholipase C, promoting the formation of the intracellular messengers, inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (Berridge, 1987). These molecules are responsible for the release of calcium from intracellular stores and the activation of protein kinase C, respectively. A change in the steady state of cytosolic calcium has been suggested to be involved in the early triggering of activation of neutrophil response (Smolen *et al.*, 1981). Activation of protein kinase C appears to stimulate neutrophil responses, degranulation and superoxide generation (Sha'afi *et al.*, 1983). In contrast, it is reported that PMA inhibits chemoattractant-induced degranulation in neutrophils and agonist-stimulated elevation of $Ca^{2+}i$ (Naccache *et al.*, 1985).

Cell-free NADPH oxidase from resting neutrophils was stimulated by PMA, and the stimulatory effect of PMA was inhibited by staurosporine but was not affected by genistein and pertussis toxin. Thus, these findings support the reports that PMA may activate protein kinase C directly by pass activation of the membrane linked-G proteins (Tauber, 1987). NADPH oxidase in a cell-free system is probably not influenced by protein tyrosine kinase. However, the activity of NADPH oxidase obtained from PMA-activated neutrophils, was inhibited by protein kinase C activation. The preactivated NADPH oxidase system may be down-regulated by the change of protein kinase C activity. The role of protein kinase C in the activation mechanism of NADPH oxidase %is controversial. Stimulus-induced superoxide generaton is partially inhibited by protein kinase C inhibitors (Berkow *et al.*, 1987). These findings indicate that other protein kinases may be involved in stimulus-induced superoxide generation. fMLP and PMA induce the tyrosine phosphorylation of proteins from the cytosolic fraction and the particulate fraction of neutrophils (Berkow and Dodson, 1990). Inhibitors of protein tyrosine kinase inhibit superoxide generation and eicosanoid production in chemoattractant-activated macrophage-cell line (Utsumi *et al.*, 1992; Glaser *et al.*, 1993). Thus, these data suggest that protein tyrosine phosphorylation is involved in activating neutrophil response.

PMA-induced superoxide generation was inhibited by staurosporine but not affected by pertussis toxin. This result confirms previous reports that PMA acts directly on protein kinase C without the intervention of G protein (Tauber, 1987). Meanwhile, genistein further stimulated PMA-induced superoxide generation in a dose-dependent fashion. This finding coincides with the investigation of Tanimura *et al.* (1992). Stimulation of protein kinase C activation-induced superoxide generation by protein tyrosine kinase inhibition suggests that the action of protein kinase C may be affected by the change of protein tyrosine kinase activity. However, the stimulatory effect of genistein was not observed in C5a-induced superoxide generation. The stimulatory effect of complement component C5a on phagocytic cells is known to be mediated by G proteins (Becker *et al.*, 1985). C5a-induced superoxide generation was inhibited by pertussis toxin, staurosporine and genistein. The activation of protein kinase C and protein tyrosine kinase appears to be involved in the C5a-activation of neutrophils. It is also suggested that the role of protein tyrosine kinase in neutrophil activation mediated by direct activation of protein kinase C may be different from receptor-mediated neutrophil activation.

A low concentration of synthetic diacylglycerols prime neutrophils and promote superoxide generation by fMLP (Bass *et al.*, 1989). The priming effect is considered to involve mechanisms distinct from classical translocation or activation of protein kinase C (Bass *et al.*, 1987). Inhibition by genistein of the priming dose of DiC_8-induced superoxide generation indicates that the activation of protein tyrosine kinase may be involved partially in the priming of neutrophils. The stimulatory effect of genistein on PMA-induced superoxide generation in DiC_8-primed neutrophils was smaller than that in nonprimed neutrophils. In the preactivated state of protein tyrosine kinase, the stimulatory effect of protein tyrosine kinase inhibitor on superoxide generation by protein kinase C activation may be decreased. The finding also suggests the interaction of protein kinase C with protein tyrosine kinase. Both staurosporine and genistein inhibited degranulation by PMA, which is insensitive to G proteins. The present data indicate that the activation mechanism of the respiratory burst may be different from degranulation (Shin *et al.*, 1989). Degranulation appears to be regulated partially by protein tyrosine kinase.

