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Rotenone Activates Phagocyte NADPH Oxidase through Binding to Its Membrane Subunit gp91^{phox}

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Abstract

Rotenone, a widely used pesticide, reproduces Parkinsonism in rodents and associates with increased risk for Parkinson's disease. We previously reported rotenone increased superoxide production through stimulating microglial phagocyte NADPH oxidase (PHOX). The present study identified a novel mechanism by which rotenone activates PHOX. Ligand-binding assay revealed that rotenone directly bound to membrane gp91^{phox}, the catalytic subunit of PHOX; such binding was inhibited by diphenyleiiodonium, a PHOX inhibitor with a binding site on gp91^{phox}. Functional studies showed both membrane and cytosolic subunits were required for rotenone-induced superoxide production in cell-free systems, intact phagocytes, and COS7 cells transfected with membrane subunits (gp91^{phox}/p22^{phox}) and cytosolic subunits (p67^{phox} and p47^{phox}). Rotenone-elicited extracellular superoxide release in p47^{phox}-deficient macrophages suggested rotenone enabled to activate PHOX through a p47^{phox}-independent mechanism. Increased membrane translocation of p67^{phox}, elevated binding of p67^{phox} to rotenone-treated membrane fractions, and co-immunoprecipitation of p67^{phox} and gp91^{phox} in rotenone-treated wild-type and p47^{phox}-deficient macrophages indicated p67^{phox} played a critical role in rotenone-induced PHOX activation via its direct interaction with gp91^{phox}. Rac1, a Rho-like small GTPase, enhanced p67^{phox}-gp91^{phox} interaction; Rac1 inhibition decreased rotenone-elicited superoxide release. In conclusion, rotenone directly interacted with gp91^{phox}; such an interaction triggered membrane translocation of p67^{phox}, leading to PHOX activation and superoxide production.

Keywords

rotenone; macrophages; NADPH oxidase; gp91^{phox}; superoxide; PHOX; NOX2; Rac1

Introduction

Parkinson's disease (PD) is a movement disorder characterized by a progressive degeneration of the nigrostriatal dopaminergic pathway [1]. Although its etiology remains unclear, many lines of evidence have indicated a close association of environmental

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exposure with this disease [2–6]. For example, a recent epidemiological study has reported that PD is positively associated with lifetime use of pesticide rotenone in a population with well-characterized pesticide exposure [7]. Rotenone replicates Parkinsonism features when chronically administered to rodents [8–11]. Mechanistically, rotenone impairs neuronal mitochondrial complex I [12, 13] and disrupts the microtubule-based transport of neurotransmitter vesicles [14, 15]. Recent evidence indicates that microglia, the resident immune cells in the brain, also participate in rotenone-induced neurodegeneration [16, 17]. We previously reported that microglial activation greatly augmented dopaminergic neurodegeneration induced by rotenone, especially at low doses [16]; the enhanced neurodegeneration elicited by rotenone was primarily mediated by microglial NADPH oxidase [18].

Phagocyte NADPH oxidase (PHOX, often referred to as NOX2), an activity-dependent enzyme complex, is widely expressed in various immune cells including microglia, macrophages, and neutrophils. It is the key extracellular superoxide-producing enzyme in phagocytes and plays a critical role in inflammation. Superoxide and its downstream products are highly reactive and they engage in diverse cellular processes. Excessive production of these free radicals increases peroxidation products that damage proteins, lipids, DNA, and RNA, leading to cell dysfunction and eventual death [19]. Oxidative stress has long been recognized as a central pathological event during chronic progression of neurodegenerative diseases including PD [20–22]. Ample evidence strongly suggests that over-activated PHOX plays a pivotal role in inflammation-mediated neurodegeneration [22, 23].

It is well established that the activation of PHOX requires assembling of its multiple subunits into a complex. Upon stimulation, cytosolic subunit p47^{phox} is phosphorylated, which initiates translocation of the cytosolic subunits (p47^{phox} and p67^{phox}) to the plasma membrane where they associate with the membrane component, flavocytochrome b558, to assemble a functional oxidase. Flavocytochrome b558 is a heme-containing heterodimer composed of gp91^{phox} and p22^{phox}. Cytosolic subunits p40^{phox} and two small G-proteins, Rac1 and Rap1A, are also involved in the activation process of PHOX [24–26]. Our previous studies demonstrated that the genetic ablation of gp91^{phox}, the catalytic subunit of PHOX, diminished rotenone-elicited extracellular superoxide release from microglia, thereby mitigating rotenone-induced neurodegeneration [18]. These findings lend strong support to an important role of PHOX activation in rotenone-mediated neurodegeneration. However, how rotenone activates this enzyme remains unknown. Here, we investigate the detailed molecular mechanisms underlying rotenone-induced activation of PHOX.

Materials and methods

Animals and cell cultures

Wild-type (C57BL/6J) and B6.129S6-Cybb^{tm1Din/J} (gp91^{phox}-deficient) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). B6.129S2-Ncf1^{tm1Shl} (p47^{phox}-deficient) mice and Fischer 334 rats were purchased from Taconic (Hudson, NY) and Charles River Laboratories (Wilmington, MA), respectively. Housing and breeding of the animals were performed in strict accordance with National Institutes of Health guidelines.

