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Involvement of Protein Phosphatase 2A in PKC-Independent Pathway of Neutrophil Superoxide Generation by fMLP

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Abstract We examined the effects of okadaic acid, a protein phosphatase 1 and 2A inhibitor, on superoxide generation in human neutrophils. Superoxide generation induced by fMLP was inhibited by low-dose okadaic acid (10–100 nM), but it had no effect on superoxide synthesis by PMA, and the fMLP-induced rise of the intracellular Ca^{2+} concentration was not affected by low-dose okadaic acid. These findings suggested that the inhibitory mechanism of okadaic acid might involve PKC-independent and Ca^{2+}-independent pathways in fMLP induced NADPH oxidase activation.

Both fMLP-stimulated phosphorylation of serine residues in p47phox and its translocation to the plasma membrane were suppressed by low-dose okadaic acid. On the other hand, PMA-induced phosphorylation and translocation of p47phox were not affected by such a low dose of okadaic acid. These findings suggested that fMLP induced phosphorylation of serine residues in p47phox was regulated by protein phosphatase 2A, and its phosphorylation was necessary for translocation and superoxide generation in fMLP-activated human neutrophils.

Key words: superoxide, p47phox, phosphorylation, okadaic acid, protein phosphatase 1 and 2A

When neutrophils are exposed to chemotaxins or phagocytic stimuli, they undergo an oxidative burst and generate superoxide. The mechanism of superoxide synthesis in neutrophils has been intensively examined during the past 10 years [Tauber, 1987; Nishizuka, 1988; Sha'afi and Molski, 1988], and NADPH oxidase is known to be a key enzyme of superoxide synthesis. There are two major signaling pathways in the formyl-methionyl-leucyl-phenylalanine (fMLP)-induced activation of NADPH oxidase, namely phorbol myristate acetate (PKC)-dependent and PKC-independent pathways [Gerard and McPhail, 1986; Berkow et al., 1987; Nishizuka, 1988; Ohsaka et al., 1988; Watoson et al., 1988; Koenderman et al., 1989; Dusi et al., 1993]. The activation of NADPH oxidase in the PKC-dependent pathway was reported to be mediated by the signal transduction mechanism composed of a receptor-linked GTP-binding protein, phospholipase C (PLC), and PKC [Tauber, 1987; Grinstein and Furuya, 1988; Nishizuka, 1988; Combadiere et al., 1990]. In fMLP stimulation, activated PLC produced inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 caused the release of calcium and acted synergistically with DAG to activate PKC. In contrast to the PKC-dependent pathway, the molecular mechanism of PKC-independent pathways has not yet been clarified.

The activation of NADPH oxidase required the interaction between the plasma membrane and certain cytosolic components. These components included the low-potential cytochrome b558, a flavoprotein, p47phox, p67phox (47, 67-kDa phagocyte oxidase factor) and the small GTP-binding protein [Grinstein and Furuya, 1988; Sha'afi and Molski, 1988; Combadiere et al., 1990]. Translocation of both p47phox and p67phox to the plasma membrane was an essential process for the activation of NADPH oxidase. This translocation mechanism has been examined, but a definitive conclusion has not been obtained.

Abbreviations: OA; okadaic acid, PP1/2A; protein phosphatase 1 and 2A, fMLP; formyl-methionyl-leucyl-phenylalanine, PMA; phorbol-myristate-acetate, $[Ca^{2+}]$; intracellular Ca^{2+} concentration, PKC; protein kinase C, p47phox and p67phox; 47-kDa and 67-kDa phagocyte oxidase factor.

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Phosphorylation and translocation of p47phox were important for the activating mechanism of NADPH oxidase. The relationships between phosphorylation of p47phox and NADPH oxidase activation were examined by using phorbol esters and receptor-mediated agonists. Protein phosphorylation was not regulated only by protein kinases, but also by protein phosphatases. Recently, protein phosphatase inhibitors including okadaic acid (OA) have been developed as a cell-permeable probe to elucidate the involvement of protein phosphatase [Murata et al., 1995]. Some investigators have reported that in PMA-activated neutrophils, OA inhibited superoxide generation. This inhibition has been explained by a suppression of increasing $[Ca^{2+}]_i$ [Garcia et al., 1992]. However, PMA-stimulated neutrophils did not show any increase in $[Ca^{2+}]_i$, and Ca^{2+}-depleted neutrophils could generate superoxide by PMA stimulation [Dusi et al., 1993]. Thereby, OA might inhibit PMA-induced superoxide generation by a Ca^{2+}-independent mechanism. In contrast to PMA, fMLP-induced superoxide generation was reported to be prolonged by pretreatment with OA [Berkow et al., 1987]. This phenomenon has been explained by an essential role of p47phox dephosphorylation in deactivation of NADPH oxidase. In these experiments, p47phox phosphorylation was detected by ^{32}P-preloaded neutrophils and autoradiograph. The molecular-weight 47-kDa protein was phosphorylated. There has been no direct evidence, however, that the phosphorylated 47-kDa protein is p47phox. It would be possible to clearly identity p47phox and p67phox by employing specific antibodies against these polypeptide [Nakanishi et al., 1992]. OA is an inhibitor of protein phosphatase 1 and 2A (PP1/2A), but the inhibitory spectrum is markedly different in the concentration of the agent used. OA should bind preferentially to PP2A, and it inhibited PP2A with an IC_{50} of 10 nM, but its IC_{50} for PP1 was about 50–100 times higher than that for PP2A [Tachibana et al., 1981; Bialojan and Takai, 1988]. Many experiments used 1–3 μM OA, which inhibited both PP1 and PP2A. The aim of this study was to examine the relationships between phosphorylation and activation of NADPH oxidase by investigating the effect of low-dose OA that could inhibit only PP2A and Western blotting of phosphoamino acids against immunoprecipitated p47phox.

