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Participation of Cofilin in Opsonized Zymosan-Triggered Activation of Neutrophil-Like HL-60 Cells through Rapid Dephosphorylation and Translocation to Plasma Membranes[1]

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We studied the roles of cofilin, an actin-binding phosphoprotein, in superoxide production of neutrophil-like HL-60 cells triggered by opsonized zymosan (OZ). OZ caused dephosphorylation of cofilin as well as a transient increase of F-actin. Both reactions were complete within 30 s. Okadaic acid (OA) magnified the OZ-triggered O_2^--production 3.3-fold at 1 μM, but inhibited it completely at 5 μM. We used these critical concentrations to study the effects of OA on changes in phosphorylation and intracellular localization of cofilin. The OZ-induced dephosphorylation of cofilin was inhibited by 5 μM OA but not by 1 μM OA. Subcellular fractionation and immunoblotting revealed that 1 μM OA increased cofilin on the phagosomal membranous fraction but 5 μM OA decreased it. At 1 μM, OA increased translocation of p47phox to membranes, which may explain in part the enhancing effect of 1 μM OA. Confocal laser scanning microscopy showed that: (i) Cofilin diffused throughout the cytosol of resting cells, but accumulated at the plasma membranes forming phagocytic vesicles in activated cells. (ii) At 1 μM, OA had little effect on the OZ-evoked translocation of cofilin, whereas 5 μM OA suppressed it completely. (iii) OA alone, which could not trigger the phagocytic respiratory burst, did not cause any change in the distribution of cofilin at such concentrations. Furthermore, in a superoxide-producing cell-free system employing membranous and cytosolic fractions, affinity-purified anti-cofilin antibody showed an enhancing effect. These results suggest that cofilin participates in the superoxide production of the OZ-activated phagocytes through dephosphorylation and translocation. The roles of cofilin in the activated leukocytes will be discussed.

Key words: actin-binding protein, CR3, okadaic acid, phagocytes, superoxide.

Phagocytic neutrophils are dormant in the absence of stimulants, but when activated by invading microorganisms or harmful substances they play a central role in host defense systems through chemotaxis, adhesion, phagocytosis, superoxide production, degranulation, and release of cytokines or lipid mediators. The mechanism of neutrophil activation has been widely studied in terms of protein phosphorylation, phospholipid metabolism, and cytoskeletal organization (reviewed in Ref. *1*). The importance of protein phosphorylation has been pointed out in regard to superoxide production particularly on p47phox (*2*), p67-phox, and substrates of tyrosine kinases (*3*). It has been hypothesized that increased phosphorylation of p47phox is a triggering event for the translocation of the cytosolic components to membranes to form an active complex of the O_2^--generating enzyme (*4*). Phorbol diester, a potent artificial activator of neutrophils, not only caused protein phosphorylation but increased the association of p47phox and p67phox with the cytoskeleton (*5-8*). Therefore, it is thought that cytoskeletal rearrangement may also be involved in the respiratory burst. However, the intracellular machinery for regulation of the O_2^--generating enzyme remains to be established. Recently, we found that okadaic acid has a concentration-dependent stimulatory or inhibitory effect on O_2^--production by neutrophil-like differentiated HL-60 cells activated with opsonized zymosan (OZ), suggesting that a cytosolic 21 kDa phosphoprotein plays an important role in the respiratory burst through dephosphorylation (*9*). The 21 kDa phosphoprotein was identified as cofilin, an actin and phosphatidylinositol 4,5-bisphosphate (PIP_2)-binding protein (*10*). Very recently, the dephosphorylation of cofilin in neutrophils was confirmed by other groups using artificial activators, formylpeptides and phorbol diester (*11-13*).

It has generally been thought that only dephosphorylated cofilin binds both actin monomer (G-actin) to sequester it from polymerization and filamentous actin (F-actin) to

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Abbreviations: DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; HRP, horse radish peroxidase; OA, okadaic acid; OZ, opsonized zymosan; PAGE, polyacrylamide gel electrophoresis; PIP_2, phosphatidylinositol 4,5-bisphosphate; SDS, sodium dodecyl sulfate.

depolymerize it (*14*) mainly based on *in vitro* experiments. However, it is unclear how cofilin is involved in the expression and regulation of phagocyte functions.

In this study, using OZ as a physiological activator, we examined the biochemical and histochemical changes of cofilin in the activated phagocytes. The essential role of cofilin in phagocyte functions will be discussed.

MATERIALS AND METHODS

Cells—HL-60 cells obtained from the Japanese Cancer Research Resources Bank were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The culture conditions have been described previously (*9*). The cells were induced to neutrophil-like form by exposure to 1.25% dimethyl sulfoxide (DMSO) for 6 days according to the method described by Collins *et al.* (*15*). Cell viability was assayed by the trypan blue dye exclusion method.

