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Original Contribution

Small GTPases Rap1 and RhoA regulate superoxide formation by Rac1 GTPases activation during the phagocytosis of IgG-opsonized zymosans in macrophages

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ABSTRACT

Phagocytic NADPH oxidase plays a critical role in superoxide generation in macrophage cells. Small GTPases, including Rac1 and Rac2, have been implicated in the regulation of NADPH oxidase activity. Rap1, which has no effect in a cell-free system of oxidase activation, recently has been proven to colocalize with cytochrome b_{558}. In addition, neutrophils from rap1A$^{-/-}$ mice reduce fMLP-stimulated superoxide production. Here, we tried to determine whether Rap1 also plays a role in the production of superoxide. IgG-opsonized zymosan (IOZ) particles treatment induced Rap1 activation and superoxide generation. Knock-down of Rap1 by si-Rap1 suppressed IOZ-induced superoxide formation. Sh-RhoA also reduced superoxide levels, but 8CPT-2Me-cAMP, an activator of Epac1 (a guanine nucleotide exchange factor (GEF) of Rap1), could recover the levels to the control value. When cells were stimulated by IOZ, Rap1 and Rac1 were translocated to the membrane, and then interacted with p22^{phox}. 8CPT-2Me-cAMP rescued sh-RhoA-induced reduction of the interaction between Rac1 and p22^{phox}, and enhanced lysophosphatidic acid (LPA)-induced increase of their interaction. Moreover, Rac1 activity was increased by both LPA and 8CPT-2Me-cAMP when treated with IOZ particles. Si-Vav2 impaired GTP-Rac1 levels in response to 8CPT-2Me-cAMP/IOZ. Phosphorylation of RhoA activates Rac1 in response to IOZ by the enhanced binding of phospho-RhoA to RhoGDI, leading to the release of Rac1 from the Rac1-RhoGDI complex. In conclusion, IOZ treatment induces Rap1 activation and phosphorylation of RhoA, which in turn cause Rac1 activation and promote Rac1 translocation to the membrane leading to binding with p22^{phox} that activates NADPH oxidase and produces superoxide.

Introduction

Professional phagocytes, including neutrophils and macrophages, engulf pathogens and damaged cells from an organism. After phagocytosis, there is an abrupt increase in superoxide formation in professional phagocytes known as the oxidative burst [1,2]. Oxidative burst is catalyzed by NADPH oxidase, which is a membrane-associated enzyme complex that generates superoxide by a one-electron reduction of oxygen, using NADPH as the electron donor [1]. The NADPH oxidase complex of phagocytes is composed of a catalytic subunit and regulatory subunits, including membrane-bound flavocytochrome b_{558} (a heterodimer of NOX2/gp91^{phox} and p22^{phox}) and cytosolic components such as p47^{phox}, p67^{phox}, and p40^{phox} [3–5]. Stimulation of cells by phagocytosis permits p47^{phox} to undergo extensive serine phosphorylation by a number of kinases, primarily protein kinase C [6], and then recruit the p40^{phox} and p67^{phox} complex. After they are assembled, cytosolic components of p47^{phox}, p40^{phox}, and p67^{phox} translocate to the membrane-associated cytochrome b_{558} [7]. In addition, the Ras-related small GTP-binding proteins Rac1/2 are essential for activating NADPH oxidase [8]. When Rac is activated, the active Rac also translocates to the membrane, associating with b_{558} and p67^{phox} [9,10]. Finally, the assembled NADPH oxidase complex is fully activated, leading to the generation of superoxide in the phagosomes.