Wild-type and transfected monkey kidney COS7 cells were gifts from Dr. Mary Dinanuer (Indiana University, IN) and cultured as described previously [27, 28]. Briefly, wild-type COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 50 U/ml penicillin, and 50 µg/ml streptomycin (Sigma, St. Louis, MO). COS7 cells stably expressed with flavocytochrome b558 (COS-cytob558 cells) were maintained in the same medium

supplemented with additional 0.8 mg/ml genectin (Invitrogen) and 10 µg/ml puromycin (InvivoGen). COS7 cells stably expressed with flavocytochrome b558, p47^{phox}, and p67^{phox} (COS-phox cells) were maintained in the above DMEM medium containing 0.8 mg/ml genectin, 2 µg/ml puromycin, and 0.35 mg/ml hygromycin B (Invitrogen).

Peritoneal macrophage and neutrophil preparation

Resting peritoneal macrophages were obtained from the peritoneal cavity of rats or mice by using a modified method described previously [29]. The peritoneal cavities of animals were flushed with 5 ml of ice-cold RPMI medium 1640 (Invitrogen). After washed twice in RPMI, cells were preincubated in no-serum medium for 1 h. The cells were then washed twice to remove nonadherent cells. Adherent macrophages were cultured in DMEM containing 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin (Sigma) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Peritoneal neutrophils were prepared as described [30]. Briefly, mouse peritoneal neutrophils were harvested 16 h after the injection of 1 ml of phosphate buffered saline (PBS) containing 0.9% casein (Sigma). The peritoneal cells were washed twice with HBSS and cell pellets were stored in -80°C until use.

Measurement of extracellular superoxide and intracellular reactive oxygen species (iROS)

The release of superoxide was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of tetrazolium salt (WST-1, 2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulphophenyl]-2H-tetrazolium) as described [31]. Primary microglia (1×10^5 /well), peritoneal macrophages (1×10^5 /well), or various COS7 cells (5×10^4 /well) were grown overnight in 96-well plates in DMEM containing 10% FBS and switched to phenol red-free HBSS (100 µl/well). Fifty microliters of HBSS containing vehicle, rotenone, or phorbol 12-myristate 13-acetate (PMA) were then added to each well, followed by 50 µl of WST-1 (1 mM) in HBSS, with and without 600 U/ml SOD. The cells were incubated for 30 min at 37°C, and the absorbance at 450 nm was read with a SpectraMax Plus microplate spectrophotometer (Molecular Devices, CA).

The production of iROS was measured by a fluorescence probe DCFH-DA as described [32]. Briefly, after cultured overnight in 96-well plates, macrophages were incubated with 10 µM DCFH-DA (Invitrogen) for 30 min at 37°C. After 2 washes with HBSS buffer, cells were switched to HBSS containing 1% FBS. After the cells were incubated with vehicle or rotenone (10 nM) at 37°C for 30 min, the fluorescence was read at 488 nm for excitation and 525 nm for emission using a SpectraMax Gemini XS fluorescence microplate reader (Molecular Devices, CA).

Cell-free NADPH-dependent superoxide production assay

Generation of superoxide was measured by determining the rate of SOD-inhibitable ferricytochrome *c* reduction as described [33, 34]. Briefly, RAW 264.7 cells (a mouse macrophage-like cell line) were suspended to a concentration of 10^8 cells/ml in ice-cold disruption buffer containing 8 mM Na,K-phosphate buffer (pH 7.0), 131 mM NaCl, 340 mM sucrose, 2 mM NaN₃, 1 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and proteinase inhibitor cocktail. Cells suspension was sonicated by 5s bursts followed by a 5s rest; the cycle was repeated six times. Sonicated cell lyses were centrifuged at 6,000g for 10 min at 4°C to remove unbroken cells or organelles (e.g. mitochondria). After ultracentrifugation at 110,000g for 2 h at 4°C, the cytosolic fraction (supernatant) and the membrane fraction (pellet) were collected. The membrane pellet was then washed by 1M KCl and suspended in activation buffer containing 65 mM Na,K-phosphate buffer (pH 6.5), 170 mM sucrose, 2 mM NaN₃, 1 mM EGTA, and 10 µM FAD.

After treatment of the membrane fraction with rotenone (10 nM) at 37°C for 5 min, the cytosolic fraction supplemented with ferricytochrome c (0.1 mM) was reconstituted with the membrane fraction; SOD (600 unit/ml), GTP γ S (10 μ M) and DPI (1 μ M) were added as indicated. The reaction was initiated by the addition of freshly prepared NADPH (final concentration of 0.1 mM); the absorbance at 550 nm was read with a SpectraMax Plus microplate spectrophotometer (Molecular Devices, CA). Rates of superoxide production were calculated and expressed as “nmol of O $^{2-}$ /min/mg protein” [33].

Plasma membrane preparation

Plasma membranes of macrophages or neutrophils were isolated followed a published protocol [35]. Briefly, cells were suspended in isolation buffer (10 mM Tris-Cl, pH 8.0, 0.25 M sucrose, 1 mM EDTA and protease inhibitor cocktails), and cell membranes were broken by Dounce homogenization. The cell lysates were centrifuged at 6,000 X g for 10 min at 4°C to remove unbroken cells, cell debris, and mitochondria; afterwards, pellets of membranes were obtained by ultracentrifugation at 100,000g for 1 h at 4°C. After washed by 1M KCl, membrane pellets were either freshly used or stored at -80°C. Mitochondrial contamination of isolated plasma membranes were detected by Western blot analysis using antibody specific for VDAC (voltage-dependent anion channel, a mitochondrial membrane marker); crude mitochondrial fractions (pellets harvested by low-speed centrifugation of homogenized cell lysates) were used as a positive control of mitochondria.