References

Bass, D. A., Gerard, C., Olbrantz, P., Wilson, J., McCall, C. E. and McPhail, L. C. (1987) *J. Biol. Chem.* **262**, 6643.

Bass, D. A., McPhail, L. C., Schmitt, J. D., Morris-Natschke,

S., McCall, C. E. and Wykle, R. L. (1989) *J. Biol. Chem.* **264**, 19610.

Becker, E. L., Kermode, J. C., Naccache. P. H., Yassin, R., Marsh, M. L., Munoz, J. J. and Sha'afi, R. I. (1985) *J. Cell Biol.* **100**, 1640.

Berkow, R. L. and Dodson, R. W. (1990) *Blood* **75**, 2445.

Berkow, R. L., Dodson, R. W. and Kraft, A. S. (1987) *J. Leuk. Biol.* **41**, 441.

Berkow, R. L., Dodson, R. W. and Kraft, A. S. (1989) *Biochim. Biophys. Acta* **997**, 292.

Berridge, M. J. (1987) *Ann. Rev. Biochem.* **56**, 159.

Cohen, H. J. and Chovaniec, M. E. (1978) *J. Clin. Invest.* **61**, 1081.

Fantone, J. C. and Ward, P. A. (1982) *Am. J. Pathol.* **107**, 397.

Gerard, C., McPhail, L. C., Marfat, A., Stimler-Gerard, N. P., Bass, D. A. and McCall, C. E. (1986) *J. Clin. Invest.* **77**, 61.

Glaser, K. B., Asmis, R. and Dennis, E. A. (1990) *J. Biol. Chem.* **265**, 8658.

Glaser, K. B., Sung, A., Bauer, J. and Weichman, B. M. (1993) *Biochem. Pharmacol.* **45**, 711.

Ingraham, L. M., Coates, T. D., Allen, J. M., Higgins, C. P., Baehner, R.L. and Boxer, L.A. (1982) *Blood* **59**, 1259.

Lowry. O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 266.

Markert, M., Andrews, P. C. and Babior, B. M. (1984) *Methods Enzymol.* (Packer, L., ed) Vol. 105, pp. 358-365, Academic Press, Inc., New York, New York.

McCarthy, S. A., Hallam, T. J. and Merritt, J. E. (1989) *Biochem. J.* **264**, 357.

Naccache, P. H., Molski, T. F. P., Borgeat, P., White, J. R. and Sha'afi, R.I. (1985) *J. Biol. Chem.* **260**, 2125.

Nishizuka, Y. (1984) *Nature* **308**, 693.

O'Flaherty, J. T., Jacobson, D. P., Redman, J. F. and Rossi, A. G. (1990) *J. Biol. Chem.* **265**, 9146.

Serhan, C., Fridovich, I., Goetzl, E., Dunham, P. B. and Weissmann, G. (1982) *J. Biol. Chem.* **257**, 4746.

Sha'afi, R. I., White, J. R., Molski, T. F. P., Shefeyk, J., Volpi, M., Naccache, P. H. and Feinstein, M. B. (1983) *Biochem. Biophys. Res. Commun.* **114**, 638.

Shin, J. H., Lee, C. S., Han, E. S., Shin, Y. K. and Lee, K. S. (1989) *Korean J. Pharmacol.* **25**, 75.

Smolen, J. E., Korchak, H. M. and Weissmann, G. (1981) *Biochim. Biophys. Acta* **677**, 512.

Tanimura, M., Kobuchi, H., Utsumi, T., Yoshioka, T., Kataoka, S., Fujita, Y. and Utsumi, K. (1992) *Biochem. Pharmacol.* **44**, 1045.

Tauber, A. I. (1987) *Blood* **69**, 711.

Utsumi, T., Klostergaard, J., Akimura. K., Edashige, K., Sato, E. F. and Utsumi, K. (1992) *Arch. Biochem. Biophys.* **294**, 271.

Verhoeven, A. J., Leusen, J.H.W., Kessels, G. C. R., Hilarius, P. M., de Bont, D. B. A. and Liskamp, R. M. J. (1993) *J. Biol. Chem.* **268**, 18593.

Watson, F., Robinson, J. and Edwards, S. W. (1991) *J. Biol. Chem.* **266**, 7432.