MATERIALS AND METHODS

Reagents

Okadaic acid and fluo 3-AM were purchased from Wako Pure Chemicals Co. (Osaka, Japan). FMLP, PMA, and lucigenin were purchased from Sigma Chemical Co. (St. Louis, MO). Antiphosphoserine and antiphosphothreonine antibodies were obtained from BioMakor (Rehevot, Israel). Antiphosphotyrosine antibody was purchased from Leinco Technologies, (St. Louis MO). Anti-p47phox and anti-p67phox antibodies were kindly provided by Dr. S. Ohmi, Institute of Medical Science, University of Tokyo, Japan.

Preparation of Neutrophils

Neutrophils were prepared from 20 ml of citrated venous blood from normal subjects. Blood was mixed with an equal amount of 3% dextran saline in a plastic syringe and left to sit vertically for 60 min at room temperature. The resultant upper phase was taken and placed gently on the same volume of Ficoll-Paque (sp. gr. 1.077). By centrifugation at 450*g* for 20 min at 4°C, neutrophils were sedimented at the bottom of the tube. After removing both the upper phases and interface, contaminating erythrocytes were lysed by a mixture with 5 ml of ice-cold distilled water for 30 sec, and then 5 ml of ice-cold 1.8% NaCl solution. After centrifugation at 100*g* for 10 min, the cells were resuspended to 1×10^7/ml with Tyrode solution buffer (135 mM, NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.6 mM glucose, pH 7.4). Neutrophils were used within 3 h after preparation.

Measurement of Superoxide Generation

Neutrophils (1×10^7/ml) were incubated at 37°C in Tyrode solution buffer (1 ml) containing 10^{-5} M lucigenin [Nishida et al., 1989]. Superoxide synthesis was determined for lucigenin-enhanced chemiluminescence with the aid of a luminescence reader BRL-301 (Aloka, Tokyo), measuring its peak height (counts per minute) recorded for 10 min. Water-insoluble compounds, including fMLP, PMA, and OA, were dissolved in DMSO or ethanol and added to the cell suspension. The final concentration of DMSO or ethanol in the assay mixture did not exceed 0.1%.

Measurement of Ca^{2+} Concentration

Neutrophils were incubated with 15 μM fluo-3 AM at 37°C for 60 min. The cells were washed once with phosphate-buffered saline (PBS) (135 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, pH 7.4) and suspended in Tyrode solution buffer without Ca^{2+}. Fluo-3 AM-loaded neutrophils were preincubated at 37°C with various concentrations of OA or vehicle for 3 min, and 10^{-7} M fMLP or 10^{-7} M PMA was added in the presence of 1 mM $CaCl_2$, which was added 3 min before stimulation. The fluorescence was continuously monitored at the settings of 480 nm (excitation) and 530 nm (emission) by a fluorescence spectrometer (F-3000, HITATI, Tokyo). The intracellular Ca^{2+}concentration was determined according to the method of Vandenberghe and Ceuppens [1990].