Antibodies and Cofilin—Rabbit anti-cofilin antiserum and *Escherichia coli* producing recombinant pig cofilin containing $(His)_6$-tag were generously provided by Drs. I. Yahara, K. Iida, and K. Moriyama (Tokyo Metropolitan Institute of Medical Science). The $(His)_6$-tagged cofilin was purified on Ni^{2+}-chelating nitrilotriacetic acid (NTA)-agarose (Qiagen, Hilden, Germany) and the antibody was purified on the recombinant cofilin-fixed Affigel 10 (Bio Rad, Hercules, CA, USA). Monoclonal anti-cofilin antibody MAB22 was kindly donated by Drs. T. Obinata and H. Abe (Chiba University, Chiba). Affinity-purified rabbit anti-p47phox antibody was prepared as described (*9*). Mouse anti-p67phox antiserum was prepared by immunizing BALB/c mouse with recombinant p67phox. 5-(4,6-Dichlorotriazinyl)aminofluorescein-conjugated $F(ab')_2$ fragment goat anti-mouse IgG(H+L) was obtained from Immunotech (Marseilles, France).

Reagents—Okadaic acid (Wako Pure Chemical, Osaka) was dissolved in DMSO to a concentration of 1.0 mM in DMSO and diluted with Hanks' balanced salt solution (HBSS). OZ was prepared according to the method of Markert *et al.* (*16*). Briefly, 700 mg of zymosan A (Sigma, St. Loius, MO) was incubated with 70 ml of human serum at 37°C for 30 min and washed with HBSS. The OZ suspended in HBSS (10 mg/ml) was divided and stored at −80°C until use. Fluorescein isothiocyanate (FITC)-phalloidin was purchased from Molecular Probes (Oregon, USA) and immunostaining chemiluminescence reagents were from DuPont NEN (Boston, MA, USA). All other chemicals used were commercial preparations of the highest purity.

Two-Dimensional Electrophoresis of Phosphoproteins—The differentiated HL-60 cells, which had been preincubated with $^{32}P_i$ at 37°C for 60 min, were stimulated with OZ (1 mg/ml) for 15 s-2.0 min. After subcellular fractionation, the cytosolic fractions were subjected to two-dimensional gel electrophoresis followed by autoradiography. The experimental conditions were as described (*2*). The amount of ^{32}P incorporated in a protein was determined by use of a Bioimage Analyzer BAS2000 (Fuji Film, Tokyo). To calculate the means of data, each value was normalized and expressed as a percentage of the control (not activated).

Staining of F-Actin with Fluorescein-Phalloidin—F-Actin in cells was stained with FITC-phalloidin and determined by flow cytometry. Differentiated HL-60 cells were stimulated with OZ at 37°C for the indicated time, then ice-cold paraformaldehyde (final 1.9%) was added, and the mixture incubated at room temperature for 30 min. The fixed cells were washed with PBS and treated with FITC-phalloidin (0.5 U/ml PBS) containing lysophosphatidylcholine (5 mg/ml) with gentle shaking at room temperature for 30 min. Fluorescence intensity was determined with a flow cytometer (FACSCalibur, Becton Dickinson) with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of Superoxide Production by Intact Cells—O_2^--production was determined by reduction of cytochrome *c* as described previously (*9*). HBSS, 500 μl, containing 1.5×10^6 cells, and OA were incubated at 37°C for 15 min. After addition of cytochrome *c* (final concentration 1 mg/ml), the cells were stimulated with OZ (final concentration 1 mg/ml) at 37°C. The exact maximum rates of O_2^--production were calculated based on time-scan data obtained with a spectrophotometer (Hitachi, Model 3200, Tokyo) equipped with a photomultiplier positioned close to the cuvette, a windmill cell mixer developed by Kakinuma *et al.* (*17*), and a temperature controller.

Subcellular Fractionation for Immunoblotting—The cells (1.5×10^7), which had been preincubated with OA as above, were activated with OZ (final concentration: 6 mg/ml) in HBSS (1 ml) at 37°C for 2 min. Activation was stopped by addition of 5 ml of ice-cold inhibitor solution (*2*) containing 5 mM diisopropyl fluorophosphate, 20 μM leupeptin, 20 μM pepstatin, 0.1 M NaF, 10 mM EDTA, 2 mM *N*-ethylmaleimide, 1 mM ammonium molybdate, 1 mM iodoacetic acid, 1 mM benzamidine, 75 mM NaCl, 2.5 mM KCl, 3 mM Tris, and 3 mM Hepes (pH 7.3), and the cells were chilled by immersing them in melting ice for 10 min. The cells were then packed by centrifugation (800 rpm, 15 min) and resuspended in 0.7 ml of 0.125 M sucrose solution containing the above inhibitors, except diisopropyl fluorophosphate. The cells were pressurized with N_2 for 20 min at 350 psi in a nitrogen bomb (model 4639, Parr Instrument, Moline, IL) according to Borregaard *et al.* (*18*), and the cavitates were centrifugated at $100,000\times g$ for 30 min to separate a cytosolic fraction and a particulate fraction containing phagosomes and plasma membranes. All the fractionation procedures were performed at 0-4°C.

