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Effects of Staurosporine and Genistein on Superoxide and HOCl Production in C5a- or PMA-activated Neutrophils

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ABSTRACT

Effects of staurosporine, genistein and pertussis toxin on superoxide and HOCl production in C5a- or PMA-activated neutrophils were investigated.

A C5a-induced superoxide and H_2O_2 production was inhibited by staurosporine, genistein and pertussis toxin. The stimulatory effect of PMA was inhibited by staurosporine but was not affected by pertussis toxin, whereas it was further promoted by genistein. Staurosporine and genistein inhibited superoxide production by sodium fluoride, but pertussis toxin did not affect it. PMA-induced H_2O_2 production was inhibited by staurosporine but was not affected by pertussis toxin. Genistein did not show a stimulatory effect on PMA-induced H_2O_2 production. Staurosporine and pertussis toxin inhibited HOCl production by C5a- or PMA, whereas genistein stimulated it. C5a- or PMA-induced myeloperoxidase release was inhibited by genistein, in this response the effect of pertussis toxin was not detected. Staurosporine did not affect the stimulatory effect of PMA on the release. Myeloperoxidase activity was markedly increased by genistein but was not affected by staurosporine and pertussis toxin.

These results indicate that the respiratory burst of neutrophils may be regulated by protein kinase C and protein tyrosine kinase. Superoxide production induced by the direct activation of protein kinase C might be affected by protein tyrosine kinase oppositely. Genistein probably promotes HOCl production by activating myeloperoxidase.

Key Words: Staurosporine, Genistein, Respiratory burst, Human neutrophils

INTRODUCTION

Neutrophils produce oxidants such as superoxide anion ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2) when stimulated by a variety of particulate and soluble stimuli (Babior, 1978; Badwey and Karnosky, 1980). They also release cytoplasmic-granule components, including myeloperoxidase. Superoxide anion rapidly dismutates to yield H_2O_2, and myeloperoxidase catalyzes oxidation of chloride (Cl^-) by H_2O_2 to yield hypochlorous acid (HOCl), a powerful oxidizing agent (Harrison and Schultz, 1976; Fantone and Ward, 1982). The respiratory burst is catalyzed by NADPH oxidase, which is dormant in resting neutrophils but acquires catalytic activity when the cells are stimulated (Babior, 1992).

The binding of chemoattractants to their receptors on the plasma membrane of neutrophils induces phospholipid hydrolysis by phospholipase C, promoting the formation of the intra-

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cellular messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG) (Dougherty *et al.*, 1984; Berridge, 1987). These molecules are responsible for the Ca^{2+} release from intracellular stores and the activation of protein kinase C, respectively. A change in the steady state of cytosolic Ca^{2+} appears to be involved in the early triggering of the activation of neutrophil responses (Smolen *et al.*, 1981). Protein kinase C (PKC) is thought to play a major role in the activation process (Nishizuka, 1984). Since activation of PKC by phorbol 12-myristate 13-acetate (PMA) can produce superoxide anion, PKC may be involved in activation of the respiratory burst (Tauber, 1987). However, receptor agonists such as N-formyl-methylleucyl-phenylalanine (fMLP) induce superoxide production much faster than PKC ligands, including PMA (Wymann *et al.*, 1987). This finding indicates that activation of NADPH oxidase does not depend soly on PKC. In addition, activation of PKC could be regulated by Ca^{2+}-independent signal (O'Flaherty *et al.*, 1990). Thus, the activation process of neutrophil responses has not been cleary elucidated.

