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Downstream components of RhoA required for signal pathway of superoxide formation during phagocytosis of serum opsonized zymosans in macrophages

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Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfony fluoride; DMPC, dimyristoylphosphatidyl choline; DPI, diphenyleneiodonium chloride; ERK1/2, extracellular signaling-regulated kinase 1/2; GST, glutathione S-transferase; HRP, horse-radish peroxidase; IPTG, isopropylthio-β-D-galactoside; isoluminol, 6-amino-2,3-dihydro-1,4-phtalazinedione; luminol, 5-amino-2,3-dihydroxy-1,4-phtalazinedione; LY294002, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; MAPK, mitogen-activated protein kinase; MEK, MAP kinase kinase; MV, methylviologen; NAC, N-acetyl-L-cysteine; PA, phosphatidic acid; PI3K, phosphatidyl inositol 3-kinase; PD98059, 2'-amino-3'-methoxyflavone; SB203580, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; SOD, superoxide dismutase; SOZ, serum-opsonized zytmosan

Abstract

Rac1 and Rac2 are essential for the control of oxidative burst catalyzed by NADPH oxidase. It was also documented that Rho is associated with the superoxide burst reaction during phagocytosis of serum- (SOZ) and IgG-opsonized zymosan particles (IOZ). In this study, we attempted to reveal the signal pathway components in the superoxide formation regulated by Rho GTPase. Tat-C3 blocked superoxide production, suggesting that RhoA is essentially involved in superoxide formation during phagocytosis of SOZ. Conversely SOZ activated both RhoA and Rac1/2. Inhibition of RhoA-activated kinase (ROCK), an important downstream effector of RhoA, by Y27632 and myosin light chain kinase (MLCK) by ML-7 abrogated superoxide production by SOZ. Extracellular signaling-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK) were activated during phagocytosis of SOZ, and Tat-C3 and SB203580 reduced ERK1/2 and p38 MAPK activation, suggesting that RhoA and p38 MAPK may be upstream regulators of ERK1/2. Inhibition of ERK1/2, p38 MAPK, phosphatidyl inositol 3-kinase did not block translocation of RhoA to membranes, suggesting that RhoA is upstream to these kinases. Inhibition of RhoA by Tat-C3 blocked phosphorylation of $p47^{PHOX}$. Taken together, RhoA, ROCK, p38MAPK, ERK1/2, and $p47^{PHOX}$ may be subsequently activated, leading to activation of NADPH oxidase to produce superoxide.

Keywords: extracellular signal-regulated MAP kinases; macrophages; NADPH oxidase; P38 mitogen-activated protein kinases; rhoA GTP-binding protein; superoxides

Introduction

Phagocytosis of pathogens and apoptotic cells by macrophages triggers the immune response and is important to host defense mechanisms. Phagocytosis is performed through the receptors such as complement receptor type 3 (CR3, referred also as CD11b/CD18, Mac-1, and integrin αMβ2), Fcγ receptors (FcγRs), and mannose receptor. CR3 recognizes the C3b/C3bi fragments coating particles like pathogens, FcγRs recognize the Fcγ domain of immunoglobulin G (IgG), and mannose receptor recognizes mannose and fucose in the capsule or on the lipopolysaccharide of invading bacteria (Brown, 1995; Chimini and Chavrier, 2000).

Subsequently, macrophage induces the abrupt increase of superoxide formation, referred to as oxidative burst, which is mediated by NADPH oxidase.

The phagocyte NADPH oxidase is a membrane-associated enzyme complex that generates superoxide (O_2^-) by the one-electron reduction of oxygen, using NADPH as the electron donor (Babior, 1999). The core enzyme of NADPH oxidase is composed of five components. Among them, $p22^{PHOX}$ and $gp91^{PHOX}$ exist in the membranes of the secretory granular vesicles, which form a heterodimeric flavohemoprotein known as cytochrom b_{558} (Rotrosen *et al*., 1993). The other components, $p40^{PHOX}$, $p47^{PHOX}$, and $p67^{PHOX}$ are located in the cytosol as a complex form (Wientjes *et al*., 1996). $p47^{PHOX}$ becomes highly phosphorylated by protein kinases (Park and Babior, 1997; Park *et al*., 1997), and the entire cytosolic complex of $p40^{PHOX}$, $p47^{PHOX}$, and $p67^{PHOX}$ translocates to the membrane, where it associates with cytochrome b_{558} to assemble the active NADPH oxidase (Heyworth *et al*., 1991; Sathyamoorthy *et al*., 1997). In addition to the core subunits, Ras- related small GTP-binding proteins, Rac1 or Rac2 is required for the activation of NADPH oxidase (Mizuno *et al*., 1992; Kreck *et al*., 1994; Kim *et al*., 2001). In resting state Rac is localized in the cytoplasm in a dimeric complex with Rho GDP dissociation inhibitor (GDI), while GTP-bound Rac translocates to the membrane along with core cytosolic complex during activation (Knaus *et al*., 1992; Quinn *et al*., 1993; Bokoch, 1994).

Rho family proteins in actin dynamics are essential for the phagocytosis and engulfment (Chimini and Chavrier, 2000). It was found that Cdc42/Rac regulated the phagocytosis mediated through FcR and the subsequent superoxide formation through FcγR activation is regulated by Rac, whereas Rho regulated phagocytosis mediated through CR3 (Caron and Hall, 1998; Massol *et al*., 1998). Besides Rac, RhoA was reported to be also involved in the production of H_2O_2 in other cell lines such as swiss 3T3 fibroblast (Koo *et al*., 1999) and Rat-2 fibroblast (Lee *et al*., 2000), when TGF and EGF stimulated them, respectively. In addition to FcR, the stimulation of CR3 by anti-CR3 antibody-coated particles (Serrander *et al*., 1999), by *Staphylococcus* particles with anti-CD18 antibodies (Lofgren *et al*., 1999), and by zymosan ingestion through CR3 (Le Cabec *et al*., 2000) induces superoxide production. Recently, it has been documented that Rho is essentially involved in superoxide formation during phagocytosis of opsonized zymosans (Kim *et al*., 2004). In this study, we elucidated the components required for signal pathway regulating superoxide formation mediated by Rho.

Materials and Methods

Materials

Zymosan A particles, FITC, BSA, isopropylthio-β-D-galactoside (IPTG), NAD^+, GDP, triethanolamine/HCl, DTT, PMSF, Y-27632, 5-amino-2,3-dihydro-1,4-phtalazinedione (luminol), 6-amino-2,3-dihydro-1,4-phtalazinedione (isoluminol), 2'-amino-3'-methoxyflavone (PD98059), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580), 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), PMA, dimyristoylphosphatidyl choline (DMPC), Triton X-100, and glutathione (GSH) were purchased from Sigma chemicals. PBS without calcium or magnesium was purchased from Bio-Whittaker. Catalase, horse-radish peroxidase (HRP), and superoxide dismutase (SOD) were purchased from Roche Molecular Biochemicals. FBS, DMEM, and penicillin- streptomycin were purchased from GibcoBRL. C3bi was from Calbiochem, and IgG against zymosan was from Molecular Probe. GSH-Sepharose bead was purchased from Pharmacia. Anti-RhoA, anti-His antibodies were purchased from Santa Cruz, and anti-phospho-extracellular signal-regulated kinase (ERK), -phospho-JNK, and -phospho-p38 antibodies and anti-p38 antibody were from Cell Signaling. Anti-$p47^{PHOX}$ antibody and pGEX-1λT plasmid containing GSH S-transferase (GST)-$p47^{PHOX}$ fused gene were kindly given from Dr. J. W. Park of Kyoungbuk National University. Tat-C3 exoenzyme was expressed in *E.coli* DH5α and purified through Ni^{2+}-IDA gel column (Novagen).