SDS-PAGE and Immunoblotting—The particulate fractions were suspended in 100 μl of 125 mM Tris-HCl (pH 6.8) containing 0.1% SDS and 2 mM $MgCl_2$ by sonication, and treated with 25 units of benzone nuclease (Merck, Darmstadt, Germany) at 37°C for 30 min to break the DNA. Then, each fraction was mixed with 10 μl of 3% SDS solution containing 15 mM dithiothreitol, sonicated for 5 min, and incubated at 70°C for 2 min. The heat-treated mixtures were centrifuged at $1,400\times g$ for 10 min to remove zymosan, and 20 μl of each sample was subjected to SDS-PAGE according to Laemmli (*19*) using 12.5% gel. Electroblotting and immunochemical detection by chemiluminescence were carried out as previously described (*9, 10*) using affinity-purified rabbit anti-cofilin, anti-p47phox antibody, or mouse anti-p67phox antiserum.

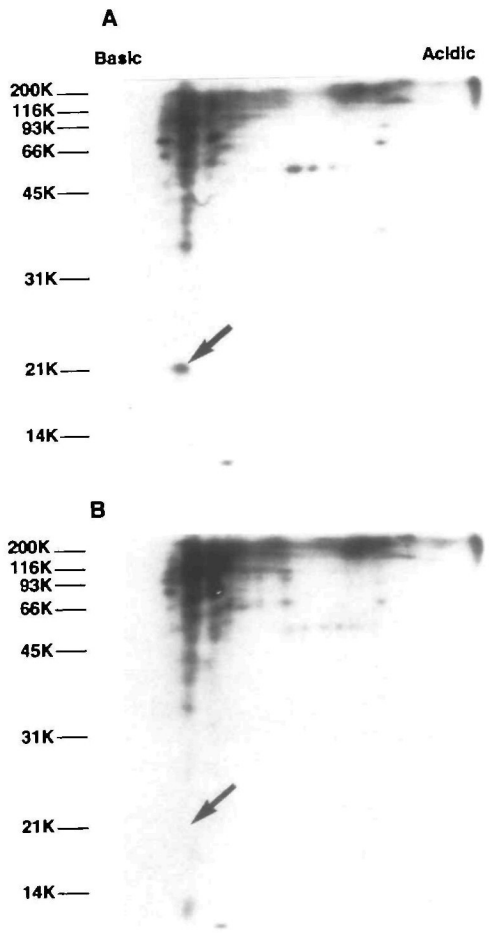
Confocal Laser Scanning Microscopy—The cells were treated with OA and activated by OZ as above for 1 min, and the activation was stopped by addition of formaldehyde (final 4.3%) and immediate chilling in melting ice for 5 min. Incubation for formaldehyde fixation was continued at

room temperature for 30 min. The fixed cells were attached to poly-L-lysine-coated slide glasses by centrifugation and stained with monoclonal anti-cofilin antibody (MAB22) and 5-(4,6-dichlorotriazinyl)aminofluorescein-conjugated $F(ab')_2$ fragment goat anti-mouse IgG(H+L). The experimental conditions were as described previously (*10*). Digitized images were generated by using a confocal laser scanning microscope (RCM8000, Nikon, Tokyo) with an excitation wavelength of 488 nm and emission wavelength of at least 520 nm. A Nikon 40X/NA1.15 water immersion objective was used.

Cell-Free Assay of NADPH-Oxidase Activation—Cytosolic and membranous fractions were prepared according to Borregaard *et al.* (*18*), and cell-free assay was carried out as described previously (*20*). Briefly, the differentiated HL-60 cells (1×10^8) which had been treated with 5 mM diisopropyl fluorophosphate in 5 ml of PBS for 10 min on ice were suspended in 0.7 ml of relaxation buffer [100 mM KCl, 3 mM NaCl, 1 mM ATP, 3.5 mM PIPES (pH 7.3), and 1.25 mM EGTA]. The suspended cells were disrupted by N_2-cavitation (350 psi, 20 min), the cavitates were centrifuged at $850\times g$ for 15 min to remove nuclei, mitochondria, and unbroken cells, and the supernatant was centrifuged at $100,000\times g$ for 30 min. The pelleted membranes were resuspended in 100 μl of 0.25 M sucrose containing 5 μM Tris-HCl (pH 7.4), 20 μM leupeptin, 20 μM pepstatin, and 20 μM APMSF. The membranous and cytosolic fractions were stored at −80°C until use. Rabbit anti-cofilin antibody was purified on a column of cofilin-fixed agarose gel and dialyzed against PBS. Normal rabbit IgG fraction (Miles Lab., Elkhart, IN, USA) was solubilized in PBS and dialyzed against PBS. The assay mixture (0.7 ml) consisted of 0.1 mM cytochrome *c*, 65 mM K, Na-phosphate buffer (pH 6.9), 1 mM EGTA, 1 mM $MgCl_2$, 2 mM NaN_3, 10 μM FAD, 10 μM GTPγS, 4 μl of membranous fraction, 40 μl of cytosolic fraction, 0.2 mM NADPH, 2.5 mM myristate, 10 μg of the antibody (when indicated), and 0.1 mM SOD (for reference). The mixture was incubated at 25°C for 5 min, and the reaction was started by the addition of NADPH. The reduction of cytochrome *c* was monitored with a spectrophotometer (Shimadzu UV-2200) at 25°C.