The inhibitors of protein kinase C including 1-(5-iso-quinoline-sulfonyl)-3-methylpiperazine (H-7) do not inhibit all neutrophil responses (Gerard *et al.*, 1986; Berkow *et al.*, 1987). It has been shown that the receptormediated responses are unaffected and occasionally even potentiated when PKC is inhibited. fMLP-induced superoxide production in a primed neutrophils is stimulated by staurosporine and H-7, inhibitors of PKC (Tanimura *et al.*, 1992). fMLP and PMA induce an increase in protein tyrosine phosphorylation (Berkow and Dodson, 1990). The protein tyrosine kinase (PTK) inhibitor genistein inhibits superoxide production in a primed neutrophils by fMLP (Tanimura *et al.*, 1992) and platelet activating factor (PAF)-stimulated prostaglandin production in a primed macrophage cell line (Glaser *et al.*, 1990). These data suggest that protein tyrosine phosphorylation is also involved in neutrophil activation.

In this study, effects of PKC inhibitor, PTK inhibitor and G protein inhibitor on the superoxide and HOCl production in C5a (or PMA)-activated neutrophils and the release and activity of myeloperoxidase were investigated.

MATERIALS AND METHODS

C5a, phorbol 12-myristate 13-acetate (PMA), staurosporine, pertussis toxin, phenylmethylsulfonyl fluoride (PMSF), ferricytochrome c, taurine and myeloperoxidase (human leukocyte) 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 (PBS) at a concentration of 1×10^7/ml. Final suspensions of neutrophils were comprised of about 97% neutrophils as judged from Wright-Giemsa stain and viability was more than 98% as judged from trypan blue dye exclusion.

After neutrophils were pretreated with cytochalasin B (5 μg/ml for 10^7 cells) for 5 min, the assay for the respiratory burst and degranulation was done.

Assay of superoxide production

The superoxide dependent reduction of ferricytochrome c was measured by the method of Markert *et al.* (1984). The reaction mixtures in plastic microfuge tubes contained 2×10^6 neutrophils, C5a (or PMA), 75 μM ferricytochrome c, 20 mM HEPES-tris and HBSS bufer, pH 7.4 in a total volume of 1.0 ml. The reactions were performed in a 37°C shaking water bath for 15 min. The reaction was then stopped by placing the tubes in melting ice, and the cells were rapidly pelleted by centrifuging at 1,500 g for 5 min at 4°C. The supernatants were taken, and the amount of reduced cytochrome c was measured at 550 nm. 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).

Assay of hydrogen peroxide production

H_2O_2 produced from activated neutrophils was measured by change of scopoletin fluorescence. The reaction mixtures 2×10^6 neutrophils, C5a (or PMA), 2.5 μM scopoletin, 5 μg/ml horse radish peroxidase, 20 mM HEPES-tris and HBSS buffer, pH 7.4 in a total volume 1.0 ml. After preincubation of 5 min at 37℃ with inhibitors, the reaction was initiated by the addition of agonist. The decrease of scopoletin fluorescence by H_2O_2 produced was read at the wavelength of excitation, 343 nm and emission, 460 nm (Root *et al.*, 1975)

Assay of HOCl production

HOCl production was determined by measuring formation of extracellular taurine chloramine (TnCl) (Shacter *et al.*, 1991). Neutrophils (2×10^6 cells/ml) were incubated in the reaction mixtures contained 15 mM taurine, 1.23 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mg/ml glucose and PBS, pH 7.4. After 15 min of incubation at 37℃, the reaction was stopped by adding 10 μl of 1 mg/ml catalase, and reaction mixtures were centrifuged at 3,000 rpm for 10 min. A 0.75 ml of supernatants were taken and then mixed with 15 μl of 1 M KI. The absorbance was read spectrophotometrically at 350 nm. The amount of HOCl produced was estimated by using an extinction coefficient of $2.29\times10^4 M^{-1}cm^{-1}$ for OCl^-.

Assay of myeloperoxidase release

A 5×10^6/ml neutrophils in HBSS buffer with or without inhibitors were stimulated by adding C5a (or PMA) at 37℃. After 15 min of incubation, 250 μl of 0.2 M phosphate buffer, pH 6.2 and 250 μl of an equal mixture of 3.9 mM O-dianisidine HCl and 15 mM H_2O_2 were added. After 10 min of reincubation, the reaction was stopped by the addition of 250 μl of 1% sodium azide. The absorbance was read at 450 nm (Spangrude *et al.*, 1985).