Expression and purification of the Tat-C3

For the preparation of Tat-C3 exoenzyme, *E. coli* BL21 (Pharmacia) transformed with pC3 or pTat-C3 constructs were grown overnight at 37°C in LB broth supplemented with 100 g/ml ampicillin. The purification of the proteins was performed following the methods described in previous reports (Park *et al.,* 2003; Kim *et al.,* 2004).

Cell culture and transduction of Tat-C3 fusion protein

Macrophage J774 cell line was cultured in DMEM containing 20 mM Hepes/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% FBS and antibiotics (100 U/ml streptomycin and 100 U/ml penicillin) at 37°C in 5% CO_2. For the transduction of Tat-C3, macrophage cells were grown to confluence on 6-well plate for 4-6 h. And then culture medium was replaced with 1 ml of fresh solution without FBS. J774 cells were treated with various concentrations of Tat-C3 for 30 min, and then were performed the assays for the phagocytosis and association of particles. For the assay of RhoA modification by Tat-C3, the cell lysates pretreated with Tat-C3 were run on SDS- PAGE and RhoA protein was analyzed by Western blot by using anti-RhoA antibody.

Preparation of FITC-zymosan

Zymosan A particles were labeled with FITC without addition of gelatin (Gelfand *et al*., 1976). The zymosan particles were then pelleted by centrifugation, washed more than 7 times, and resuspended in PBS (adjusted to 5×10^8 zymosan particles/ml). Aliquots were stored at -70°C and thawed immediately before use (Gelfand *et al*., 1976). Zymosans were opsonized with 1 mg/ml mouse serum (SOZ), C3bi, and IgG (Allen and Aderem, 1996). C3b in the serum was found to rapidly fix onto zymosan, and about 80% of the C3b to be converted to C3bi when zymosan was opsonized with fresh FBS at 37°C (Newmans and Mikus, 1985).

Assay of phagocytosis and association of zymosan on the surface of macrophages

Cells were plated onto 35-mm dishes at a density of 2×10^5 cells and grown overnight, and the cells were incubated in DMEM media without FBS for 16 h at 37°C in CO_2 incubator. Cells were incubated for 30 min with 5×10^5 of FITC-conjugated zymosan particles previously resuspended in fresh mouse serum to 1/4 dilution. The cells were washed with PBS three times to clear the unbound zymosan particles and detached from dishes with 2 ml PBS. The phagocytosis was assayed by measuring the intensity of FITC engulfed into the cells. FITC was excited at 490 nm and the emission fluorescence of it was immediately measured at 520 nm by fluorescence spectrophotometer (Kontron SFM25). The fluorescence of adherent FITC-zymosan on the surface of the cells was quenched by addition of crystal violet to 10 M of final concentration (Hed *et al*., 1987). The association of FITC-zymosans on the cell surface was evaluated by subtracting the value of net translocated fluorescence intensity from total fluorescence intensity (Kim *et al*., 2003).

Determination of superoxide

For the measurement of extracellular superoxide, J774 cells (2×10^3) were harvested and washed 3 times with PBS. And then the cells were resuspended in 1 ml of modified Krebs-Ringer buffer (KRG: 120 mM NaCl, 5 mM KCl, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, 10 mM glucose, 1 mM $CaCl_2$, and 1.5 mM $MgCl_2$) containing 4 U/ml HRP and 0.05 mM isoluminol (Dahlgren and Karlsson, 1999). For the measurement of intracellular superoxide, J774 cells (2×10^3) were harvested and washed 3 times with PBS, and resuspended in 1 ml of KRG buffer containing 50 U/ml SOD, 2000 U/ml catalase and 0.05 mM luminol (Dahlgren and Karlsson, 1999). In both cases, the reaction of superoxide formation was started by addition of opsonized zymosan particles (2×10^4), and the subsequent chemiluminiscence was measured by Luminometer (Lumat LB 9507, EG & G, Berthold, Germany). To examine the effect of Tat-C3 on the formation of superoxide, purified Tat-C3 (10 g/ml) was pretreated to the J774 cell for 30 min at 37°C (Kim *et al*., 2003).

Expression and purification of GST-RhoA and GST-Rac1 fusion proteins, and measurement of GTP-binding to the fusion proteins, and of superoxide formation induced by GST-RhoA-GTP

For the preparation of GST-RhoA and GST-Rac1, pGEX4T1-RhoA, and pGEX4T1-Rac1 plasmids were transformed into *E. coli* DH5α and grown. And then the expressions of proteins were induced by the addition of 0.1 mM IPTG as the preparation of Tat-C3. GST-RhoA and GST-Rac1 proteins were purified using affinity of GSH-Sepharose beads to GST-fusion proteins. The proteins were eluted with 5 mM GSH and the GSH was eluted out by dialysis against a buffer (50 mM Hepes, pH 7.4, 1 mM DTT, 2 g/ml aprotinin and 2 g/ml leupeptin). For the binding of GTP to the fusion proteins, 1 g GST-RhoA and GST-Rac1 were incubated with 0.1 M [γ-^{35}S] GTP in 50 l of GTP-binding buffer (10 mM Hepes, pH 7.4, 0.5 M $MgCl_2$, 1 mM DTT, and 1 mM DMPC) containing 30 g BSA as a carrier protein for 10 min at 30°C following Kikuchi's method with a little modification (Kikuchi *et al.,* 1988). The GTP-binding activities were stopped by addition of 1 ml of ice-cold stop buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, and 25 mM $MgCl_2$). The reaction mixtures were filtered immediately on a BA85 membrane (Schleicher & Schuell) and the membranes were washed 5 times with 1 ml of cold stop buffer. Radioactivity of dried membranes in 5 ml cocktail (Beckman, Ready Safe) was measured by using a liquid scintillation counter (Beckman LS5000TD). For the measurement of superoxide formation induced by RhoA *in vitro*, various concentration of GST-RhoA was incubated in the presence of 0.1 mM GDP or GTP in 1 ml KRG buffer containing 50 M luminol with cell lysates which were prepared by sonication. The reaction of superoxide formation was started by addition of 0.1 mM NADPH, and the subsequent chemiluminiscence was measured using Luminometer.