RESULTS

Opsonized Zymosan-Dependent Rapid Dephosphorylation of Cofilin and Changes in Cytoskeleton—Zymosan, an



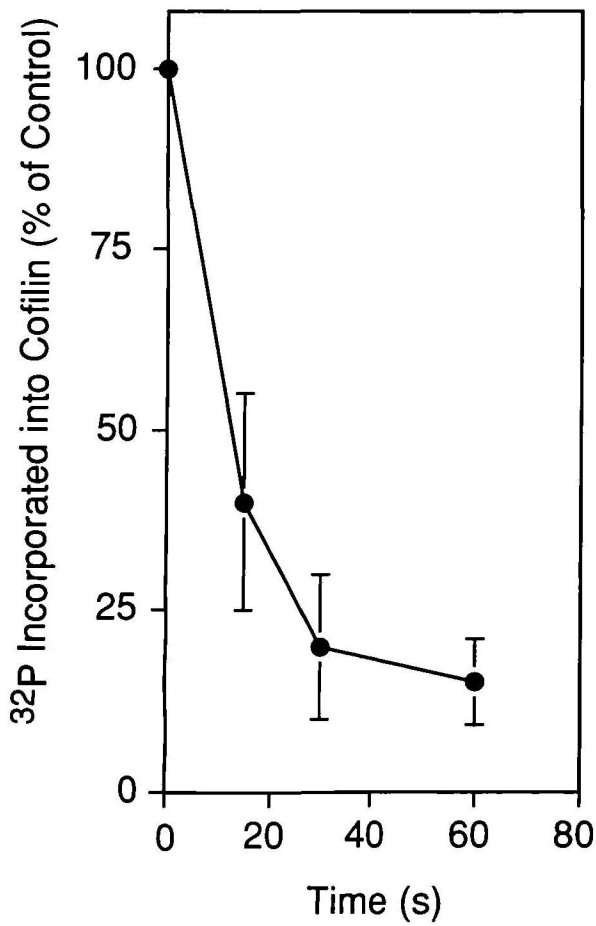


Fig. 1. **Opsonized zymosan-triggered rapid dephosphorylation of cofilin.** Left: $^{32}P_i$-loaded neutrophil-like HL-60 cells were exposed to HBSS (A) or opsonized zymosan (B) at 37°C for 30 s. The cytosolic fraction derived from 1×10^6 cells was subjected to two-dimensional electrophoresis and autoradiography. The arrow in each photograph indicates cofilin. Right: Time courses of dephosphorylation of cofilin. The amount of ^{32}P incorporated into cofilin was determined by radioluminography and is expressed as a percentage of control (not activated) on the vertical axis. The means of three experiments are expressed with standard deviations.

insoluble polysaccharide particle derived from yeast, was coated with activated complement C3bi by treatment with human serum to be opsonized. Throughout this study, the opsonized zymosan (OZ) was used as a physiological stimulant, which activated the phagocytes *via* complement receptor CR3, a member of the $\beta 2$ integrin family (*21, 22*). OZ triggered remarkable dephosphorylation of a 21 kDa protein, which had been identified as cofililn by sequencing and immunoblotting (*10*). The results are summarized in Fig. 1, which is similar to the previous reports (*9, 10*). The reaction was rapid, and more than 60% of the ^{32}P incorporated to serine (*9*) was lost within 15 s after the addition of OZ. Because cofilin is an actin-regulating protein, time-dependent changes in F-actin in the OZ-stimulated cells were investigated by flow cytometry. The content of F-actin stained with fluorescein-phalloidin changed rapidly. It increased at 15 s after stimulation and decreased to the original level at 30 s. A typical result is shown in Fig. 2. This transient increase was reproducible and essentially consistent with previous reports in which FMLP (*23*) and IL-8 (*24*) were used. The dephosphorylated cofilin may be involved in the rapid reorganization of cytoskeleton, because cofilin is a major actin-regulating protein and only the dephosphorylated form of cofilin can bind F- and G-actin *in vitro* (*14*). No significant changes in intracellular pH, which affect F-actin-depolymerizing activity of cofilin (*14*), were observed in the OZ-stimulated HL-60 cells (data not shown).