Assay of myeloperoxidase activity

Myeloperoxidase activity was assayed for HOCl formation using the mixtures contained 62.9 mU/ml myeloperoxidase, inhibitor, 137 mM NaCl, 5 mM taurine, 20 μM H_2O_2 and 200 mM NaH_2PO_4, pH 7.4 (Shacter *et al.*, 1991). The reaction was started by adding H_2O_2, and the incubation was done for 2 h at 37℃. The reaction was terminated by the addition of 20 μg/ml catalase, and the absorbance was read at 350 nm.

RESULTS

Effects of staurosporine and genistein on superoxide and hydrogen peroxide production

Role of protein kinase C and protein tyrosine kinase in the activation of respiratory burst was investigated. Superoxide production in 11.6 nM C5a or 0.1 μg/ml PMA-stimulated neutrophils was inhibited by 100 nM staurosporine (Fig. 1). The inhibitory effect of staurosporine on PMA- stimulated superoxide production was greater than its effect on C5a. In contrast, genistein differently affected superoxide production in activated neutrophils. PMA-induced superoxide production was further stimulated by 10 μM genistein, whereas the stimulatory effect of C5a was inhibited by it. At the stated concentration, genistein showed a 59% of stimulation. Pertussis toxin (0.1 μg/ml) inhibited C5a-induced superoxide production but did not affect the stimulatory effect of PMA.

A 20 mM sodium fluoride, a G protein activator, stimulated superoxide production, and the stimulatory effect of sodium fluoride was inhibited by staurosporine and genistein, whereas the effect of pertussis toxin was not detected (Fig. 2).

H_2O_2 is produced from the dismutation of superoxide anion (Fridovich, 1975). Fig. 3 shows that C5a-timulated H_2O_2 production was inhibited by staurosporine, genistein and pertussis toxin. H_2O_2 production in PMA-stimulated neutrophils was inhibited by staurosporine but was not affected by genistein and pertussis toxin (Fig. 4).

Effects of staurosporine and genistein on HOCl production

HOCl is formed from the oxidation of Cl^- with H_2O_2 catalyzed by myeloperoxidase. Effects of kinase inhibitors on HOCl production were

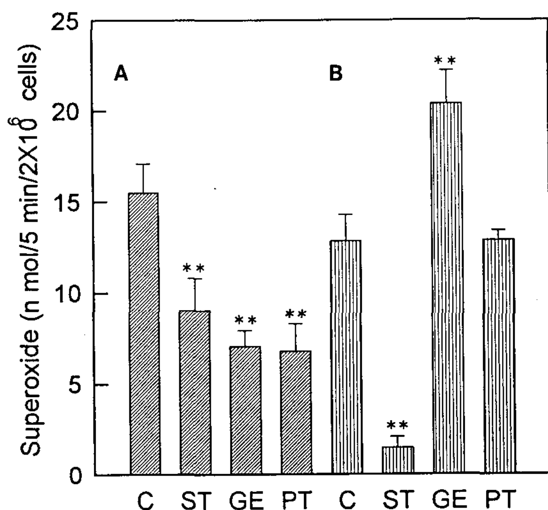

Fig. 1. Effects of staurosporine and genistein on superoxide production in activated neutrophils. Neutrophils (2×10^6 cells/ml) were stimulated with 11.6 nM C5a (or 0.1 μg/ml PMA) in the presence of inhibitors. Values are means±SD, n=4-6. C, no addition; ST, 100 nM staurosporine; GE, 10 μM genistein; PT, 0.1 μg/ml pertussis toxin with C5a(A) or PMA (B). ** $p<0.01$ by Student *t*-test.