Translocation of RhoA and $p47^{PHOX}$ proteins

J774 macrophages (2×10^6 cells) were treated with 50 M LY294002, 50 M PD98059, 30 M SB203580, and 10 g/ml Tat-C3 for 30 min at 37°C. The cells were harvested and lysed with a sonication in 50 l lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaF, 2

mM $NaVO_4$, 20 mM $Na_4P_2O_7$, 50 g/ml PMSF, 2 g/ml aprotinine, 2 g/ml leupeptin, 2 g/ml pepstatin A, and 0.05% Triton-X 100) (Hippenstiel *et al.,* 1998). The cell membranes were harvested by a table-top ultracentrifuge for 30 min at 100,000 × *g*, 4°C, and the membranes were resuspended in 50 l the lysis buffer. For the measurement of translocation of $p47^{PHOX}$, both the membrane and the supernatant solution were run on 14% SDS-PAGE, and Western blot was performed by using anti-$p47^{PHOX}$ antibody.

GST pull-down assay for activated RhoA, Rac1, and Rac2

Briefly, a total of 2 × 10^6 cells cultured in 100-mm plates were washed in ice-cold PBS and harvested. The cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 500 mM NaCl, 10 mM $MgCl_2$, 5 g/ml each of leupeptin and aprotinin, and 1 mM PMSF). After centrifugation (15,000 rpm, 15 min, 4°C), aliquots of the supernatant were added to GST-Rho binding domain of Rhotekin (GST-RBD), or GST-GTPase binding domain of p21-activated kinase-1 (PAK-1) (GST-PBD), which was previously incubated for 1 h with 50 g of GSH-Sepharose beads for GST-fusion protein or Ni-NTA His-Bind resins for His-fusion protein. The beads were incubated with cell lysates and washed, and the proteins on the beads were run on SDS-PAGE. RhoA, Rac1, and Rac2 were determined by Western blotting using each antibody (Ren *et al*., 2000; Kim *et al*., 2004).

Scanning electron microscopy (SEM)

Control J774 cells were collected from the Petri dishes, centrifuged (1,000 × *g* for 5 min) and fixed in suspension with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 20 min. After washing twice with the same buffer, the cells were seeded on glass coverslips coated with fibrinogen for 1 h at room temperature. Cells were challenged with SOZ or IOZ on glass coverslips for 30 min and also fixed with 2.5% glutaradehyde for 20 min at room temperature and washed twice with the 0.1 M cacodylate buffer. All samples were post-fixed in 1% osmium tetroxide for 30 min at room temperature, dehydrated through graded ethanols, critically dried in CO_2 and gold coated by sputtering. The samples were examined with a Cambridge 360 scanning electron microscope.

Expression and purification of GST-$p47^{PHOX}$, and kinase assay of recombinant GST-$p47^{PHOX}$ fusion protein

Recombinant GST- $p47^{PHOX}$ fusion protein as a substrate for kinase was prepared following the procedure described by Park and Babior (Park and Babior, 1997). Briefly, *E. coli* transformed with pGEX-1λT containing an insert of $p47^{PHOX}$ cDNA were grown in the presence of 1 mM IPTG and lysed. The

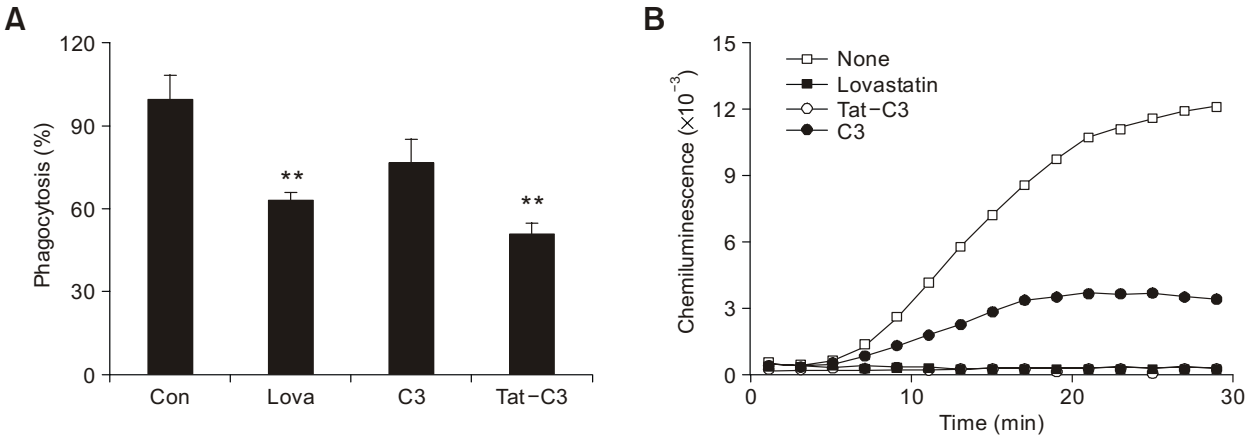

Figure 1. Involvement of Rho in the superoxide formation during phagocytosis of SOZ particles in macrophages. J774 cells (2 × 10^5) were treated with 50 M lovastatin, 50 g/ml of C3, 10 g/ml Tat-C3 for 1 hr at 37°C. For the phagocytosis, the cells were incubated with 5 x 10^5 of FITC-conjugated zymosan particles previously opsonized in fresh serum for 30 min. The phagocytosis was assayed by measuring the intensity of fluorescence of FITC bound to zymosan by using a fluorescence spectrophotometer. The fluorescence of adherent FITC-zymosan on the surface of the cells was quenched by adding crystal violet to 10 M of final concentration. The values were means ± SE (n = 3, $^{**}P < 0.01$) (A). For the measurement of intracellular superoxide, J774 cells (2 × 10^3) were resuspended in 1 ml of KRG buffer containing 50 U/ml SOD, 2000 U/ml catalase and 50 M luminol. The reaction of superoxide formation was started by addition of opsonized zymosan particles (2 × 10^4), and the subsequent chemiluminiscence was measured by using Luminometer. 50 g/ml of C3 (●), 10 g/ml purified Tat-C3 exoenzyme (○), 50 M lovastatin (■), and none (□) were pretreated to the J774 cells for 30 min at 37°C (B).

GST-p47^{PHOX} fusion protein in the cell lysate was purified on GSH-Sepharose. The purity and identity of the fusion protein were assayed by 10% SDS-PAGE and immunoblotting. Kinase assay of recombinant GST-p47^{PHOX} fusion protein was performed following Yammamori's method (Yammamori *et al*., 2000). Briefly, macrophages (2×10^7) in Hanks' balanced salt solution containing 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$ (HBSS+) were incubated for 5 min at 37°C and then stimulated with 1 mg/ml of SOZ for 15 min at 37°C. The reaction was stopped by centrifugation and the cells were suspended in 50 l of ice-cold lysis buffer (20 mM Hepes, pH 7.7, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100, 0.5 mM DTT, 20 mM L-glycerophosphate, 0.1 mM Na_3VO_4, 2 g/ml of leupeptin, and 100 g/ml PMSF). Cells were then lysed by sonication and the extracts were obtained by centrifugation. The extracts were mixed with 75 l of GSH-sepharose suspension to which 15 g of GST-p47^{PHOX} was bound, and rotated for 3 h at 4°C. After the sepharose beads were washed four times with Hepes binding buffer (20 mM Hepes, pH 7.7, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, and 0.05% Triton X-100), they were resuspended in 30 l of kinase buffer (20 mM Hepes, pH 7.6, 20 mM $MgCl_2$, 20 mM L-glycerophosphate, 0.1 mM Na_3VO_4, and 2 mM DTT) containing 20 M ATP and 10 Ci of [γ-^{32}P]ATP. After incubation for 20 min at 30°C, the beads were washed with Hepes binding buffer for stopping kinase reaction. Laemmli sample buffer was added to the beads, which were boiled to separate GST-p47^{PHOX} from them. The separated GST-p47^{PHOX} was subjected to 10% SDS-PAGE. After drying the gel, autoradiography of phosphorylated GST-p47^{PHOX} was performed.