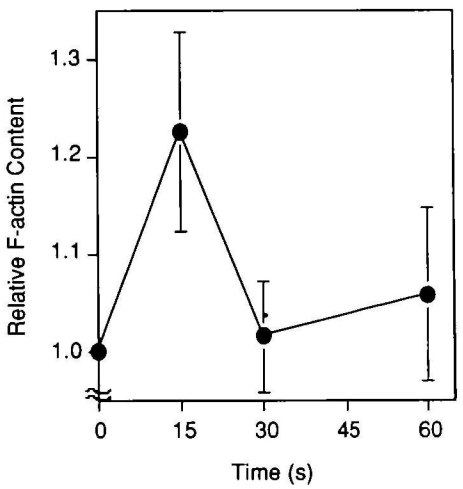


Fig. 2. **Change in F-actin content in OZ-stimulated HL 60 cells.** Neutrophil-like HL-60 cells were activated by OZ at 37°C for the indicated time. F-actin in the cells was stained with fluorescein-phalloidin and its fluorescence intensity was measured with a flow cytometer. The averages of four experiments are expressed with standard deviations. Experimental details are described in "MATERIALS AND METHODS."

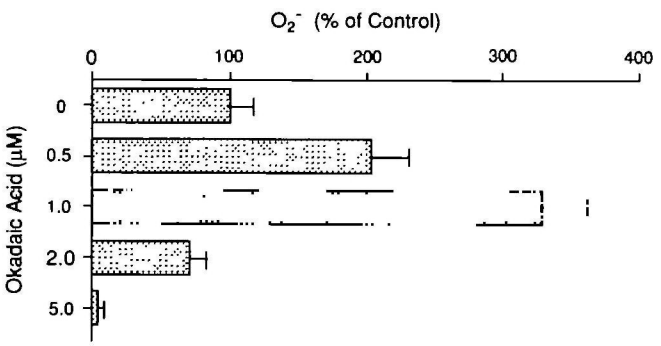


Fig. 3. **Biphasic effect of okadaic acid on opsonized zymosan-stimulated superoxide production by neutrophil-like HL-60 cells.** After incubation in the presence of OA at the indicated concentrations for 15 min, the cells were activated by OZ (1 mg/ml). The rates of superoxide production were measured by time-scanning of reduction of cytochrome *c*. In the absence of okadaic acid, the rate of superoxide production was 1.1 nmol/min/10^6 cells. The data are represented as means ± SD of four experiments.

Enhancing and Inhibitory Effects of OA on Superoxide-Production—In other cells, cofilin is not only dephosphorylated but also translocated to nuclei (*25, 26*) or to the membrane region (*10, 27*), depending on cell type and on type of stimulation (*28*). We therefore investigated the changes in intracellular distribution of cofilin in the OZ-stimulated phagocytes, employing okadaic acid (OA) as a unique modulator for OZ-dependent activation of the neutrophil-like HL60 cells. As shown in Fig. 3, OA showed a unique opposing effect on superoxide release by differentiated HL-60 cells triggered by OZ in a narrow micromolar range of concentrations. In this study, we adopted 1 μM as an enhancing concentration of OA on OZ activation,

TABLE I. **Effect of okadaic acid on phosphorylation of cofilin and p47phox.** The differentiated HL-60 cells, which had been loaded with ^{32}P, were preincubated with okadaic acid for 15 min and stimulated with OZ for 1 min. ^{32}P-incorporated proteins were separated by two-dimensional gel electrophoresis and radioactivities of cofilin and p47phox were determined by a bioimage analyzer and expressed as photon-stimulated luminescence (PSL). Relative degrees of phosphorylation of each protein are shown in parenthesis as percent of control (without OA and OZ). One typical result of four similar experiments is shown.

OA	Cofilin		p47phox	
	−OZ	+OZ	−OZ	+OZ
0	2,508 (100)	401 (16)	756 (100)	1,021 (135)
1 μM	2,252 (90)	451 (18)	1,141 (151)	1,739 (230)
5 μM	2,759 (110)	2,734 (109)	2,124 (281)	2,102 (278)

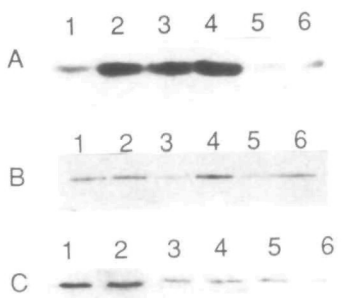


Fig. 4. **OA and OZ-induced changes in cofilin, p47phox, and p67phox in particulate fractions.** The cells were treated with HBSS (lanes 1 and 2), 1 μM OA (lanes 3 and 4), or 5 μM OA (lanes 5 and 6) and exposed to HBSS (lanes 1, 3, and 5) or OZ (lanes 2, 4, and 6). The particulate fractions containing phagosomes and plasma membranes were obtained, and an aliquot of each fraction was subjected to SDS-PAGE. Cofilin (A), p47phox (B), and p67phox (C) were detected by immunoblotting. A typical result of five experiments is shown. Details are described under "MATERIALS AND METHODS."

and 5 μM as an inhibitory concentration. Although the results were similar to that of the endpoint assay described previously (*9*), the exact maximum rates of O_2^--production were determined by time-scanning. OA alone failed to induce the generation of active oxygen as previously described (*9*). No cytotoxic effect of OA was observed by dye exclusion test during the experiments.