[3 H] labeled-rotenone binding assay

Rotenone's binding was mainly performed using plasma membranes.. Briefly, membrane pellets were suspended in binding buffer (50mM Tris-Cl, pH 8.0, 100 mM NaCl, 1% BSA and 1 mM PMSF) and divided into aliquots of 0.25 mg protein per tube. Protein concentrations were determined by BCA Protein Kit (Pierce, Rockford, IL). For binding assay, [3 H]-labeled rotenone ([3 H] Dihydrorotenone, 50 Ci/mmol, American Radiolabeled Chemicals Inc, St Louis, MO) was added into membrane aliquots at a final concentration of 10 nM. For saturation studies, the concentration of [3 H]-labeled rotenone ranged from 1 to 80 nM, and nonspecific binding was determined in the presence of additional 100 μ M rotenone. Binding assay was terminated by filtration through glass microfibre filters (GF/C, Whatman) after binding samples were incubated on a rotator for 2 h at 4°C. Filters were immediately washed six times using binding buffer and transferred to the scintillation vials. After the filters were solubilized in 1 M NaOH, radioactivity was counted with a liquid scintillation counter (Perkin Elmer, MA).

Since rotenone is a highly lipophilic molecule and easily penetrates and is incorporated into biological membranes, rotenone shows high non-specific binding in various binding assays. In order to encounter these intrinsic obstacles in binding assay of rotenone, we combined immunoprecipitation using anti-gp91 antibody and radiolabeled binding assay (IP-binding). Specifically, we prepared plasma membranes from wild-type and gp91^{phox}-deficient macrophages, COS7WT cells lacking endogenous PHOX, and COS7 cells stably expressed with gp91^{phox} and p22^{phox}. Membrane pellets were suspended in 0.5 ml IP buffer (10 mM HEPES [pH 7.4], 1% Triton X-100, 5 mM Mg $_2$ Cl, 10 mM KCl, 1 mM PMSF, 1 mM dithiothreitol [DTT], 10% glycerin and protease inhibitor cocktails). After pre-incubation with [3 H]-labeled rotenone (10 nM) for 30 min, membrane aliquots (0.25 mg protein) were incubated overnight with the addition of control IgG or anti-gp91^{phox} polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA) at 4°C. Immunoprecipitation was performed by protein A/G beads (Santa Cruz Biotech, Santa Cruz, CA) saturated in 100 μ M rotenone. After 4-hour incubation at 4°C, protein beads were washed 8 times by IP buffer. Then the beads were eluted twice by 0.5 ml glycine buffer (0.1 M at pH 2.5), and radioactivity was

counted with a liquid scintillation counter. The binding capacity was determined by subtracting the binding of control IgG from that of gp91-antibody.

Cell-free membrane binding assay

Isolated membrane pellets (100 μ g) from resting peritoneal macrophages were suspended in 100 μ l binding buffer containing protease inhibitors and were incubated with vehicle or rotenone (50 nM) for 30 min at 37 °C. Samples were then incubated with corresponding cell cytosol extracts at 37°C for another 30 min. G-protein stimulator GTP γ S (10 μ M) was added in some groups. Since SDS has been reported to induce p47^{phox} binding to cell membranes [36] and to stimulate PHOX-mediated superoxide production in cell-free systems [37], we used SDS (0.1 mM) as a positive control. The pellets of treated membranes were re-collected by centrifugation at 100,000 g for 1 h and then analyzed by western blotting. Membrane binding capacities of p67^{phox} and p47^{phox} were determined, and gp91^{phox} was used as a membrane marker.

Immunoprecipitation for determining interactions between gp91^{phox} and p67^{phox}

Immunoprecipitation studies were performed using a method described previously [38]. Peritoneal macrophages were homogenized in extraction buffer (10 mM Hepes, pH 7.4, 0.25 M sucrose, 1 mM EDTA and protease inhibitor cocktails). Differential centrifugation of cell lysates removed debris and mitochondria; membrane pellets and cytosol were obtained by ultracentrifugation at 100,000g for 1 h. Isolated membrane pellets (250 μ g) were suspended in 0.5 ml lysis buffer (10 mM HEPES [pH 7.4], 2% Triton X-100, 5 mM Mg₂Cl, 10 mM KCl, 1 mM PMSF, 1 mM dithiothreitol [DTT] and protease inhibitor cocktails). After pre-incubation with vehicle or rotenone (50 nM) at 37°C for 30 min, solubilized membrane proteins were incubated with 0.5 ml cytosol extract. Immunoprecipitation was performed by the addition of anti-gp91^{phox} polyclonal antibody and protein A/G beads (Santa Cruz Biotech, Santa Cruz, CA) saturated in BSA. After 4-hour incubation at 4°C, protein beads were washed 5 times; bound proteins were denatured in Laemmli sample buffer by boiling at 100°C for 5 min.

Gel electrophoresis and Western blotting analysis

Membranes of peritoneal macrophages or various cultured cells were homogenized in SDS lysis buffer (2% SDS and 50 mM Tris-HCl, pH 7.5) or RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 1:100 protease inhibitor cocktails), sonicated and heated to 100°C for 10 min. Extracted protein samples were resolved on SDS polyacrylamide gels, and immunoblot analyses were performed using antibodies against gp91^{phox}, p67^{phox}, or p47^{phox}. Monoclonal anti-GAPDH antibody was included as an internal standard to monitor loading errors.