Results

Intracellular formation of superoxide during phagocytosis of SOZ reached maximal extent level in 30 min when it was measured with luminol (Figure 1B). However, the formation of extracellular superoxide, which was measured with isoluminol, was delayed in comparison with that of intracellular superoxide (data not shown). To attempt to confirm whether Rho is involved in the superoxide formation, the effect of the inhibition of Rho activity of J774 macrophages on superoxide formation was examined. First the macrophages were treated with C3 that specifically inhibit Rho, and with Tat-C3 that was readily transduced into cells resulting in specific inhibition of Rho. Both C3 and Tat-C3 inhibited superoxide formation in macrophages induced by SOZ particles, but Tat-C3 much more efficiently inhibited it (Figure 1A) since C3 may have a limitation for the translocation into the cells (Park *et al*., 2003). Tat-peptide itself did not inhibit the superoxide formation (data not

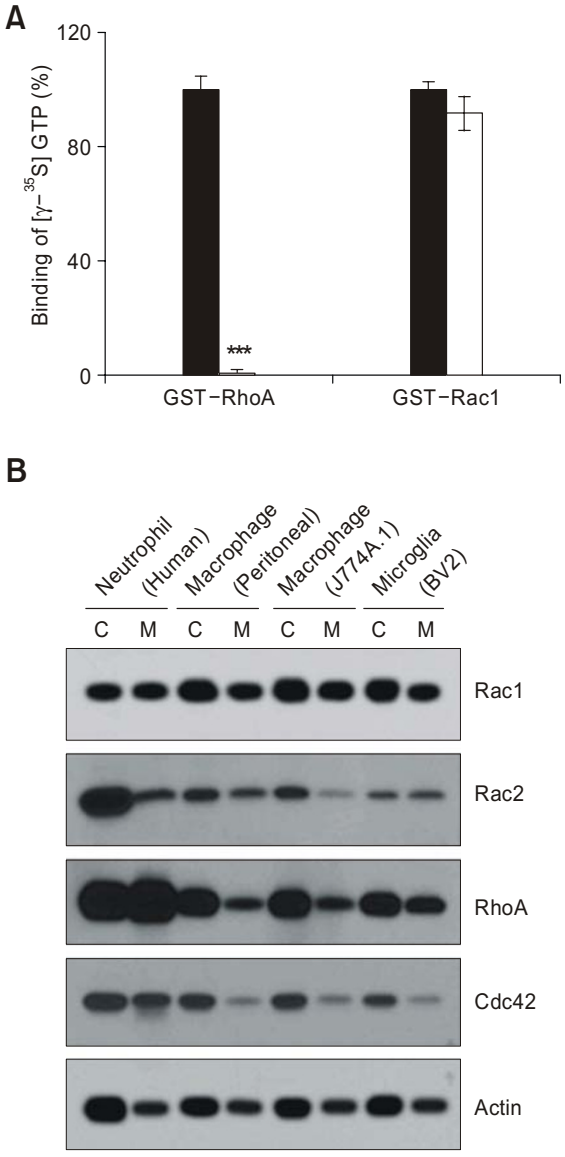

Figure 2. Inhibition of RhoA by Tat-C3 and expression of Rho GTPases in the leukocytes cell types. For ADP-ribosylation of GST-fusion proteins, 10 g/ml of Tat-C3 (□) or none (■), 3 M NAD^+, 6 M GDP, 1 g/ml GST-RhoA or GST-Rac1 in 50 l buffer were premixed on ice. ADP-ribosylation reaction was started with the addition of the GST-RhoA protein or -Rac1 and incubated for 1 h at 37°C. For the binding of GTP, GST-RhoA and -Rac1 were incubated with 0.1 M [γ-^{35}S]-GTP or in 50 l of GTP-binding buffer at 30 °C for 10 min. The GTP-binding activities were assayed by membrane filtering methods. The values were means ± SE (n = 3, ***P < 0.01) (A). Immunoblot was performed with extracts prepared from freshly isolated neutrophils (human), macrophage (peritoneal or J774A.1 cell line), and microglia (BV2 cell line), by using anti-Rac1, anti-Rac2, anti-RhoA, anti-Cdc42 or anti-Actin antibody, as indicated (B).

shown). Moreover, lovastatin that inhibits the synthesis of prenyl group also inhibited superoxide formation. It has been known that RhoA GTPase has a geranygeranyl group at Cys residue of C-terminus (Glomst and Farnsworth, 1994). These results indicate that Rho is essential in the superoxide formation induced by SOZ particles.

To eliminate the possibility that Tat-C3 inhibits Rac1, the GTP-binding activity of RhoA and Rac1 was assayed with or without pretratment of Tat-C3. Tat-C3 completely inhibited GTP-binding to GST-RhoA, whereas it did not abrogate GTP-binding to Rac1, suggesting that Tat-C3 specifically inhibits RhoA activity. In addition to the evidence that Tat-C3 did not inhibit Rac1, we compared the relative amounts of Rac1 and Rac2 in several cells and cell lines. Apparent Rac1 amount seems to be higher in J774 cells than in neutrophils, whereas Rac2, RhoA and Cdc42 seem to be higher in neutrophils than in J774 cells, peritoneal macrophages, and BV2 cells (Figure 2).