Immunoprecipitation and Phosphorylation of p47phox

Neutrophils (1×10^7/ml) stimulated by 10^{-7} M fMLP pretreated with various agents were suspended in 3 ml of relaxation buffer (10 mM Pipes, KCl 100 mM, NaCl 3 mM, $MgCl_2$ 3.5 mM, EGTA 1.25 mM, pH 7.3) containing 20 μg/ml leupeptin and 20 mM PMSF. The cells were then disrupted in an ice-water bath for 30 sec by a Branson sonicator at index 7. After centrifugation at 500*g* for 5 min, the soluble fraction was mixed with 30 μl of protein A–Sepharose CL4B suspension (50%, w/v) and incubated for 30 min at 4°C under rotation. The gells were pelleted by centrifugation (450*g*, 3 min), and the resultant supernatant was incubated with 10 μl of specific anti-p47phox antibody overnight. The samples were then incubated with 100 μl of protein A–Sepharose CL4B suspension for 2 h. After centrifugation, the pellet was washed three times with 800 μl of relaxation buffer. The immunoprecipitated proteins were dissolved in 300 μl of SDS-sample buffer (4% SDS, 4% mercaptoethanol, 20% glycine, 0.06 M. Tris–HCl, pH 6.8, 0.001% BPB) and subjected to electrophoresis on a 4–20% gradient gel. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (BioRad Lab, Richmond, CA) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell apparatus (BioRad Lab, Richmond, CA). PVDF membranes were incubated overnight at 4°C with a monoclonal antibody against phosphoserine (PSR-45), phosphothreonine (PTR-8) (BioMakor, Rehevot, Israel) [Hunter and Cooper, 1985; Heffetz D et al., 1991] or phosphotyrosine (PY20, Leinco Tech, St. Louis, MO) [Glenny et al., 1988, Ruff-Jamison et al., 1991]. Samples were developed using alkaline phosphatase-conjugated with goat anti-mouse IgG, 5-bromo-4-chloro-3-imdolyphosphate (BCIP) and nitroblue tetrazolium (NBT).

Neutrophil Fractionation and Immunoblotting of p47phox and p67phox

The soluble fractions of neutrophils were prepared by centrifugation (500*g*, 5 min) from the cell lysate as described above. The samples were further centrifuged at 110,000*g* for 6 min at 4°C. The membrane fraction (pellet) was then resuspended with 200 μl of relaxation buffer and subsequently mixed with 100 μl of SDS sample buffer. After electrophoresis on 4–20% gradient gel, immunoblotting of p47phox and p67phox was performed as described above, using anti-p47phox and anti-p67 antibodies. All measurements were performed at least three times and presented as means ±SE or as representative traces. Unless otherwise indicated, the significance of differences was calculated using the unpaired *t*-test.

RESULTS

Effect of OA on Superoxide Generation in fMLP- and PMA-Stimulated Neutrophils

Figure 1 showed the time plots of the intensity of the chemiluminescence upon various stimulation to neutrophils. The intensity of the chemiluminescence increased immediately after stimulation. As this increase of the intensity had disappeared by superoxide dismutase, the increased intensity was due to the formation of superoxide. In fMLP-activated neutrophils, superoxide generation was observed immediately after stimulation. Incubation with OA followed by stimulation with fMLP resulted in an inhibition of superoxide production in a dose- and time-related manner (Fig. 2). This inhibitory effect was observed by incubation with a very low dose of OA (10 nM), which could only inhibit PP2A. There were no differences between 0.1 and 1 μM OA, and complete inhibition of superoxide generation was observed by 0.1 μM OA, which was not enough to inhibite PP1 completely. By contrast, OA exhibited no effect on neutrophils stimulated by PMA, normally a potent activator of NADPH oxidase through PKC

(Fig. 3). These findings indicated that PP2A might be involved in fMLP-induced superoxide generation.

Effect of OA on $[Ca^{2+}]_i$

The influence of OA in $[Ca^{2+}]_i$ was examined. Figure 4 showed that OA did not affect the resting $[Ca^{2+}]_i$ in neutrophils (left). FMLP induced a transient rise in $[Ca^{2+}]_i$ after stimulation was decreased by only a high dose of OA (1–2 μM) (right). The increasing of $[Ca^{2+}]_i$ stimulated by fMLP was not affected by 0.1 μM OA, which was enough to suppress the superoxide generation. These findings suggested that the inhibitory mechanism of OA in superoxide generation by fMLP was Ca^{2+} independent.

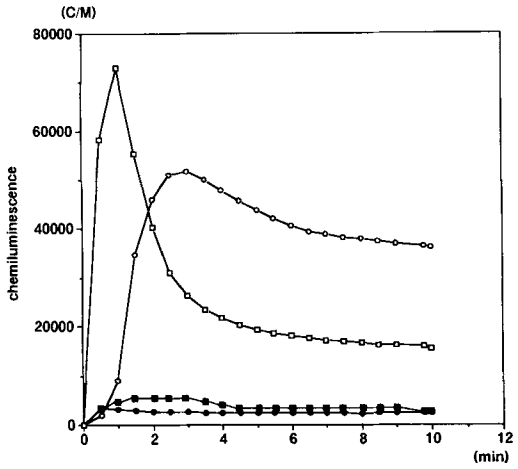

Fig. 1. Superoxide generation on lucigenin-enhanced chemiluminescence in neutrophils stimulated by fMLP and PMA. Human neutrophils (1×10^7/ml) preincubated for 3 min at 37°C in the presence (●) or absence (○) of 200 U/ml SOD were stimulated by 10^{-7} M PMA or in the presence (■) or absence (□) of 200 U/ml SOD stimulated by 10^{-7} M fMLP for 10 min at 37°C with gentle shaking. Intensity of the chemiluminescence was measured using 10^{-5} M lucigenin as described in Materials and Methods.