Small GTPases are switch proteins that control a wide variety of signaling pathways in cells in response to extracellular stimulation. Depending on their structure, GTPase families have two

Abbreviations: DHE, dihydroethidium; DMEM, Dulbecco's modified Eagle medium; Epac, exchange protein directly activated by cAMP; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAP, GTPase activating proteins; GDI, guanosine nucleotide dissociation inhibitor; GDS, guanine nucleotide dissociation stimulator; GEF, guanine nucleotide exchange factor; GSH, glutathione; GST, glutathione *S*-transferase; IOZ, IgG-opsonized zymosan; LAP,, lysophosphatidic acid; MAPK, mitogen-activated protein kinases; PBD, Rac/Cdc42-binding domain of PAK; PAK, p21-activated kinase; PBS, phosphate-buffered saline; PKA, protein kinase A; PMSF, phenylmethanesulfonyl fluoride; RalGDS-RapBD, Rap-binding domain of Ral guanine nucleotide dissociation stimulator; RBD, Rho-binding domain of Rhotekin; ROCK, Rho-associated, coiled-coil containing protein kinase; SOZ, serum-opsonized zymosan.

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conformational states: the GTP-bound active state and the GDP-bound inactive state. Regulation of these two states is achieved by two protein families: guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). In addition, GDP dissociation inhibitor (GDI) binds to inactive GTPases in the cytosol to inhibit the exchange between GDP and GTP [11]. In particular, Rho GTPases, including RhoA, Cdc42, and Rac1, regulate cell morphology governed by dynamic changes in cytoskeletal structures. It was reported that RhoA participates in integrin αMβ2 (CD11b/CD18, MAC-1)-mediated phagocytosis of C3bi-opsonized particles and that Cdc42 and Rac1 participate in Fcγ receptor-mediated phagocytosis of IgG-opsonized particles [2,12]. Furthermore, the RhoA protein is also involved in superoxide generation during the phagocytosis of IgG-opsonized zymosan (IOZ) particles [13]. Besides RhoA and Rac1/2, another small GTPase, Rap1, seems to be involved in a more complicated regulation of superoxide production. Whereas specific cytosolic protein, $p47^{phox}$, $p67^{phox}$, and Rac are necessary to activate the NADPH oxidase in a cell-free system [14,15], Rap1A has no effect on the activation of NADPH oxidase in a cell-free system [16]. However, other studies that were performed on intact cells demonstrated that Rap1A is colocalized with cytochrome b_{558} both in the plasma membrane and in some specific granule membranes, even in unstimulated human neutrophils [17,18]. More than half of the specific granule-associated Rap1A translocates to the plasma membrane in PMA-stimulated cells [18]. Rap1A can be phosphorylated at its Ser 180 residue by cAMP-dependent protein kinase (PKA). This phosphorylation can disrupt its interaction with cytochrome b_{558} [19]. In addition, Maly et al. found that both constitutively active and dominant-negative mutants of Rap1A inhibited phorbol ester-stimulated superoxide generation [20], indicating that Rap1A serves its functions in a dynamic cycle as opposed to a unidirectional pathway; this is similar to another small GTPase, Rab, which directs trafficking between endocellular membranes [21]. In addition, Li recently found that neutrophils from *rap1A*$^{-/-}$ mice reduce fMLP-stimulated superoxide production as well as a weaker initial response to phorbol ester but increased Fc receptor-mediated phagocytosis [22]. The results obtained from *in vivo* studies complement *in vitro* observations showing that although Rap1A cannot support NADPH oxidase in a cell-free system, Rap1 may play an essential role in NADPH oxidase-dependent superoxide generation *in vivo*. Here, we confirmed that Rap1 indeed participates in IOZ particle-induced superoxide generation. Moreover, Rap1 and RhoA have complementary or additive functions in regulating superoxide production by activating Rac1, subsequently increasing the translocation and binding of Rac1 to NADPH oxidase, including the $p22^{phox}$subunit.

Materials and experimental procedure

Plasmids and reagents

Zymosan A particles, phenylmethylsulfonyl fluoride (PMSF), Triton X-100, luminol, 8CPT-2Me-cAMP, dihydroethidine, and lysophosphatidic acid (LPA) were purchased form Sigma Chemicals (St. Louis, MO). Protein A-agarose beads were from Thermo Scientific (Rockford, IL). IgG against zymosan was from Molecular Probes (Eugene, OR). Scrambled control si-RNA, si-Rap1, and si-Epac1 (exchange protein directly activated by cAMP) were from Bioneer (Taejeon, Korea). Sh-RhoA and scrambled control sh-RNA were constructed in plasmids following the protocol of a previous report [23]. Scrambled control si-RNA and si-Vav2, si-Tiam1, anti-RhoA, anti-$p22^{phox}$, anti-Rap1, anti-RhoGDIα, anti-Vav2, and anti-Tiam1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Rac1 antibody was purchased from Upstate (Lake Placid, NY). The anti-Epac1 antibody was purchased from Cell Signaling (Beverly, MA, USA). The anti-phospho-RhoA (phospho-Ser188) antibody was purchased from Abcam (Cambridge, MA).