Concentration-Dependent Effect of OA on Phosphorylation of Cofilin and p47phox—Because OA is a potent inhibitor of phosphatases, the effect of OA on protein phosphorylation in the OZ-treated cells was investigated. As shown in Table I, OZ-induced dephosphorylation of cofilin was not inhibited by 1 μM OA but inhibited by 5 μM OA. The p47phox was heavily phosphorylated in the 5 μM OA-treated cells, which did not generate superoxide in response to OZ (Fig. 3). These results are essentially consistent with our previous observation (*9*). There is a possibility that not only phosphorylation of p47phox but dephosphorylation of cofilin is required for the respiratory burst. In the 1 μM OA-treated cells, OZ-dependent increase in phosphorylation of p47phox was observed, which may explain the enhancing effect of 1 μM OA on the OZ-triggered respiratory burst.

Effect of OA on Subcellular Distribution of Cofilin—We then investigated the effect of OA on subcellular localization of cofilin. The cells were disrupted by N_2 cavitation, and changes in cofilin in the phagosome-containing particulate fraction were studied by immunoblotting. In the absence of OA, OZ increased the amount of cofilin in the particulate fraction (Fig. 4A, lane 2), consistent with our earlier observations (*10*). OA, 1 μM, which enhanced OZ-triggered O_2^--production (Fig. 3), increased the amount of membrane-associated cofilin with or without OZ-stimulation, while 5 μM OA strongly inhibited the association of