Effect of OA on Phosphorylation of p47phox

Since phosphorylation of p47phox has been shown to play a key role in superoxide generation in neutrophils, p47phox was immunoprecipitated by its specific antibody; the phosphorylation was examined in detail by using antibodies against phosphoserine, phosphothreonine and phosphotyrosine. In the presence of 1 mM $CaCl_2$, phosphorylation of p47phox was detected by the antiphosphoserine antibody, but not by other antibodies (Fig. 5). This fMLP induced phosphorylation of serine residues in p47phox was inhibited by the pretreatment with OA. The inhibitory effect on p47phox phosphorylation was closely related to superoxide generation. Both EGTA used to suppress Ca^{2+} influx and H-7, a protein kinase inhibitor including PKC, also inhibited phosphorylation of p47phox. In contrast to fMLP, PMA-induced phosphorylation in p47phox was inhibited only by pretreatment with H-7, while OA and EGTA had no effect.

Effect of OA on Translocation of p47phox and p67phox

To clarify the inhibitory mechanisms in superoxide generation by OA, translocation of p47phox

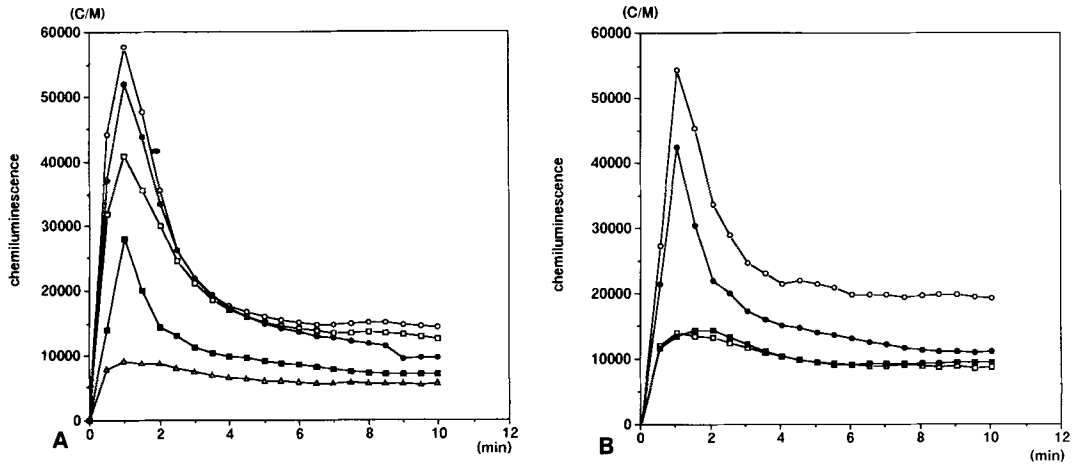

Fig. 2. Effect of OA on superoxide generation in fMLP-stimulated neutrophils. Human neutrophils (1×10^7/ml) preincubated for various times (○: OA(−), ●: 0 min, □: 30 sec, ■: 1 min, △: 3 min) with 0.1 μM OA were stimulated by 10^{-7} M fMLP for 10 min at 37°C. (Fig. 2.A). Human neutrophils (1×10^7/ml) incubated with various amounts of OA (○: 0 μM, ●: 10 nM, □: 0.1 μM, ■: 1 μM) for 3 min were stimulated by 10^{-7} M fMLP for 10 min at 37°C. (Fig. 2.B)

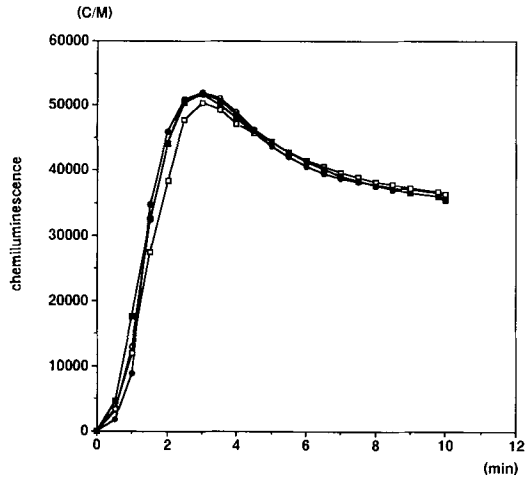

Fig. 3. Effect of OA on superoxide generation in PMA-induced superoxide generation. Human neutrophils (1×10^7/ml) incubated with various amounts of OA (○: 0 μM, ●: 2 μM, □: 1 μM, ■: 0.1 μM) for 3 min were stimulated by 10^{-7} M PMA for 10 min at 37°C.