Statistics analysis

Data were expressed as mean $\pm$ S.E.M. Statistical significance was assessed by ANOVA followed by Bonferroni's t test using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). A value of $p < 0.05$ was considered statistically significant.

Results

Rotenone-induced superoxide release from phagocytes required the presence of PHOX

Like PMA (an activator of PHOX; a positive control), rotenone (10 nM) induced superoxide release in both primary microglia (Fig.1A) and peritoneal macrophages (Fig.1D) from wild-type mice, but failed to do so in cells from gp91^{phox}-deficient mice. These data indicated that PHOX was indispensable for rotenone-induced extracellular superoxide release from

microglia and macrophages. Similar dose-response curve of superoxide production elicited by rotenone was observed in primary microglia (Fig. 1B) and macrophage (data not shown). The EC₅₀ value of rotenone was 5.34×10^{-9} M. Ten nanomolar rotenone was used in all following studies, since at this concentration rotenone reached the 83% of the plateau of superoxide production. In contrast to rotenone, MPP⁺ (1-methyl-4-phenylpyridinium ion, 5 μ M) did not produce superoxide in microglia (Fig. 1C) or macrophages (data not shown), although both toxins are well-known inhibitors of mitochondrial complex I [39]. The dose of rotenone (10 nM) used in the present study is much lower than the doses used for mitochondrial inhibition and mitochondrial ROS production (100–2000 nM) [40–42], which further implied that mitochondria did not participate in the PHOX activation and consequent extracellular superoxide release elicited by a low dose of rotenone. In order to definitely confirm that rotenone-induced PHOX activation is independent of mitochondria, we measured NADPH-dependent superoxide production in a cell-free system. The results showed that rotenone (10 nM) induced superoxide production in the cell-free system reconstituted from wild-type microglial membrane and cytosolic fractions but not from gp91^{phox}-deficient microglial membrane and cytosolic fractions.

To further elucidate the mechanism underlying the activation of PHOX by rotenone, we used peritoneal macrophages and neutrophils instead of microglia in the present study for the following reasons: 1) microglia are considered as the macrophages in the brain, and both cells are derived from bone marrow monocytes; 2) in response to rotenone stimulation, the production of extracellular superoxide is qualitatively similar in peripheral macrophages and in microglia (Fig. 1); 3) macrophages and neutrophils have high levels of PHOX activity; 4) it is difficult to generate a great amount of primary microglia required for this study.

Rotenone directly interacted with flavocytochrome b558

We isolated plasma membranes of peritoneal neutrophils from wild-type and gp91^{phox}-deficient mice for binding assay, since PHOX was much abundant in neutrophils. The results revealed that more rotenone bound to wild-type neutrophil membranes than bound to gp91^{phox}-deficient neutrophil membranes (Fig. 2A). After enrichment of gp91^{phox} protein from wild-type primary macrophage membranes by immunoprecipitation using anti-gp91 antibody, the radiolabeled rotenone binding showed greater differences in the presence and absence of gp91^{phox} (Fig. 2A). We also compared the binding capacity of rotenone to plasma membranes prepared from COS7 cells that lack endogenous expression of p22^{phox} and gp91^{phox} [27] or COS7 cells stably transfected with flavocytochrome b558 alone (COS-cytob558) or in combination with p47^{phox} and p67^{phox} (COS-phox). The reason for the co-transfection of gp91^{phox} and p22^{phox} is that p22^{phox} facilitates stable expression of gp91^{phox} in plasma membrane of COS7 cells [43]. Western blotting assay using VDAC (voltage-dependent anion channel, a mitochondrial membrane marker) antibody did not show detectable mitochondrial contamination in isolated membranes (Fig. S1A). [³H]-labeled rotenone binding assay showed that plasma membranes from COS-cytob558 and COS-phox cells had 50~60% higher binding capacity to rotenone than membranes from wild-type COS7 cells (Fig. 2B). After immunoprecipitation using anti-gp91 antibody, about 2.5 folds increase in the binding of [³H]-labeled rotenone to immunoprecipitated COS7-cytob membrane proteins compared with immunoprecipitated COS7WT membrane proteins (Fig. 2B). These binding results together implied that rotenone could directly interact with flavocytochrome b558, and specially, subunit gp91^{phox}. The saturation study was performed by using wild-type COS7 and COS-cytob558 cells. In wild-type cells, no significant specific binding was observed since the total binding and the non-specific binding displayed very similar linear curves (Fig. 2C). On the contrary, the specific binding was clearly observed in COS-cytob558 transfected cells (Fig. 2D). [³H]-labeled rotenone binding was saturable with an affinity in the low nanomolar range, and the calculated K_d value was 21.18 ± 3.47 nM.