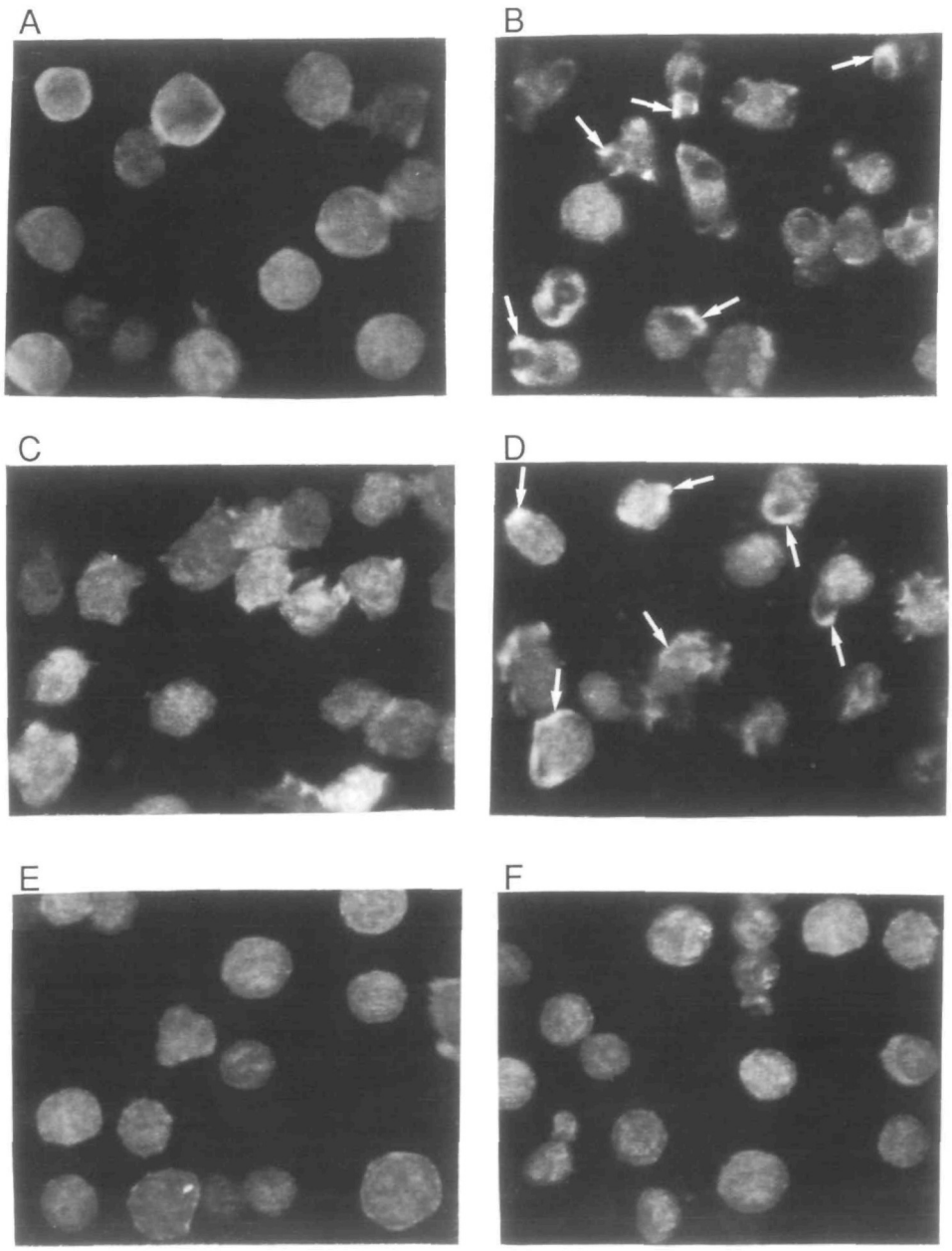


Fig. 5. **Confocal laser scanning microscopy of differentiated HL-60 cells stained with anti-cofilin antibody and dichlorotriazinyl amino fluorescein-conjugated second antibody.** The cells were treated with HBSS (A and B), 1 μM OA (C and D), or 5 μM OA (E and F) and exposed to HBSS (A, C, and E) or OZ (B, D, and F). Typical areas of accumulated cofilin are indicated by arrows.

cofilin with the membrane fraction (Fig. 4A, lanes 3-6). Though it is unclear why 1 μM OA alone increased the membranous cofilin (lane 3), the deformed membrane induced by OA (Fig. 5C) might interact with cofilin. The association of p47phox and p67phox with the membranes was also investigated in the presence or absence of OA. In the membranes of resting cells, a considerable amount of p67phox was observed, which may associate with phosphoinositides (*29*). A significant amount of p47phox was also detected in the membrane fraction. The OZ-induced increase in p47phox in the membrane fraction was about 15%. The effect of OA was quite different from its effect on cofilin. The membranous p47phox and p67phox decreased in response to OA (Fig. 4, B and C, lanes 3 and 5). However, the amount of p47phox increased in the 1 μM OA and OZ-treated particulate fraction (Fig. 4B, lane 4). This result and OZ-stimulated increase in phosphorylation of p47phox (Table I) may explain at least partly the enhancing effect of 1 μM OA on the O_2^--production. These results also suggest that the associations of p47phox and p67phox with the membranes were differently controlled.

Translocation of Cofilin in the Phagocytic Cells—To visualize the effect of OA on changes in the localization of cofilin, cellular cofilin was indirectly stained with fluorescence-labeled antibody and observed by confocal laser scanning microscopy. As shown in Fig. 5, cofilin was diffusely distributed in the cytosol and nuclear regions of the resting cells, but activation by OZ caused the accumulation of cofilin beneath the raised membranes and around forming phagocytic vesicles. OA alone induced no significant change in the cofilin distribution at 1 or 5 μM, but at 1 μM it produced morphological changes (Fig. 5C). The OA-induced morphological changes seemed to be consistent with previous observations (*30, 31*). The OZ-induced translocation of cofilin to the plasma membrane region was also observed in the presence of 1 μM OA. However, 5 μM OA inhibited OZ-dependent translocation of cofilin completely. Although cofilin has a nuclear translocation sequence, no nuclear translocation was observed in the OZ and/or OA-treated phagocytes. Such changes in intracellular distribution of cofilin were clearly observed in the fluorescence-stained unbroken cells, while stimuli-dependent translocation of p47phox, p67phox, and rac has not been observed by the fluorescence-staining method, probably because only a small part (less than 10%) of each of them was translocated to the plasma membranes (*3*).

On the other hand, OA alone had little effect on the expression of OZ receptors (CD11b/CD18) on the phagocytes as already mentioned (*9*).

Based on the results of immunoblotting and confocal microscopy, OA is a potent modulator of the localization of cofilin, and the dual effect of OA on the superoxide production may be correlated with the redistributed cofilin.

Enhancing Effect of Anti-Cofilin Antibody on Superoxide Production by Cell-Free System—To examine the roles of cofilin *in vitro*, the effect of purified anti-cofilin antibody on superoxide production was tested employing a cell-free system. As shown in Fig. 6, the antibody enhanced the O_2^--production. Endogenous activity of cytochrome *c* reductase (fp2), which was determined in the presence of SOD, was below 10% of the SOD-inhibitable superoxide production. The mechanism of the enhancing effect of the antibody is unclear, while actin that was sequestered by cofilin may be released by the added antibody, because it has been reported that G-actin enhances the superoxide production in the cell-free system (*32*).