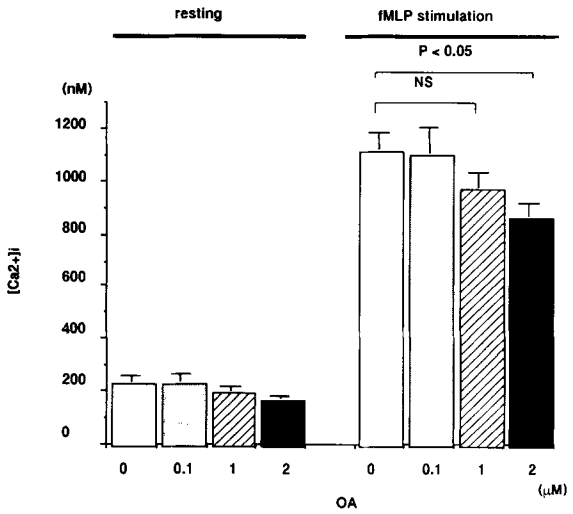

Fig. 4. Effect of OA on the intracellular Ca^{2+} concentration. After fluo-3 AM-loaded neutrophils were preincubated at 37°C with various amounts of OA or vehicle for 3 min, 10^{-7} M fMLP was added in the presence of 1 mM $CaCl_2$. The intracellular Ca^{2+} concentration was determined using fluo-3 AM as described in Materials and Methods. Each point and vertical bar represent the mean ±SE of three separate experiments.

and p67phox to the plasma membrane was studied. The translocation of p47phox and p67phox was detected in the plasma membrane fraction by Western blotting, using anti-p47phox and anti-p67phox antibodies at 5 min after fMLP and PMA stimulation (Fig. 6). In fMLP stimulation, pretreatment of OA suppressed their translocation to plasma membrane. EGTA and H-7 exhibited a similar inhibitory effect. In the case of PMA stimulation, translocation of p47phox was suppressed only by pretreatment with H-7.

Relationship Between Phosphorylation and Translocation of p47phox

OA inhibited the phosphorylation and translocation of p47phox induced by fMLP, but the relationship between the phosphorylation of p47phox and its translocation to the plasma membrane remains unknown. To investigate this, we examined the dose-dependent effect on phosphorylation and translocation of p47phox. Pretreatment with various amounts of OA inhibited both translocation and phosphorylation in a dose-dependent manner (Fig. 7). Densimetrical analysis showed that phosphorylation and translocation of p47phox were closely related (Fig. 8).

DISCUSSION

We examined the effect of OA on fMLP-induced superoxide generation. OA is a fatty acid derivative, cell-permeable PP1 and PP2A inhibitor [Tachibana et al., 1981]. OA inhibited PP2A with an IC_{50} of 10 nM, but its IC_{50} for PP1 was about 50–100 times higher than that for PP2A [Bialojan et al., 1988]. We showed in Figure 2 that OA inhibited fMLP induced superoxide generation in a dose- and time-dependent manner. This inhibitory effect was observed by pretreatment with a low concentration of OA (10 nM), which could inhibite only PP2A. There were no differences between 0.1 and 1 μM OA on the inhibitory effect. Both PP1 and PP2A was inhibited by 1 μM OA, but 0.1 μM OA was not a high enough dose to inhibite PP1. Although it was not easy to determine whether PP1 or PP2A was the relevant enzyme acting on a particular phosphoprotein in vivo from the result of the dose-dependent inhibition, these findings suggested that PP2A might be involved fMLP-induced superoxide generation. In contrast to fMLP, superoxide synthesis induced by PMA, a direct activator of PKC, was not affected by the same dose of OA.

Receptor mediated signal pathways including fMLP have not been clarified yet. The PKC-dependent signal pathway is generally believed to play a major role in NADPH oxidase activation. However, recent studies suggest the existence of PKC-independent pathways for a receptor-mediated agonist such as a fMLP, based on the findings from the studies with PKC inhibitors [Gerard and McPhail, 1986; Berkow et al.,