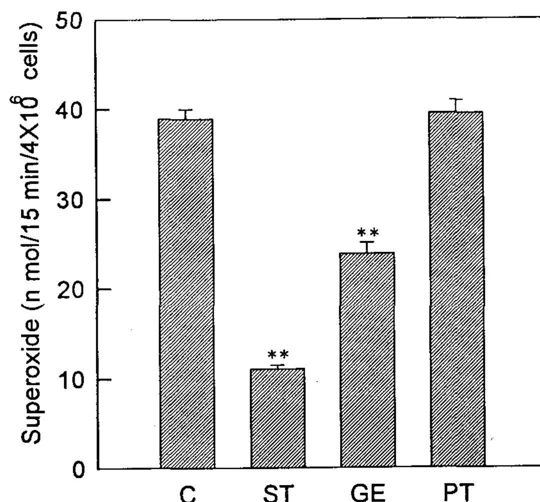

Fig. 2. Inhibitory effects of staurosporine and genistein on sodium fluoride-induced superoxide production. Neutrophils (4×10^6 cells/ml) were stimulated with 20 mM sodium fluoride in the presence of inhibitors. Values are means±SD, n=5. C, no addition; ST, 100 nM staurosporine; GE, 10 μM genistein; PT, 0.1 μg/ml pertussis toxin with sodium fluoride. ** $p<0.01$ by Student *t*-test.

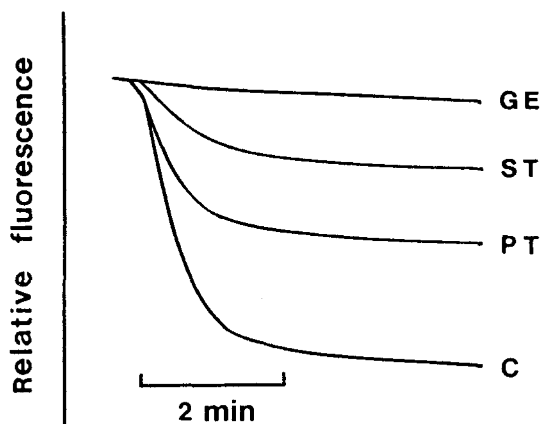

Fig. 3. Inhibition of hydrogen peroxide production in C5a-activated neutrophils by staurosporine and genistein. Neutrophils (2×10^6 cells/ml) were stimulated with 20 nM C5a in the presence of inhibitors. C, no addition; ST, 100 nM staurosporine; GE, 10 μM genistein; PT, 0.1 μg/ml pertussis toxin with C5a.

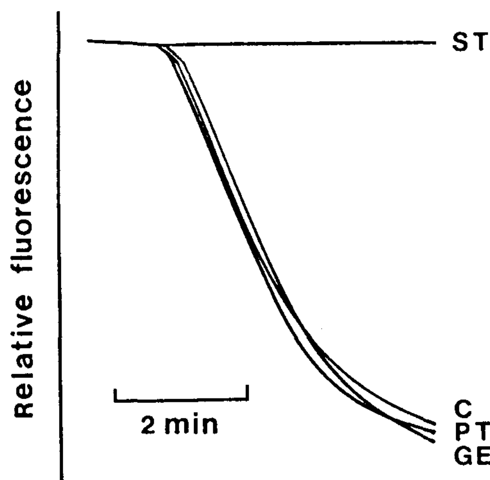

Fig. 4. Effects of staurosporine and genistein on PMA-induced H_2O_2 production. Neutrophils (2×10^6 cells/ml) were stimulated with 0.1 μg/ml PMA in the presence of inhibitors. C, no addition; ST, 100 nM staurosporine; GE, 10 μM genistein; PT, 0.1 μg/ml pertussis toxin with PMA