To confirm directly whether Rho is involved in the formation of superoxide *in vitro*, macrophage cell lysates were incubated with the purified recombinant GST-RhoA in the presence of GDP or GTP. It was found that GST-RhoA stimulated the superoxide formation in the concentration-dependent manner in the presence of GTP, whereas it did not in the presence of GDP (data not shown). In addition, RhoA was translocated to the membranes during phagocytosis of SOZ particles (Figure 3A), suggesting that RhoA may be involved in the superoxide formation and be activated during phagocytosis of SOZ particles. As controls, localization of RalA and caveolin on cell membranes was not changed in response to phagocytosis of SOZ or treatments of Tat-C3 in contrast to RhoA (Figure 3B). Both SOZ and IOZ activated RhoA, Rac1, and Rac2, and the time courses of their activation were a little different: active RhoA and Rac1 were continuously increased, whereas Rac2 was trended to be activated in early time, and sustained (Figure 3C). We assessed the morphology of macrophages by scanning electric microscope to test the relevance of RhoA to an interaction of macrophages with SOZ particles. SOZ induced well-developed dorsal membrane ruffles, whereas Tat-C3 blocked it and induced spreading of cells resulting in attachment of peripheral mem-

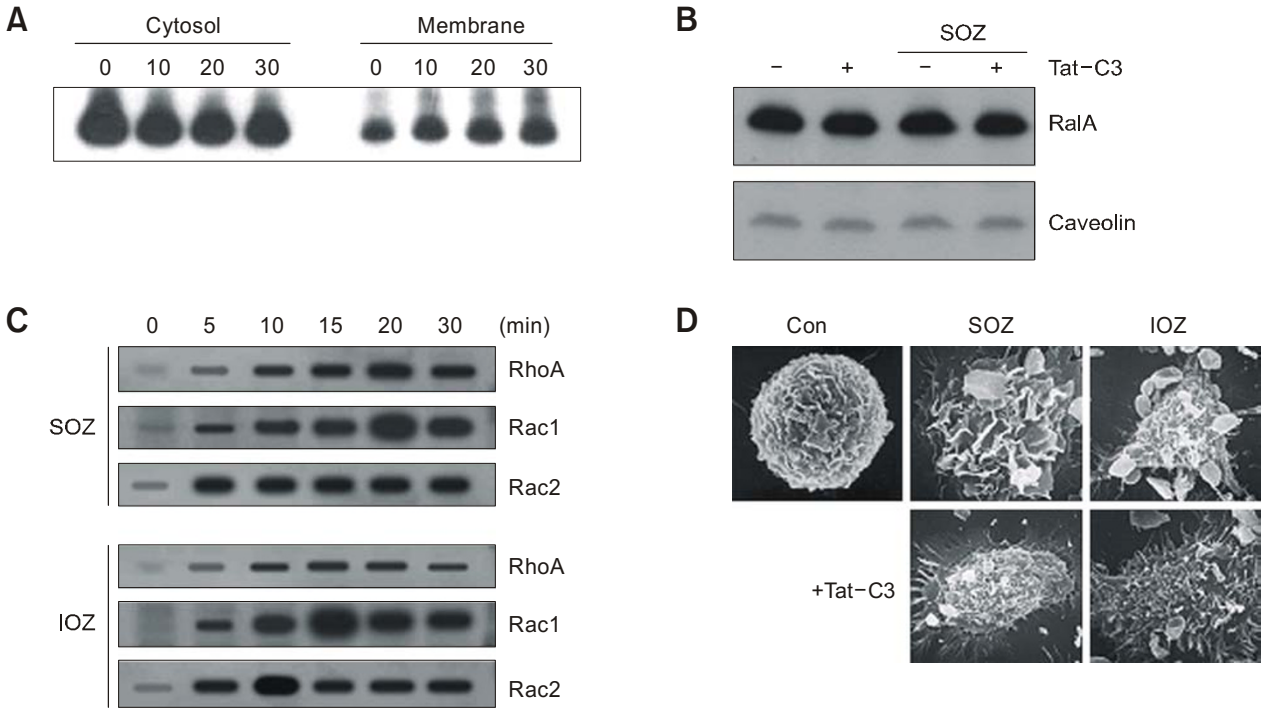

Figure 3. Activation of Rho GTPases during phagocytosis of SOZ or IOZ particles in macrophages. To measure the translocation of RhoA, the cells of phagocytosis were ruptured by using a sonicator, and the membrane and cytosolic fractions were separated using ultracentrifugation at 100,000 × *g*, 4°C for 30 min. RhoA of the membranes and the cytosol were detected by Western blot by using anti-RhoA (A). RalA and caveolin of membrane fraction were measured by using anti-RalA and anti-caveolin antibodies after phagocytosis of SOZ for 30 min in macrophages pretreated with or without 10 g/ml Tat-C3 for 30 min (B). Macrophages (2×10^5 cells) were challenged with SOZ or IOZ particles (5×10^5) for indicated times. Cells were harvested and lysed with a buffer containing 1% Triton X-100. Activated Rho GTPases levels were detected by GST pull down assay and Western blot (C). SEM picture of microphages were challenged with SOZ or IOZ. 10 g/ml Tat-C3 were pretreated to the J774A.1 cells for 30 min (D).