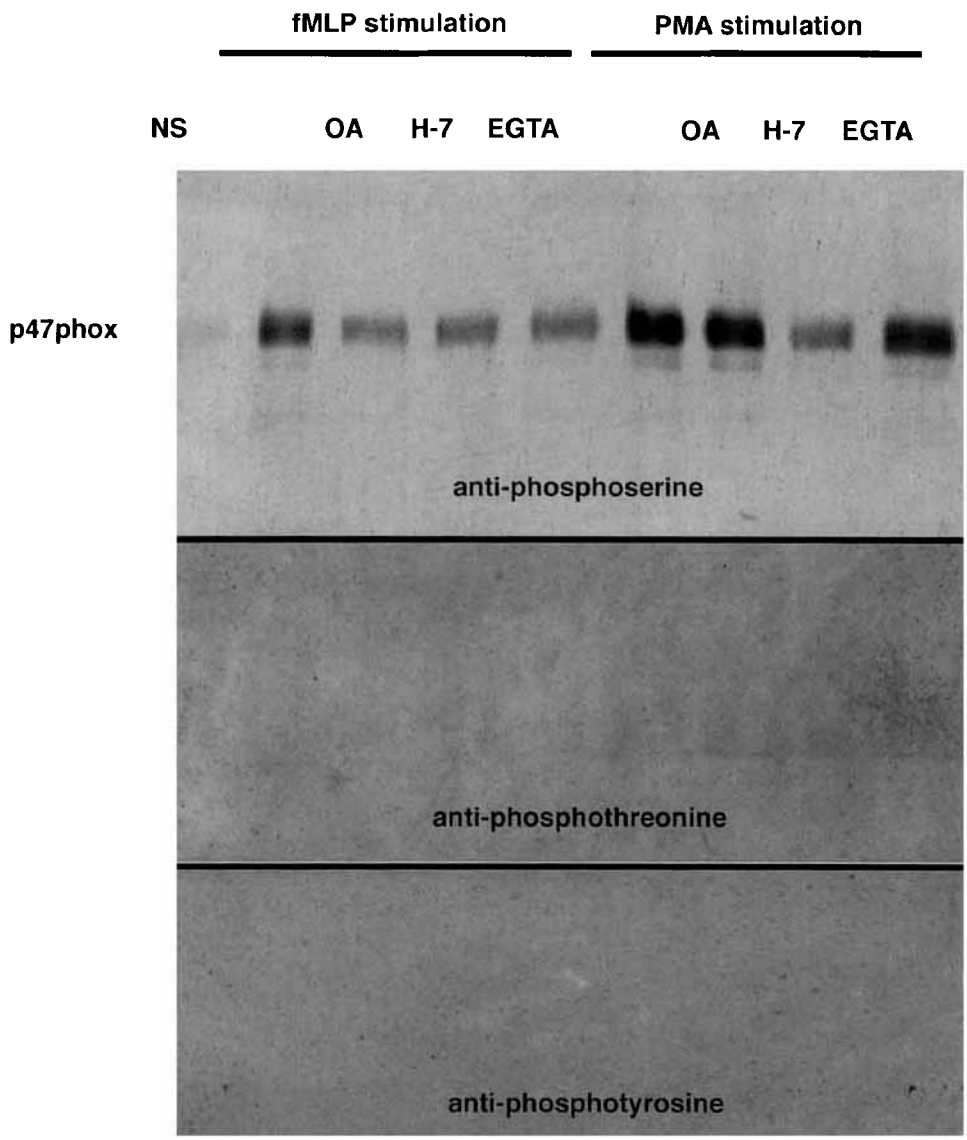

Fig. 5. Effect of OA on phosphorylation of p47phox. Neutrophils were incubated at 37°C with vehicle (line 2, 6) or 0.1 μM OA (line 3, 7) or 100 μM H-7 (line 4, 8) or 5 mM EGTA (line 5, 9) for 3 min. After 5-min stimulation with 10^{-7} M fMLP or 10^{-7} M PMA, neutrophils were sonicated and immunoprecipitated and immunoblotted with antiphosphoserine, antiphosphothreonine, and antiphosphotyrosine antibody. NS (line 1) was not stimulated.

1987; Watoson et al., 1991], Ca^{2+}-depleted cells [Dusi et al., 1993], electropermeabilized cells [Grinstein and Furuya, 1988] or the kinetic studies [Wymann et al., 1987; Ohsaka et al., 1988; Koenderman et al., 1989]. In the PKC-dependent pathway, activated neutrophils by FMLP produced IP_3 and DAG. IP_3 mobilized Ca^{2+} from intracellular Ca^{2+} storage sites and acted synergistically with DAG to activate PKC.

Our studies showed that OA (0.1–2 μM) had no inhibitory effect on PMA-induced superoxide generation. These findings suggested that inhibitory site of OA in fMLP-induced superoxide generation was upstream of PKC activation in PKC-dependent pathway or other signal pathways. To examine the effect of OA in fMLP-induced PKC activation, $[Ca^{2+}]_i$ was examined. The transient increase of $[Ca^{2+}]_i$ due to mobilization from Ca^{2+} storage sites was observed in neutrophils stimulated with fMLP. OA failed to suppress this $[Ca^{2+}]_i$ increase. These findings suggested that OA did not affect directly the process of degradation of phosphatidylinositol bisphosphate to IP_3 and DAG. Thus, we postulated that OA had no effect on PKC activation induced by fMLP and that OA inhibited the