It is known that diphenyleneiodonium (DPI) can suppress gp91^{phox} activity through interacting with C-terminal flavin-binding domain of gp91^{phox} [44]. Although it blocks multiple flavin-containing enzymes, DPI has been widely used as a NADPH oxidase inhibitor. It is because that DPI shows more potent inhibition on NADPH oxidase than on other flavin-containing targets, such as flavin components of mitochondrial electron transport chain [45] and nitric oxide synthases [46]. Our results revealed that pre-incubation of membranes with DPI (10 μ M) for 30 min at 4°C decreased the binding capacity of [³H]-rotenone to gp91^{phox}-expressed membranes isolated from wild-type macrophages and COS7-cytob558 cells. DPI failed to attenuate [³H]-rotenone binding to membranes that lack gp91^{phox} (gp91^{phox}-deficient macrophage membrane or COS7-WT cell membrane) (Fig. 2E, F). Western blotting assay using VDAC (voltage-dependent anion channel, a mitochondrial membrane marker) antibody did not show detectable mitochondrial contamination. No mitochondrial contamination in isolated membranes (Fig. S1A), combined with the finding that DPI could not affect the binding of [³H]-rotenone at 10 nM concentration to mitochondrial fractions (Fig. S1B), further excludes the involvement of mitochondria in DPI-induced reduction of [³H]-rotenone binding to gp91^{phox}-expressed membranes. These data together demonstrated an interaction between rotenone and membrane gp91^{phox}. Here, the binding inhibition by DPI is not necessary to imply that rotenone and DPI compete for the same binding site. It is possible that rotenone and DPI have different binding sites on gp91^{phox}. Conformational changes of gp91^{phox} after DPI binding may reduce rotenone's binding to gp91^{phox}. It remains to be determined whether rotenone binds to heme, the C-terminal flavin domain, or any specific domain of gp91^{phox}.

Rotenone failed to induce superoxide production in COS7 cells transfected with flavocytochrome b558

To investigate the possibility that rotenone directly activates gp91^{phox} to initiate the production of superoxide, we measured extracellular superoxide release in wild-type COS7 cells, COS7 cells transfected with flavocytochrome b558 (COS-cytob558), and COS7 cells transfected with flavocytochrome b558 and p47^{phox}/p67^{phox} (COS-phox). The expression levels of various subunits in different cells were shown in Fig. 3A. As expected, PMA (40 nM) stimulated extracellular superoxide release only in COS-phox cells (Fig. 3B), since it is well known that PMA activated PHOX through a p47^{phox}-dependent pathway in which the phosphorylation of p47^{phox} is an essential step. Rotenone (10 nM) also induced superoxide release only in COS7-phox cells, which indicated that rotenone-induced activation of PHOX required the attendance of cytosolic subunit(s): p47^{phox}, p67^{phox}, or both.

Rotenone induced superoxide production in p47^{phox}-deficient macrophages

We next used peritoneal p47^{phox}-deficient macrophages generated from p47^{phox}-knockout mice to determine the role of cytosolic subunit p47^{phox} in rotenone-induced PHOX activation. As expected, both rotenone and PMA induced superoxide production in wild-type macrophages. In contrast, differential effects of PMA and rotenone were observed in p47^{phox}-deficient macrophages. Specifically, rotenone, but not PMA, was still capable of increasing the production of superoxide even in the absence of p47^{phox} (Fig. 4A). Rotenone treatment led to significantly less production of iROS in gp91^{phox}-deficient macrophages than in wild-type and p47^{phox}-deficient macrophages (Fig. 4B). The dose of rotenone (10 nM) used in this study is much lower than the doses used for mitochondrial inhibition and ROS production (100–2000 nM) [40–42]. Thus, iROS production induced by a low dose of rotenone was mainly from PHOX. Collectively, these data indicated that p47^{phox} was not indispensable for PHOX activation induced by rotenone, suggesting a p47^{phox}-independent pathway.

Rotenone induced translocation of cytosolic subunits to cell membranes

As described above, both membrane and cytosolic subunits were required for rotenone-elicited PHOX activation. Therefore, we explored how these subunits interacted after rotenone treatment. While it is well established that translocation of cytosolic subunits to the membrane is essential for PHOX activation, the involvement of individual cytosolic subunit in this process remains unclear. Here, we used PMA as positive control for the translocation study. PMA is known to activate protein kinase C (PKC), leading to phosphorylation of p47^{phox}. Phosphorylated p47^{phox} forms a complex with p67^{phox} and the complex translocates to cell membrane. The distribution of p67^{phox} in cell membranes isolated from rotenone-treated wild-type and p47^{phox}-deficient macrophages indicated that rotenone could trigger p67^{phox} translocation to membranes (Fig. 5A). Interestingly, although p47^{phox} was not required for rotenone-induced activation of PHOX (Fig. 4), rotenone treatment caused translocation of p47^{phox} to membrane in wild-type macrophages, as shown by western blotting (Fig. 5A and quantified intensity in Fig. S2A) and immunofluorescence staining (Fig. S2B). Considering the existence of p47^{phox}-independent pathway, we hypothesized that the membrane translocation of p67^{phox} may play a key role in rotenone-induced PHOX activation. Worth noting is elevated levels of gp91^{phox} and p67^{phox} in p47^{phox}-deficient macrophages (Fig 5B). This result may suggest a compensatory role of p67^{phox} and gp91^{phox} in the absence of p47^{phox}.