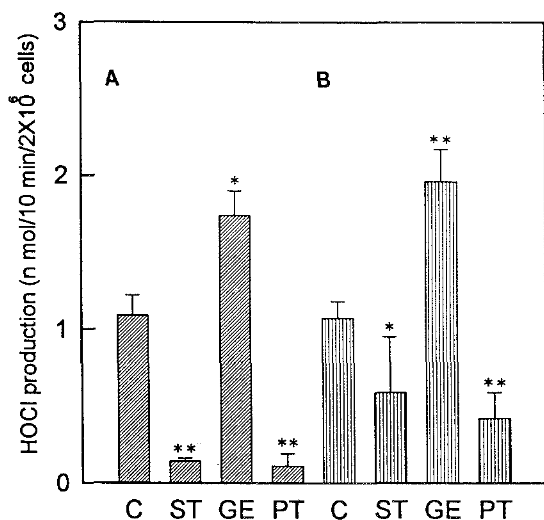

Fig. 5. Stimulatory effect of genistein on HOCl production. Neutrophils (2×10^6 cells/ml) were stimulated with 20 nM C5a (or 0.1 μg/ml PMA) in the presence of inhibitors. Values are means±SD, n =4-5. C, no addition; ST, 100 nM staurosporine; GE, 10 μM genistein; PT, 0.1 μg/ml pertussis toxin with C5a (A) or PMA (B). **$p<0.01$, *$p<0.05$ by Student *t*-test.

examined. As shown in Fig. 5, staurosporine inhibited HOCl production in both C5a- and PMA-activated neutrophils. On the contrary, genistein further promoted HOCl production by C5a or PMA. The stimulatory effect of genistein on PMA-induced superoxide production was greater than its effect on C5a. Influence of pertussis toxin on HOCl production was detected.

Effets of staurosporine and genistein on the release and activity of myeloperoxidase

Role of PKC and PTK in myeloperoxidase release from neutrophils was examined. As can be seen in Fig. 6, C5a-induced myeloperoxidase release was inhibited by staurosporine and genistein but was not affected by pertussis toxin. The stimulatory effect of PMA was inhibited by genistein, whereas the effects of staurosporine and pertussis toxin were not detected.

Direct effects of inhibitors on the activity of myeloperoxidase, a purified form, were examined. Fig. 7 showes that 10 μM genistein significantly stimulated myeloperoxidase activity and

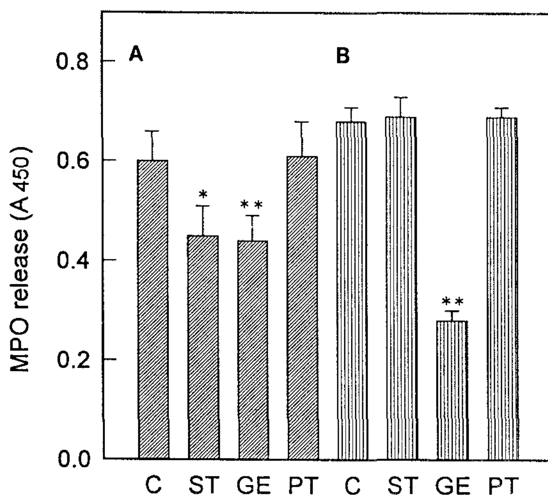

Fig. 6. Effects of staurosporine and genistein on myeloperoxidase (MPO) release. Neutrophils (5×10^6 cells/ml) were stimulated with 20 nM C5a (or 0.1 μg/ml PMA) in the presence of inhibitors. Values are means±SD, n=5 and are expressed as absorbance change. C, no addition; ST, 100 nM staurosporine; GE, 10 μM genistein; PT, 0.1 μg/ml pertussis toxin with C5a (A) or PMA (B). **$p<0.01$, *$p<0.05$ by Student *t*-test.