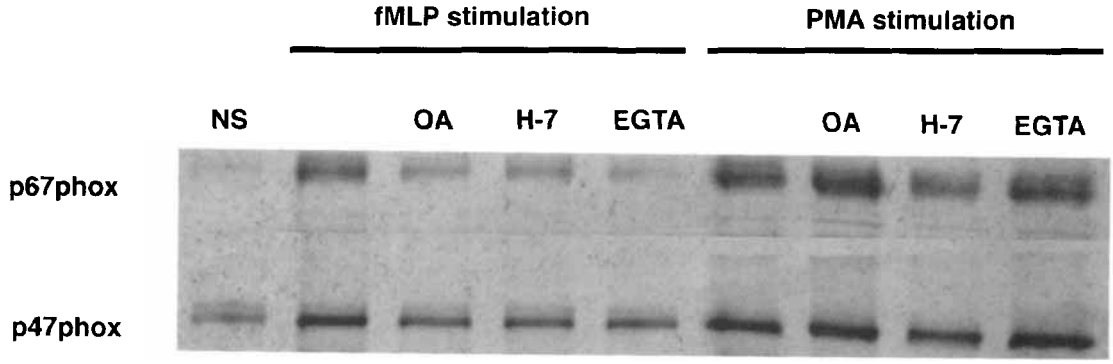

Fig. 6. Effect of OA on p47phox and p67phox translocation into the plasma membrane. Only the membrane fractions are shown. Neutrophils were incubated at 37°C with vehicle (line 2, 6), 0.1 μM OA (line 3, 7), 100 μM H-7 (line 4, 8), or 5 mM EGTA (line 5, 9) for 3 min. After 5-min stimulation with 10^{-7} M fMLP or 10^{-7} M PMA, neutrophils were sonicated and fractionated, and immunoblotting was performed with anti-p47phox and p67phox antibodies. NS (line 1) was not stimulated.

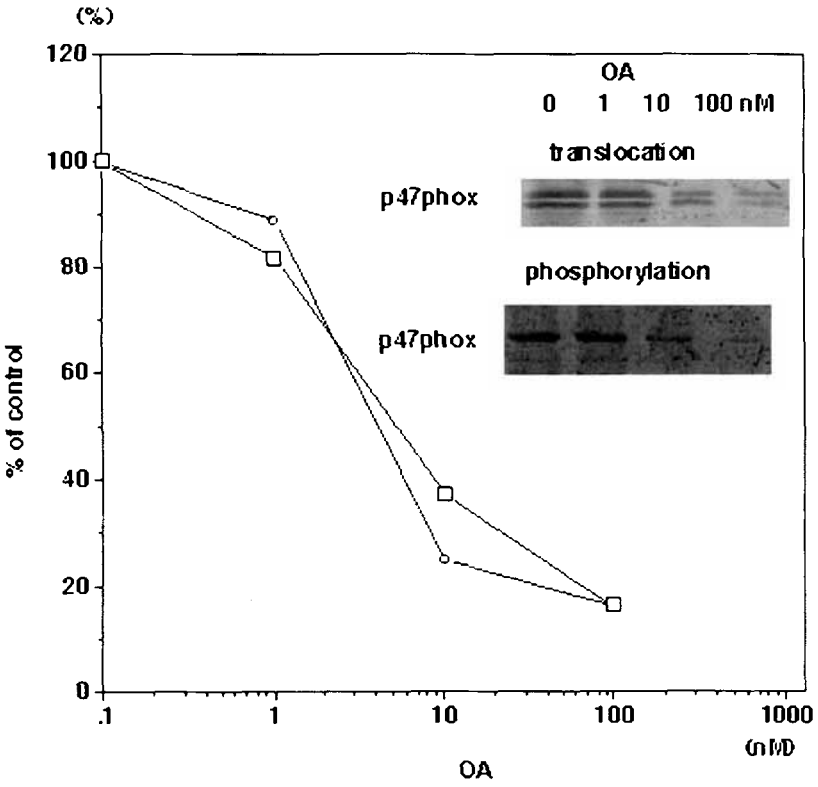

Fig. 7. Dose dependency of OA in p47phox phosphorylation and translocation. Neutrophils were incubated at 37°C with various amounts of OA for 3 min. Phosphorylation and translocation were examined by immunoprecipitation and Western blotting as described in Fig. 5. ○, p47phox translocation; □, p47phox phosphorylation. The experiments were carried out twice; the data show the results of one representative experiment.

fMLP-induced superoxide generation of neutrophils in the PKC-independent pathway.

The fMLP activated both PKC-dependent and -independent pathway of neutrophil superoxide generation. Which was the main pathway in neutrophil superoxide generation induced by fMLP? We could not answer this question because superoxide generation induced by fMLP was not inhibited completely by pretreatment with both OA and H-7 (data not shown). This question remains.