Cell culture

Mouse macrophage Raw264.7 cells were cultured in Dulbecco's modified Eagle medium-F12 (DMEM-F12) containing 5% (v/v) fetal bovine serum (FBS) and antibiotics (100 units/ml streptomycin and 100 units/ml penicillin) at 37 °C in 5% (v/v) CO_2.

Determination of superoxide

To measure intracellular superoxide, Raw264.7 cells (2×10^3) were harvested, washed three times with phosphate-buffered saline solution (PBS), and resuspended in 1 ml of modified Krebs–Ringer glucose (KRG) buffer (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 50 μM luminol [24]. The reaction was started by adding opsonized zymosan particles (2×10^4), and the chemiluminescence generated was measured with a luminometer (Lumat LB 9507, EG&G, Berthold, Germany).

Transfection of Raw264.7 cells

Raw264.7 cells were transfected using Attractene transfection reagent according to the manufacturer's instructions (Qiagen). In brief, cells were seeded 24 h before transfection at a density of 3×10^5 cells per well in 6-well plates. For transient transfection, 4.5 μl of reagent was mixed with 100 nM si-RNA, 1.2 μg sh-RNA, or 1.2 μg DNA plasmid in 100 μl of serum-free medium. The complexes were incubated at room temperature for 15 min and were then added to the cells. Tests of the transfection were performed 24 h after transfection with sh-RhoA or 72 h after transfection with si-RNA.

Phagocytosis assay

Cells were seeded in 6-well dishes at a density of 2×10^5 cells. After incubating in DMEM/F-12 medium without FBS overnight, cells were treated with fluorescein isothiocyanate (FITC)-conjugated IOZ particles (5×10^5) for 30 min. After the cells were washed and resuspended with PBS, phagocytoses were determined by measuring the fluorescence intensity of the cells at an excitation wavelength of 490 nm and an emission wavelength of 520 nm (Kontron SFM25 Spectrometer) [25].

Translocation assay

Raw264.7 cells were cultured in 100-mm dishes and treated with IOZ particles for 30 min. After being washed with PBS and resuspended, cells were lysed and the cytosolic and membrane fractions were prepared with a ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) according to the manufacturer's instructions. Western blotting with anti-Rac1 and anti-Rap1 antibodies was employed to measure the translocation of Rac1 and Rap1.

GST pull-down assay for activated RhoA, Rac1, and Rap1

A GST pull-down assay was conducted as previously described [26,27]. Briefly, a total of 2×10^6 cells cultured in 100-mm plates was washed with ice-cold PBS and harvested. The cells were lysed in a lysis buffer (25 mM Tris-HCl, pH 7.5,150 mM NaCl, 5 mM $MgCl_2$, 1% (v/v) NP-40, 1 mM DTT, and 5% (v/v) glycerol, 1 μg/ml each of leupeptin and aprotinin, and 1 mM PMSF). After centrifugation at 12,000 *g* for 20 min at 4 °C, aliquots of the supernatant were incubated with the GST-Rho-binding domain of Rhotekin (GST-RBD), the GST-GTPase-binding domain of p21-activated kinase-1 (PAK-1) (GST-PBD), or His-Rap-binding domain of Ral guanine nucleotide dissociation stimulator (GDS) (RalGDS-RapBD) that had been preincubated for 1 h with 50 μg of GSH-Sepharose beads for GST-fusion proteins or with Ni-NTA/His-Bind resins for the His-fusion protein. The beads were incubated with the cell lysates