Rotenone induced cytosolic p67^{phox} binding to gp91^{phox}

Immunoprecipitation assay was employed to examine the interaction between p67^{phox} and gp91^{phox} after rotenone treatment. As shown in Fig. 6A, p67^{phox} was co-immunoprecipitated with gp91^{phox} by anti-gp91^{phox} antibody. No immunoprecipitation by control IgG and minimal nonspecific immunoprecipitation by anti-gp91^{phox} antibody in gp91^{phox}-deficient cell lysates confirmed the observed binding between gp91^{phox} and p67^{phox} was specific. Cell-free binding assay was used to further validate rotenone-elicited p67^{phox}-gp91^{phox} interaction. As shown by Figure 6B, rotenone-treated macrophage membranes, prepared from rats and wild-type mice, exhibited higher binding capacity for p67^{phox} than vehicle-treated membranes. The binding capacity was further increased by the addition of GTP γ S, a non-hydrolyzable G-protein-activating GTP analog. These findings implied that p67^{phox} interacted with rotenone-bound gp91^{phox} and G-proteins facilitated and stabilized such an interaction (Fig. 6B). Similar results were observed in membranes from p47^{phox}-deficient macrophages (Fig. 6C). The binding of p47^{phox} to macrophage membranes was not significantly changed after the addition of vehicle, rotenone, or GTP γ S (Fig. 6B,C). Collectively, the binding of rotenone to gp91^{phox} could facilitate p67^{phox}-gp91^{phox} interaction in a p47^{phox}-independent manner, and such p67^{phox}-gp91^{phox} interaction was a critical step in PHOX activation by rotenone. The finding that GTP γ S enhanced p67^{phox}-gp91^{phox} interaction in a cell-free binding assay suggests that Rac1 or Rap1A (two small G-proteins that have been implicated in NADPH oxidase activation) may play some role in rotenone-induced PHOX activation.

Rac1 participates in rotenone-induced PHOX activation

We next discern the role of Rac1, a Rho-like small GTPase, in rotenone-elicited PHOX activation. As seen in Fig. 7A, GTP γ S (10 μ M) increased rotenone-induced NADPH-dependent superoxide production in a cell-free system, and such superoxide production was blocked by DPI. Inhibition of Rac activity by NSC 23766, a Rac1 inhibitor, significantly attenuated rotenone-induced extracellular superoxide release from primary microglia (Fig. 7B). These results together revealed an important role of Rac1 in rotenone-induced PHOX activation through enhancing p67^{phox}-gp91^{phox} interaction.

Discussion

In the present study, we elucidated a unique mechanism by which rotenone activates PHOX. We found that rotenone directly interacted with gp91^{phox} (the catalytic subunit of PHOX), which facilitated the interaction between gp91^{phox} and cytosolic subunit p67^{phox}, thus triggering the formation of activated enzyme complex in phagocytes. While Rac1 enhanced rotenone-elicited superoxide production through stabilizing p67^{phox}-gp91^{phox} interaction, the genetic deletion of p47^{phox} did not affect rotenone-induced superoxide production. The later finding indicates that p47^{phox} was not required for functional enzyme assembly in the presence of rotenone. Thus, rotenone could activate PHOX through a p47^{phox}-independent mechanism.

Recent case-control and epidemiological studies suggests that exposure to pesticide rotenone is associated with an increased risk for PD [5, 7]. However, the mechanism of rotenone-induced neurotoxicity is still unclear. Although potential relevance of mitochondrial complex I impairment to PD pathogenesis has been demonstrated in several *in vivo* models created by rotenone [8, 11], the doses of rotenone used were not sufficient to alter mitochondrial respiration in isolated brain mitochondria [11]. Doses of rotenone for PHOX activation range from 1 to 20 nM (Fig. 1B), which is consistent with our previous studies [16, 18]. The doses used for mitochondrial inhibition and mitochondrial ROS production are much higher (100–2000 nM) [40–42]. Thus, rotenone neurotoxicity must not result solely from mitochondrial complex I inhibition and subsequent bioenergetic defect. In rotenone-infusion rodents, relatively selective oxidative stress in the midbrain and olfactory bulb was observed [10, 47]. Antioxidants rescue the behavioral deficits and attenuate dopamine neuronal loss in drosophila and rats exposed to rotenone [48, 49].

Reactive oxygen species (ROS) generated by leakage from mitochondria was considered as major source of oxidative stress in rotenone-induced PD animal models [47], since impaired complex I activity enhances ROS formation [50, 51]. A recent study has reported that cultured dopaminergic neurons from wild-type and mitochondrial complex I-deficient (*ndufs4*^{-/-}) mice exhibited similar vulnerability to rotenone neurotoxicity [52], which implies that rotenone may damage neurons through alternative targets other than mitochondrial complex I. It is noticeable that in the rat rotenone PD model, selective microglial activation has been observed in the striatum and the substantia nigra, the main brain regions damaged in PD patients [17]. In addition, our previous *in vitro* studies using cells from PHOX-deficient (*gp91*^{phox}^{-/-}) mice suggested that microglial PHOX-derived superoxide markedly enhanced rotenone-induced degeneration of dopaminergic neurons [18]. Superoxide and its downstream free radical products caused oxidative damages to neurons, eventually leading to neuronal death. Thus, PHOX is perhaps an alternative target of rotenone. In the present study, we explored this possibility and elucidated how rotenone activated PHOX. The results showed that rotenone directly interacted with gp91^{phox} subunit (Fig. 2, 6). Rotenone (10 nM) elicited superoxide production in both intact wild-type phagocytes and the cell-free system reconstituted from phagocyte membrane and cytosolic fractions from wild-type mice; in the absence of gp91^{phox}, rotenone did not induce superoxide production in these systems (Fig. 1 & 7). However, the finding that rotenone failed to stimulate superoxide production in COS7 cells stably transfected with flavin cytochrome b558 containing gp91^{phox} and p22^{phox} (Fig. 3) indicated that rotenone did not directly activate gp91^{phox} and cytosolic subunits or other cofactors were required for rotenone-induced PHOX activation (Fig. 1–3).