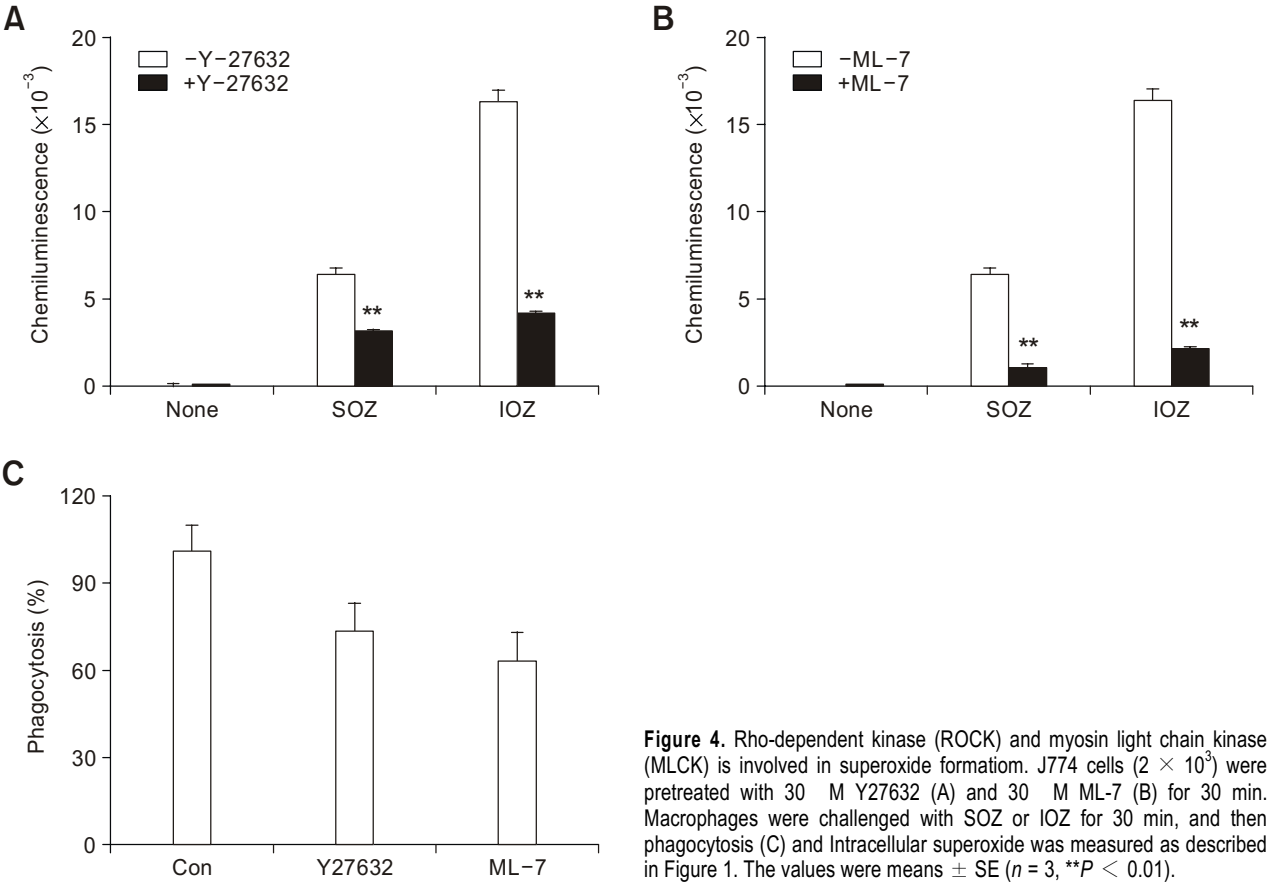

Figure 4. Rho-dependent kinase (ROCK) and myosin light chain kinase (MLCK) is involved in superoxide formatiom. J774 cells (2×10^3) were pretreated with 30 M Y27632 (A) and 30 M ML-7 (B) for 30 min. Macrophages were challenged with SOZ or IOZ for 30 min, and then phagocytosis (C) and Intracellular superoxide was measured as described in Figure 1. The values were means ± SE (n = 3, $^{**}P < 0.01$).

branes to substratum. On the other hand, IOZ did not induce membrane ruffles, and Tat-C3 induced more number of membrane protrusions (Figure 3D).

Next, we tried to reveal the downstream component to Rho A in the regulation of superoxide production. It was well known that RhoA-activated kinase (ROCK) is a typical effector protein of RhoA and Y-27632 is a specific inhibitor of ROCK. Y-27632 inhibited supeoxide formation, which was stimulated by both SOZ and IOZ, by about 50 and 75%, respectively (Figure 4A). In addition, ML7, a specific inhibitor of myosin light chain kinase (MLCK) also inhibited superoxide formation, which was stimulated by both SOZ and IOZ particles, by about 85% (Figure 4B). Both ROCK and MLCK phosphorylate myosin light chain, leading to actomyosin assembly and contraction of cells. Thus we assessed effects of Y-27632 and ML-7 on phagocytosis to reveal the correlation of phagocytosis with superoxide production. Y-27632 and ML-7 inhibited phagocytosis of SOZ by about 30 and 35%, respectively. This suggests that the reduction of superoxide by Y-27632 and ML-7 is partially dependent on the reduction of phagocytosis. ROCK and MLCK may be also involved in direct regulation of superoxide generation in that the inhibition extent of superoxide production by the inhibitors was greater than inhibition of phagocytosis.

Thus we attempted to elucidate how Rho is implicated with the superoxide formation during phagocytosis. PMA [an activator of protein kinase C (PKC)] stimulated the phagocytosis and superoxide formation, whereas PD98059 [an inhibitor of MAP kinase kinase (MEK)], SB203580 [an inhibitor of p38 mitogen-activated protein kinase (MAPK)], and LY294002 [an inhibitor of phosphatidyl inositol-3 kinase (PI3K)] significantly inhibited the phagocytosis (Figure 5A). Additionally, the superoxide formation was seriously inhibited at the same concentration of inhibitors as the phagocytosis experiments were performed (Figure 5B), which is in accord with previous report (Yamamori *et al*., 2000). These indicate that PKC, extracellular signaling-regulated kinase (ERK1/2), PI3K, and p38 MAPK are relevant to the superoxide formation during phagocytosis. Thus, we attempted to elucidate which MAPK among ERK1/2, p38 MAPK, and c-Jun N-terminal kinase (JNK) was critical for the superoxide formation concerning the linkage of Rho to these intermediate molecules. It was found that Tat-C3