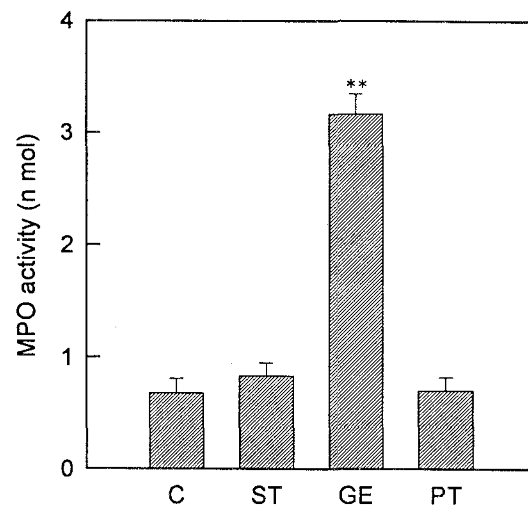

Fig. 7. Stimulation of myeloperoxidase activity by genistein. The reaction mixtures contained 62.9 mU/ml myeloperoxidase (MPO), 137 mM NaCl, 20 μM H_2O_2 and 5 mM taurine. Effects of inhibitors on myeloperoxidase activity were measured by the formation of HOCl. Values are means±SD, n=3. C, no addition; ST, 100 nM staurosporine; GE, 10 μM genistein; PT, 0.1 μg/ml pertussis toxin. ** $p<0.01$ by Student *t*-test.

showed an approximately 4.6 times of stimulation. On the other hand, staurosporine and pertussis toxin had not effect on the activity.

DISCUSSION

The bindings of chemoattractants, C5a and fMLP to cell surface receptors on neutrophils initiate a series of ionic and molecular changes in the plasma membrane and intracellular components, which induce aggregation, lysosomal enzyme release and superoxide production (Fantone and Ward, 1982). The superoxide-forming enzyme system constitutes a cyanide insensitive pyridine nucleotide oxidase, which utilizes NADPH as the electron donor (Babior, 1978). In the resting neutrophil, the NADPH oxidase system exists in an inactive state. Exposure of neutrophil to immune complexes and phorbol esters results in the activation of the oxidase (Tauber, 1987).

PKC is thought to play a major role in signal transduction and cellular processes. PKC is activated by DAG, phospholipids and Ca^{2+} (or without Ca^{2+}) (Nishizuka, 1984; O'Flaherty *et al.*, 1990). Activation of PKC by PMA and 1-oleyl-2-acetylglycerol stimulates the respiratory burst (Bass *et al.*, 1987; Tauber, 1987). PMA-induced superoxide and H_2O_2 production was inhibited by a PKC inhibitor, staurosporine. This finding supports that PMA shows a PKC-mediated response in neutrophils. Inhibition of complement C5a-stimulated superoxide and H_2O_2 production by staurosporine indicates that C5a stimulates neutrophil responses through the activation of PKC.

However, an inhibitor of PKC, H-7 fail to inhibit neutrophil responses induced by fMLP (Sha'afi *et al.*, 1988). In granulocyte-macrophage colony stimulating factor (GM-CSF)-primed neutrophils, fMLP-induced superoxide production is stimulated by staurosporine and H-7 (Tanimura *et al.*, 1992). Thus, it is suggested that other transduction mechanisms are involved in the activation of neutrophils. The PTK inhibitors, including genistein inhibits both tyrosine phosphorylation and eicosanoid production (Glaser *et al.*, 1993). These data indicate that PTK is involved in the activation of neutrophil function. A C5a-activated respiratory burst may be due to the activation of PTK. On the contrary, in PMA-stimulated neutrophils the effect of genistein on superoxide production was different from its effect on H_2O_2 production. PMA- induced superoxide production was significantly promoted by genistein (Fig. 1). The finding is coincided with the investigation of Tanimura *et al.* (1992). However, genistein did not affect H_2O_2 production by PMA. This proposes that H_2O_2 is not entirely provided from superoxide anion. In PMA-activated neutrophils, lag time to produce superoxide anion was much shorter than lag time for H_2O_2 production (data not shown). The finding partly may support the above view. It is also suggested that PKC system could be affected by the change of PTK activity.