Translocation of p47phox and p67phox to the plasma membrane is an essential process for the activation of NADPH oxidase, and translocation

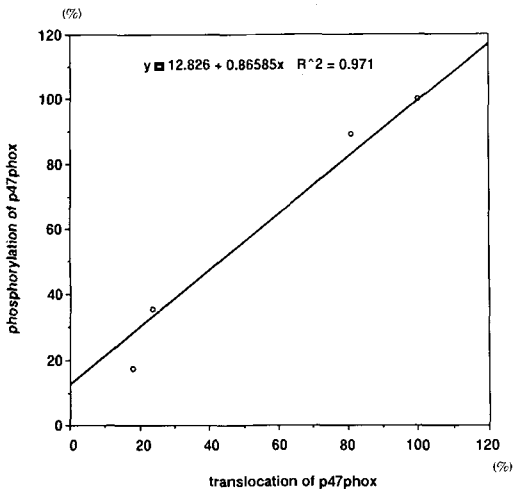

Fig. 8. Relationship between phosphorylation of p47phox and its translocation. Densimetrical analysis was performed on data in Fig. 7. The experiments were carried out twice; the data show the results of one representative experiment.

of p47phox is preceded by phosphorylation [Garcia et al., 1992; Nauseet et al., 1991]. To determine the effect of OA on p47phox phosphorylation, phosphorylation of p47phox was examined using immunoprecipitation against its antibody and Western blotting for phosphoamino acids. FMLP-induced phosphorylation of p47phox was only detected in serine residues (Fig. 5). Recently, the cDNA of p47phox has been cloned [Volpp et al., 1989]. The molecular structure of p47phox contains two SH3 domains, arginine-rich C-terminal domain and several serine residues. Six serine residues that are surrounded by basic residues are potential sites for phosphorylation by protein kinase [Volpp et al., 1989; Edelman et al., 1987]. Our experience showed that this phosphorylation of serine residues in p47phox was inhibited by OA. The details of serine/threonine protein kinase responsible for this p47phox phosphorylation remains unknown. This phosphorylation in PKC-independent pathway was regulated by PP2A. In neutrophils, four uncharacterized serine/threonine protein kinases with a molecular weight of 69, 63, 49, and 40 kDa have been identified, which were activated by stimulation with fMLP, not by PMA and calcium ionophore A23187 [Ding and Bedwey, 1993]. These kinases could catalyze the phosphorylation of a peptide that corresponds to residues of the p47phox. In addition, calyculin A, which is a protein phosphatase inhibitor as is OA, blocked the activation of the 63- and 40-kDa protein kinases. We speculated that the inhibitory effect of phosphorylation of serine residues in p47phox suppressed these 63- and 40-kDa protein kinases by OA. H-7 and EGTA exhibited a similar inhibitory effect on fMLP-induced superoxide generation. H-7 inhibited the direct activation of PKC, and EGTA inhibited the Ca^{2+}-dependent pathway due to suppression of the Ca^{2+} influx. This inhibitory mechanism was different from that of OA.

We also examined the effect of OA on the translocation of p47phox to the plasma membrane, as its translocation to the plasma membrane was an essential process for the activation of NADPH oxidase. OA inhibited not only the phosphorylation of serine residues, but also its translocation to the plasma membrane in a dose-dependent manner (Figs. 6, 7). There were no differences between the control and OA-treated cell groups on the amount of p47phox detected by Western blotting in the whole cell (data not shown). This result suggested that OA reduction in p47phox translocation was not due to an effect on the amounts of p47phox protein in the cell. Furthermore, translocation of p47phox to the plasma membrane was closely related to its phosphorylation (Fig. 8). The relationship between p47phox phosphorylation and its translocation remains unknown. Some investigators speculate on this relationship. Serine phosphorylation in p47phox neutralizes the cationic charge of the arginine-rich C-terminal domain and thereby facilitates its association with the cytoplasmic domain of the membrane-bound cytochrome b558 [Nauseef et al., 1993]. There is other speculation. SH_3 domains interact with the proline-rich regions of their target proteins. Therefore, the SH_3 domains of p47phox are masked by the proline-rich region in its C-terminal region through an intramolecular interaction [Sumitomo et al., 1994]. Arachidonic acid and SDS break this interaction of p47phox with the C-terminal region of this protein, leading to interaction with p22phox composed of cytochrome b558 and p67phox in the cell free system. In the intact cells, the unmasking of p47phox may be caused by a protein modification, such as protein phosphorylation. In this study, we could not clarify whether the translocation mechanism of p47phox was to neutralize the cationic charge or to change the conformation of p47phox, but phosphorylation of serine residues in p47phox played an important

role in the translocation to the plasma membrane and generation of superoxide in fMLP-stimulated neutrophils.

In conclusion, PP2A might be involved in the fMLP-induced phosphorylation of p47phox in the PKC-independent pathway. Translocation of p47phox and activation of NADPH oxidase were inhibited by low-dose OA, which suppressed phosphorylation of p47phox.

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