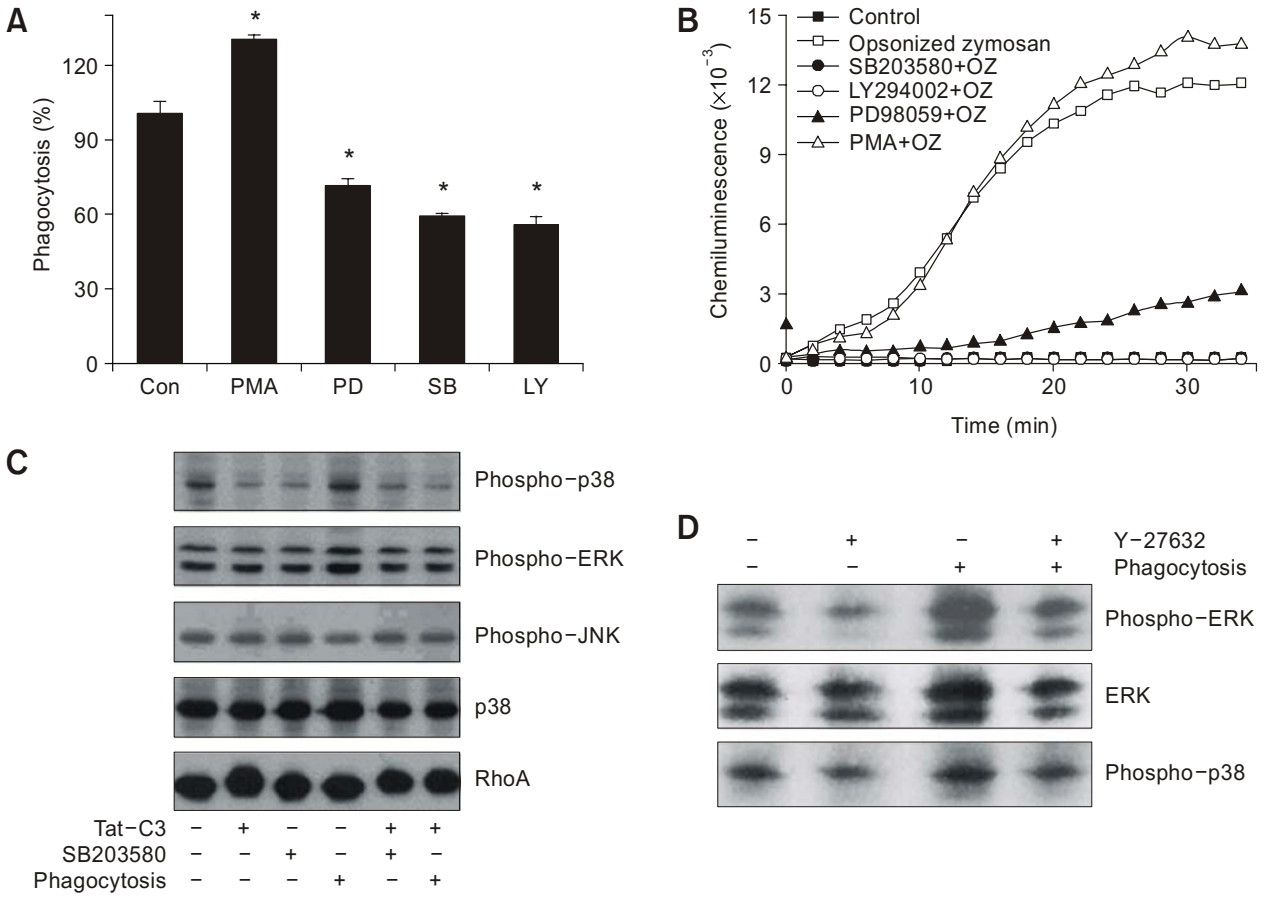

Figure 5. Implication of p38 MAPK and ERK1/2 to superoxide formation induced by RhoA. J774A.1 cells were pretreated with 10 nM PMA, 50 M PD98059, 30 M SB203580, and 50 M LY294002 for 30 min, and phagocytosis was performed. The phagocytosis of SOZ particles was measured as in Figure 1. The values were means ± SE (n = 3, *P < 0.05) (A). The superoxide formation during phagocytosis was measured from the cells pretreated with the same concentration as in (A) of PMA (△), PD98059 (▲), SB203580 (●), LY294002 (○), and none (■) (B). J774 cells were pretreated with 10 g/ml of Tat-C3 exoenzyme or 30 M SB203580 for 30 min, and then phagocytosis was performed. The cell lysates (20 g protein) were run on SDS-PAGE and Western blots were performed by using anti-p38, -phospho-p38, -phospho-ERK, -phospho-JNK, and -RhoA antibodies. It was observed that Tat-C3 exoenzyme retarded the mobility of RhoA on SDS-PAGE (C). J774 cells were pretreated with 50 M Y-27632 for 30 min, and then phagocytosis was performed. The cell lysates (20 g protein) were run on SDS-PAGE and Western blots were performed by using, anti-phospho-ERK, -ERK1/2, -phospho-p38 MAPK antibodies (D).

strongly inhibited the phosphorylation of p38 MAPK, and slightly that of ERK1/2, but not JNK (Figure 5C), suggesting that p38 MAPK may be mainly involved in the superoxide formation mediated through Rho during phagocytosis of SOZ particles. It was found that the mobility of RhoA from the cells treated with Tat-C3 was retarded on SDS-PAGE, indicating that Tat-C3 modified the RhoA (Figure 5C), whereas Tat-C3 did not change mobility of RalA and caveolin as controls (Figure 3B). In addition, Y-27632 blocked the activation of both ERK1/2 and p38 MARK, indicating that ROCK is upstream to ERK1/2 and p38 MARP (Figure 5D).

Since p38 MAPK and ERK have been reported to regulate superoxide formation by phosphorylating $p47^{PHOX}$, a cytosolic component of NADPH oxidase (El Benna *et al*., 1996), we attempted to reveal a hierarchy of RhoA and other kinases tested in the superoxide formation mediated through RhoA. SOZ induced translocation of RhoA from cytosol to membrane, suggesting that SOZ activates RhoA. Consistently, Tat-C3 blocked the translocation of RhoA, whereas SB203580 and PD98059 and LY294002did not significantly block it (Figure 6A), indicating that RhoA may be an upstream component of p38MAPK, ERK1/2, and PI3K. However, Tat-C3 did not affect the translocation of Rac1 (Figure 6B). Then the phosphorylation of $p47^{PHOX}$ was assayed by incubating GST-$p47^{PHOX}$, [γ-^{32}P]ATP, and the lysates from the J774 cells performing phagocytosis. The lysates from the cells excuting phagocytosis of SOZ stimulated the phosphorylation of $p47^{PHOX}$, whereas

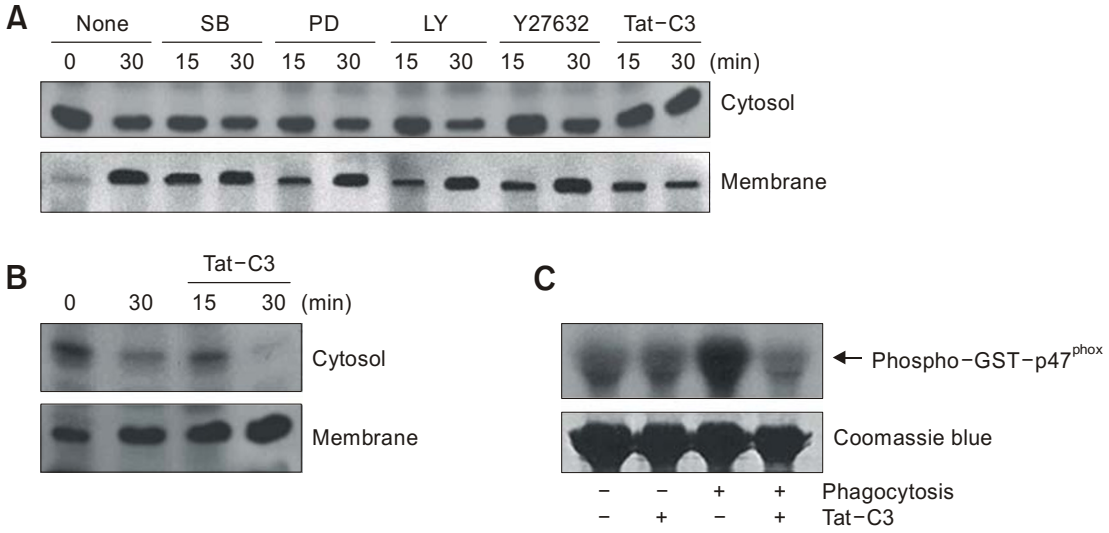

Figure 6. Inactivation of GST-p47^{PHOX} by Tat-C3 exoenzyme. J774 macrophages (2 × 10^6 cells) were treated with 50 M LY294002, 50 M PD98059, 30 M SB203580, and 10 g/ml Tat-C3 exoenzyme for 30 min at 37°C. The cells were harvested and lysed with a sonication in 50 l buffer. The cell membranes were harvested and the membranes were resuspended in 50 l buffer. Both the membrane and the supernatant solution were run on 14% SDS-PAGE, and Western blot was performed by using anti-RhoA (A) and anti-Rac1 antibodies (B). Macrophages (2 × 10^6 cells) were stimulated with or without SOZ (5 × 10^6) after cells were treated with or without 10 g/ml Tat-C3. The cells were then ruptured by sonication, and the cell extracts were mixed with GSH-Sepharose suspension to which GST-p47^{PHOX} was bound. The mixture was rotated for binding of GST-p47^{PHOX} and GSH-beads, and the washed beads were in 30 l of kinase buffer containing 10 Ci of [γ-^{32}P]ATP. The phosphorylated GST-p47^{PHOX} was analyzed on 10% SDS-PAGE and autoradiography (C).

the lysates from the cells pretreated with Tat-C3 heavily inhibited the phosphorylation p47^{PHOX} (Figure 6C).

Discussion

Rho is essential for the superoxide formation in macrophages

Rho proteins are Ras-related GTP-binding proteins that have been shown to regulate a variety of cellular functions such as cytoskeleton organization, membrane trafficking, transcriptional activation, cell growth control, and development (Hall A, 1998). Although Rac has been known to be essential for the superoxide formation in macrophage during phagocytosis (Kleinberg *et al*., 1990), there was a report supporting a possibility that RhoA exerts regulatory function in superoxide formation through NADPH oxidase during phagocytosis of SOZ (Kim *et al*., 2004). In this study, we attempted to reveal the downstream components of RhoA in signaling of superoxide generation, and observed that ROCK, MLCK, ERK1/2, p38MAPK were relevant to this process.