In the resting state, p47^{phox} separates from p67^{phox} and is functionally inactive [53]. Upon stimulation, p47^{phox} is phosphorylated, and the auto-inhibition region (AIR) of p47^{phox} was released, allowing its interaction with p22^{phox} and p67^{phox}. The complex of p47^{phox} and

p67^{phox} translocates to cell membrane, where p47^{phox} binds to membrane p22^{phox} and p67^{phox} binds to gp91^{phox}, leading to PHOX activation [24]. In fact, p47^{phox} serves as an organizer, while p67^{phox} is considered as an activator [24, 54]. Traditionally, p47^{phox} was considered essential for PHOX activation. Mutants in p47^{phox} gene have been linked to some patients with chronic granulomatous disease (CGD), a diverse group of hereditary diseases with deficiency in PHOX activity [55]. Consistent with previous publications [56], we found that p47^{phox} was required for PMA-induced free radical production (Fig. 4). However, it has also been reported that p47^{phox} is not indispensable for the activation of PHOX in some cell-free studies [57–59]. For instance, the pioneer study conducted by Freeman and Lambeth has demonstrated that in the absence of p47^{phox}, the PHOX activity is still preserved, but this requires the presence of p67^{phox} and Rac1; Rac1 is considered to stabilize the interaction between gp91^{phox} and p67^{phox}. Our findings that rotenone was able to activate PHOX and led to production of free radicals in p47^{phox}-deficient macrophages (Fig. 4–6) indicated the existence of a p47^{phox}-independent pathway in rotenone-induced activation of PHOX in intact phagocytes.

We then pinpointed a crucial role of cytosolic subunit p67^{phox} in rotenone-induced PHOX activation. Firstly, rotenone activated PHOX in COS7-phox cells that contain cytosolic subunits p47^{phox} and p67^{phox} (Fig. 3), while p47^{phox} was not indispensable (Fig. 4). Secondly, rotenone caused membrane translocation of p67^{phox} in both wild-type and p47^{phox}-deficient macrophages (Fig. 5). Thirdly, membrane binding assay and immunoprecipitation study showed that rotenone-bound gp91^{phox} exhibited higher binding affinity to p67^{phox} than vehicle-treated gp91^{phox} (Fig. 6). Therefore, rotenone-induced interaction between gp91^{phox} and p67^{phox} was crucial for assembling the active enzyme complex and for producing superoxide. It is thought that the direct protein-protein interaction between gp91^{phox} and p67^{phox} may require a conformational change of gp91^{phox} [60]; this change can be induced by p47^{phox} binding to p22^{phox} or activated-Rac1 binding to gp91^{phox} [59]. Rotenone-bound gp91^{phox} may undergo a similar conformational change. Such a conformational change might be provided by rotenone's interaction with C-terminal of gp91^{phox}, since rotenone's binding to isolated plasma membrane was inhibited by DPI that binds to flavin-binding domain in C-terminal of gp91^{phox}. In addition, C-terminal phosphorylation of gp91^{phox} has been reported to regulate the assembly of the enzyme complex possibly through some conformational changes [38]. However, at present, it remains to be determined whether rotenone binds to heme, the C-terminal flavin domain, or any specific domain of gp91^{phox}. Taken together, our findings suggest that rotenone's binding may induce a conformational change of gp91^{phox}, which facilitated the interaction between gp91^{phox} and p67^{phox} leading to PHOX activation. Thus, rotenone provided an opportunity to bypass the regulatory role of p47^{phox} and activated PHOX through a novel mechanism. These findings imply that the interaction between p67^{phox} and gp91^{phox} is the major event in oxidase activity, while p47^{phox} may not be indispensable for certain stimulators.

Although we identified a p47^{phox}-independent pathway in rotenone-induced PHOX activation, we also observed the membrane translocation of p47^{phox} in wild-type macrophages after rotenone treatment (Fig 5A, Fig S2). Additionally, in cell-free system, maximal activation of PHOX still requires the attendance of p47^{phox} [58]. As described previously, activation of PHOX has the feature of propagation [59] and superoxide-mediated superoxide production may keep sustained activation of PHOX [61]. Thus, it is possible that superoxide produced by rotenone-elicited macrophages through a p47^{phox}-independent mechanism may stimulate multiple downstream oxidative signaling pathways, which could induce p47^{phox} membrane translocation to participate in PHOX activation. However, the precise mechanism of p47^{phox}-dependent activation of PHOX by rotenone warrants further investigation.