C5a stimulates neutrophil responses via the activation of G proteins which are linked with cell surface receptors (Becker *et al.*, 1985). Pertussis toxin, a G protein inhibitor, inhibited the stimulation of respiratory burst in C5a-activated neutrophils. Meanwhile, no inhibitory effect of pertussis toxin on PMA-stimulated respiratory burst confirms previous reports that PMA acts directly on PKC without the intervention of G proteins (Tauber, 1987). Sodium fluoride is known to stimulate neutrophil response by activating guanine nucleotide regulatory proteins, but neutrophil responses to fluoride are not inhibited by pertussis toxin (English *et al.*, 1989). Sodium fluoride-stimulated respiratory burst was inhibited by staurosporine and genistein but was not affected by pertussis toxin. Fluoride-induced functional activation may be regulated by the activation of both PKC and PTK.

Activated neutrophils release oxidizing agent, H_2O_2 and secrete granule enzymes including myeloperoxidase into the intracellular phagolysosome compartment and the extracellular medium (Fantone and Ward, 1982). Myeloperoxidase catalyzes the oxidation of Cl^- with H_2O_2 to form HOCl. C5a- and PMA-induced HOCl production appears to be regulated by PKC and PTK (Fig. 5). In C5a-activated neutrophils, myeloperoxidase release may not be regulated by PKC and G proteins. The PTK appears to

affect the release of lysosomal enzyme caused by either surface receptor binding or direct PKC activation. Genistein inhibited myeloperoxidase release but stimulated the activity of myeloperoxidase and the production of HOCl. The enhancement of HOCl production in activated neutrophils by genistein may be ascribed to its marked stimulatory effect on the activity of myeloperoxidase rather than the inhibitory effect on the release. It is unlikely that myeloperoxidase release is regulated by pertussis toxin-sensitive G proteins.

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=국문초록=

Staurosporine과 Genistein이 C5a 또는 PMA에 의하여 활성화된 호중구에서의 Superoxide와 HOCl 생성에 나타내는 영향

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C5a 또는 PMA에 의하여 활성화된 호중구에서의 superoxide와 HOCl 생성에 나타내는 staurosporine, genistein과 pertussis toxin의 효과를 관찰하였다. C5a에 의한 superoxide과 H_2O_2의 생성은 staurosporine, genistein과 pertussis toxin에 의하여 억제되었다. PMA의 자극효과는 staurosporine에 의하여 억제되었으나 pertussis toxin에 의하여 영향을 받지 않았으며, 한편 이는 genistein에 의하여 더 촉진되었다. Staurosporine과 genistein은 sodium fluoride에 의한 superoxide 생성을 억제하였으나 pertussis toxin은 영향을 나타내지 않았다. PMA에 의한 H_2O_2의 생성은 staurosporine에 의하여 억제되었으나 pertussis toxin의 영향은 받지 않았다. Genistein은 PMA에 의한 H_2O_2 생성에 자극효과를 나타내지 않았다. Staurosporine과 pertussis toxin은 C5a 또는 PMA에 의한 HOCl 생성을 억제하였으나, 이에 반하여 genistein은 자극하였다. C5a와 PMA에 의한 myeloperoxidase 유리는 genistein에 의하여 억제되었으나, pertussis toxin의 효과는 나타나지 않았다. Staurosporine은 유리에 대한 PMA의 자극효과에 영향을 주지 않았다. Myeloperoxidase 활성은 genistein에 의하여 현저하게 증가되었으나 staurosporine과 pertussis toxin의 영향은 받지 않았다.

이상의 결과는 호중구의 respiratory burst가 protein kinase C와 protein tyrosine kinase에 의하여 조절된다고 제시한다. Protein kinase C의 직접적인 자극에 따른 superoxide 생성은 protein tyrosine kinase의 영향을 역으로 받을 것으로 추정된다. Genistein은 아마도 myeloperoxidase를 활성화하여 HOCl 생성을 촉진할 것으로 시사된다.