For this purpose, we used Tat peptide fused C3 exoenzyme (Tat-C3) to block RhoA. There was another possibility that Tat-C3 might also inhibit the Rac (Kreck *et al*., 1994), which has been reported to be a stimulatory factor for NADPH oxidase. To exclude this possibility, we compared the GTP-binding activities of RhoA and Rac1 when they were treated with Tat-C3 in the presence of NAD$^+$ *in vitro*, and found that Tat-C3 completely abolished the GTP-binding activity of only RhoA, but not that of Rac1 (Figure 2).

We found that Tat-C3, which inhibits specifically the activity of Rho, moderately inhibited the phagocytosis, but extensively suppressed the superoxide formation induced by sOZ particles (Figure 1). These results suggest that Rho is esentially required for superoxide formation in CR3-mediated process (Figure 1). This indicates that the inhibition of superoxide formation is not a consequence of the reduction of phagocytosis. Rho may regulate the superoxide formation at least partially independently of phagocytosis. However, it is also true that phagocytosis stimulates the superoxide formation in macrophages in that the stimulation of the receptors by C3bi protein ligands alone in macrophages did not induce the oxidative burst to full extent (Kim *et al*., 2004).

Additionally, it was shown that GST-RhoA stimulated the formation superoxide in the cell lysates and GTP (data not shown) although the stimulation extent was not high probably because of using

recombinant RhoA instead of prenylated RhoA. This suggests that RhoA may be in fact involved in the superoxide formation *in vitro*. However, it cannot be excluded to consider a possible role of RhoB and C for the involvement in superoxide formation, because C3 was also found to modify and inhibit both Rho B and C (Chardin *et al*., 1989; Just *et al*., 1995).

Conversely, RhoA was activated by the stimulation of SOZ: RhoA was translocated from cytosol to membrane, GTP-bound form of RhoA was well as both Rac1 and Rac2 was increased (Figure 3C). Furthermore, SOZ heavily induced dorsal membrane ruffles that might enclose and engulf the particles into inside of the cells (Figure 3D) (Park, 2003).

Next, we clarify whether superoxide produced by SOZ could increase control phagocytosis, since it was reported that reactive oxygen species (ROS), perhaps H_2O_2, acts as an intracellular signal mediator for NGF-induced neuronal differentiation and that NGF-stimulated ROS production is regulated by Rac1 and a flavoprotein-binding protein similar to the phagocytic NADPH oxidase (Suzukawa *et al*., 2000). In macrophages, superoxide scavenger or generator did not change the phagocytosis (data not shown; Kim *et al*., 2004), suggesting that superoxide does not act as an intracellular signal mediator for the phagocytosis.

Mechanism for the superoxide formation mediated through Rho in macrophages

To get a clue how RhoA is implicated in the superoxide formation during phagocytosis in macrophages, we examined the effects of the reagents that activate or inhibit specific steps in signal pathways. PKC was involved in stimulation of phagocytosis and association of SOZ particles in accordance with a previous report (Yamamori *et al*., 2000). Moreover, we found that p38 MAPK, ERK1/2, and PI3K were slightly involved in the phagocytosis process. In addition, the stimulation of PKC increased the superoxide formation, whereas the inhibition of ERK1/2 significantly reduced the superoxide formation, furthermore the inhibition of p38 MAPK and PI3K almost abolished it (Figure 5B). It could be suggested that p38 MAPK and PI3K are essential for the superoxide formation, and that phagocytosis and superoxide formation are performed through different signal-pathways. Furthermore, Tat-C3 heavily inhibited the phosphorylation of p38 MAPK and slightly that of ERK1/2, suggesting that p38 MAPK and ERK1/2 may be involved in the formation of superoxide mediated though Rho (Figure 5C).

It was found that the onset of respiratory burst during phagocytosis was linked to the phsophorylation of $p47^{PHOX}$ and its trnaslocation to the phagosome (DeLeo *et al*., 1999). p38 MAPK was known to activate $p47^{PHOX}$ by phosphorylation (Yamamori *et al*., 2000; Herlaar and Brown, 1999). The lysates from the cells executing phagocytosis of SOZ stimulated the phosphorylation of GST-$p47^{PHOX}$, and those from the cells treated by Tat-C3 blocked phosphorylation of GST-$p47^{PHOX}$ (Figure 6C), suggesting that Rho is related to the phosphorylation of $p47^{PHOX}$. The inhibition of $p47^{PHOX}$ through Tat-C3 appears to subsequently diminish the superoxide formation. Consequently, the sequence of 'Rho → ROCK → p38 MAPK → ERK1/2 (→ ?) $p47^{PHOX}$ → superoxide' can be considered as a signal pathway of the activation of superoxide formation.

Cytoskeleton reorganization may be involved in the superoxide formation. Inhibition of superoxide formation by cytochalasin D (data not shown) suggests a necessary role of cytoskeleton in the signaling pathway that activates the oxidase (Serrander *et al*., 1999). RhoA has been known as an activator for ROCK, which phosphorylates myosin light chain (MLC) phosphatase to be inactive (Kawano *et al*., 2000), resulting in the formation of stress fiber. ML7, an inhibitor of MLCK, inhibited that superoxide formation and phagocytosis in our system (Figure 4) and in other laboratories (Kimura *et al*., 1996; Mansfield *et al*., 2000), suggesting that cytoskeleton reorganization is important for the superoxide formation, although it has to be also considered that the inhibition of superoxide formation by ML7 may arise from the reduction of the phagocytosis. Moreover, ML7 was known to reduce the phosphorylation and the translocation of $p47^{PHOX}$ to the membranes (Heyworth *et al*., 1995).

RhoA has been also known to be an activator of phospholipase D (PLD) (Malcolm *et al*., 1994). Thus, the possibility of the formation of phosphatidic acid (PA) formation in the membranes could be a regulator of superoxide formation, for example, a regulator of translocation of NADPH oxidase. It was reported that Rac/RhoGDI complex can be disrupted in the presence of various lipids like arachidonic acid, PA, and phosphatidylinositol (Chuang *et al*., 1993). We found that the addition of exogenous PLD to the macrophages induced the enhancement of the superoxide formation (results not shown), suggesting that PLD activity is required for the superoxide formation. It has been shown that $p22^{PHOX}$ can be phosphorylated through PLD, suggesting that PLD activity is required for the superoxide formation (Regier *et al*., 2000). Moreover, the activation of PLD is tightly coupled to the phagocytosis of opsonized zymosan by human macrophages (Fallman *et al*., 1993; Kusner *et al*., 1996).

Despite of such multiple events associated to superoxide formation, it remains to be studied in detail how Rho regulates the processes including the activations of p38 MAPK and ERK1/2, and the phosphorylation of $p47^{PHOX}$.

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