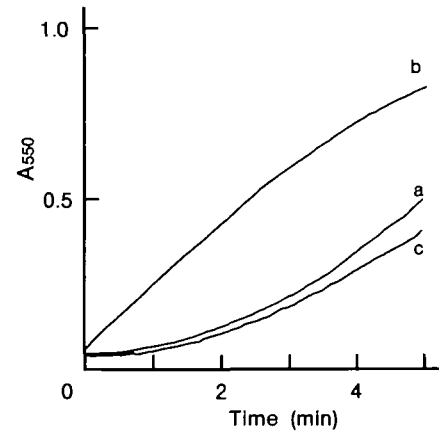


Fig. 6. **Effect of anti-cofilin antibody on superoxide production in cell-free system.** The cytosolic and membranous fractions were preincubated with either vehicle (PBS) (a), anti-cofilin antibody (b), or normal rabbit IgG (C). NADPH was added to the mixture and time-dependent reduction of cytochrome *c* was monitored. Experimental details are described in "MATERIALS AND METHODS."

DISCUSSION

We studied the time-dependent changes in phosphorylation and intracellular distribution of cofilin in neutrophil-like HL-60 cells activated by OZ, a physiological stimulant. We observed that: (i) OZ triggered rapid dephosphorylation of cofilin on two-dimensional gels. (ii) OZ caused rapid and transient increase in F-actin on flow cytometry profiles. (iii) OZ induced translocation of cofilin from cytosol to plasma membrane regions that were forming phagocytic vesicles. Therefore, cofilin seemed to participate in the phagocyte activation *via* rearrangement of cortical cytoskeleton through dephosphorylation and translocation. In particular, it was confirmed that superoxide production was highly correlated with changes in phosphorylation and localization of cofilin by using OA, a unique modulator of the respiratory burst. The results of confocal microscopy of the fluorescein stained cells and immunoblotting of particulate fractions revealed that: (i) 1 μM OA, which enhanced OZ-induced superoxide production, allowed the OZ-triggered dephosphorylation of cofilin and its translocation to plasma membranes. (ii) 5 μM OA, which inhibited the OZ-triggered superoxide production, suppressed both the dephosphorylation of cofilin and its translocation to plasma membrane regions. Other data also indicate the involvement of cofilin in the respiratory burst. The results obtained with a cell-free system using the cytosolic and membranous fractions derived from the phagocytes indicated that anti-cofilin antibody enhanced superoxide production. A part of cofilin was coimmunoprecipitated with cytosolic subunits of the respiratory burst oxidase (*9*).

The molecular mechanisms of action of cofilin are unclear, while one of the roles of cofilin may be to control the amount of G-actin, which mediates the enhancing effects of cytochalasins (*33*) and botulinum C_2 toxin (*34*) on superoxide production by neutrophils or by a cell-free system (*32*).

Recently, it was reported that cofilin controls the turnover rate of F-actin *in vitro* (*35*) and in yeast (*36*). Also in the phagocytes, cofilin may be involved in both increase and decrease of F-actin which were observed in the OZ-activated phagocytes. The OZ-triggered dephosphorylation of cofilin was more rapid than that of other artificial stimulants such as phorbol ester or formyl peptides (*10-12*). The OZ-receptor CR3, a $\beta 2$ integrin, may send a more direct signal for dephosphorylation of cofilin than the other receptors.

Cofilin is a widely distributed actin-binding protein, and it has been thought that only the unphosphorylated cofilin is capable of depolymerizing actin filaments, binding to the actin monomer, and bundling stress fibers (*37*). Various stimuli induce dephosphorylation and translocation of cofilin, and its role differs depending on the type of cell and tissue. In fibroblasts (*25*) and T-cells (*26*), cofilin is dephosphorylated and translocates to the nuclei where it forms actin/cofilin rods, whose functions are unknown. However, nuclear translocation of cofilin was not observed in the phagocytic leukocytes we tested, while its accumulation was observed just beneath the plasma membranes. On the other hand, Abe *et al.* described that a mutant of cofilin which could not be phosphorylated accumulates in the membranous regions of developing embryo of *Xenopus laevis* (*27*). These findings together suggest that the translocated cofilin is of dephosphorylated form. In conclusion, cofilin is a major actin-regulating protein and appears to be involved in various cellular functions including superoxide production and phagocytosis. It is important to clarify the machinery which regulates the phosphorylation/dephosphorylation of cofilin. Recently, it was reported that LIM-kinase phosphorylates cofilin (*38, 39*).

Interestingly, cofilin also binds PIP_2 specifically at the actin-binding sites (*40, 41*), and PIP_2 is known to be a preferential substrate for signal-activated phospholipase C (*22*). Liscovitch *et al.* reported that PIP_2 can activate phospholipase D directly (*42*). Cofilin might thus play an important role not only in remodeling the cytoskeleton but in generating phosphatidic acid and diacylglycerol, which induce the respiratory burst (*43, 44*). It was also observed that granulocyte colony stimulating factor (G-CSF) increased both the superoxide-producing activity (*45*) and the contents of cofilin during the DMSO-induced differentiation of HL-60 cells (data not shown). This observation may also support the notion that cofilin is important to phagocyte functions. Further studies are required to address the functions of cofilin in the course of leukocyte activation.

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