Certainly, in addition to p67^{phox} and p47^{phox}, other cytosolic subunits might be also involved in rotenone-induced activation of PHOX. We noticed that GTP γ S significantly increased the binding of p67^{phox} to isolated macrophage membranes after rotenone treatment (Fig. 6B, C). GTP γ S is a non-hydrolyzable G-protein-activating GTP analog. Rac-1 and Rap1A, two small GTPases, are considered as the regulatory subunits of PHOX. Upon stimulation, Rac-1 most likely moves to membrane from cytosol, and perhaps interacts with gp91^{phox} [25, 62]. GTP-bound Rac-1 also interacts with tetratricopeptide (TPR) domain of p67^{phox}, which is proposed to stabilize the interaction between p67^{phox} and gp91^{phox} [59]. The following findings from the present study indicate that Rac1 play an important role in rotenone-induced PHOX activation through enhancing p67^{phox}-gp91^{phox} interaction: 1) GTP γ S enhanced the binding between p67^{phox} and gp91^{phox} after rotenone treatment in a cell-free binding assay (Fig. 6B,C); 2) GTP γ S increased rotenone-induced NADPH-dependent and DPI-inhibitable superoxide production in a cell-free system (Fig. 7A); 3) Rac1 inhibition decreased rotenone-elicited extracellular superoxide release from microglia (Fig. 7B).

In summary, this study demonstrates that PHOX is a novel target of rotenone. Through direct interaction with gp91^{phox} (the catalytic moiety of PHOX), rotenone may induce conformational changes of gp91^{phox} in phagocytes. These changes facilitated and stabilized the binding of cytosolic subunit p67^{phox} to membrane gp91^{phox}, which led to assembling of a functional oxidase and consequent superoxide generation. Thus, this study identified a distinct p47^{phox}-independent mechanism in rotenone-induced activation of PHOX. Findings from this study advance our understanding of rotenone-induced oxidative stress and neurotoxicity, providing a potential mechanism of environmental toxin-associated neurodegeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviations

MPP⁺	1-Methyl-4-phenylpyridinium ion
WST-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
DPI	diphenyleneiodonium
PD	Parkinson's disease
PHOX	phagocyte NADPH oxidase
PMA	phorbol 12-myristate 13-acetate
ROS	reactive oxygen species
SOD	superoxide dismutase

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Fig. 1. Rotenone induced extracellular superoxide release in wild-type, but not gp91^{phox}-deficient phagocytes

Extracellular superoxide release from primary microglia (A) and peritoneal macrophages (D) of wild-type and gp91^{phox}-deficient mice was measured by the SOD-inhibitable reduction of WST-1 after treatment with vehicle, rotenone (10 nM) or PMA (40 nM). (B) Dose-response curve of superoxide production in primary microglia induced by rotenone. Curve-fitting analyses were done with GraphPad Prism. (C) Unlike rotenone (10 nM), another mitochondrial complex I inhibitor MPP⁺ (5 μ M) did not produce superoxide in microglia. (D) Rotenone (10 nM) induced superoxide production in the cell-free system reconstituted from wild-type microglial membrane and cytosolic fractions but not from

gp91^{phox}-deficient microglial membrane and cytosolic fractions. Data were shown as mean $\pm$ SEM from three independent experiments in triplicate. *, $P < 0.05$ compared with corresponding vehicle-treated control.

Fig. 2. Rotenone directly interacted with gp91^{phox} and this interaction was attenuated by DPI Plasma membranes isolated from wild-type and gp91^{phox}-deficient neutrophils and macrophages (A), wild-type COS7 cells (COS7-WT), COS7 cells stably expressed with flavocytochrome b558 alone (COS7-cytob558) or in combination with p47^{phox} and p67^{phox} (COS-phox) (B) were used to perform the radiolabeled binding directly (PM-binding) or to conduct immunoprecipitation using anti-gp91 antibody and the radiolabeled binding assay (IP-binding assay). Data were shown as a percentage of the wild-type control. Results were presented as mean $\pm$ SEM from three independent experiments in duplicate. *, $P < 0.01$ compared with wild-type. (C, D) Saturation curves of $[^3\text{H}]$ -rotenone binding were performed by using wild-type COS7 (C) and COS-cytob558 cells (D). Total binding (●) was measured

by the inclusion of [^3H]-labeled rotenone at concentrations ranging from 1 to 80 nM, while nonspecific binding ($\square$) was determined in the presence of additional 100 μM rotenone. Saturation isotherms were analyzed in Graphpad prism 5. Specific binding ($\blacktriangle$) was calculated by the difference of total binding and nonspecific binding. This experiment was performed three times with similar results. (E, F) DPI inhibited the binding of [^3H]-rotenone only in gp91^{phox}-expressed membranes. Plasma membranes prepared from wild-type and gp91^{phox}-deficient neutrophils (E), COS7-WT cells or COS7-cytob558 cells (F) were incubated with [^3H]-rotenone (10 nM) containing DPI (10 μM) or vehicle for 2 h at 4 °C. Data were shown as a percentage of wild-type control after normalized to vehicle-treated wild-type membranes. Results were presented as mean $\pm$ SEM from three independent experiments in duplicate. *, $P < 0.05$ compared with vehicle-treated wild-type membrane. **, $P < 0.05$ compared with vehicle-treated COS7-cytob558 membrane.

Fig. 3. Both membrane and cytosolic subunits were required for rotenone-induced extracellular superoxide release

(A) Western blotting shows expression of PHOX subunits in COS7, COS-cytob558 (COS7 cells stably transfected with flavocytochrome b558), and COS-phox cells (COS7 cells stably transfected with both flavocytochrome b558 and cytosolic subunits p47^{phox} and p67^{phox}).

(B) Extracellular superoxide release was measured by the SOD-inhibitable reduction of WST-1 after treatment with vehicle, rotenone (10 nM), or PMA (40 nM). Data were shown as mean $\pm$ SEM from three independent experiments in triplicate. *, $P < 0.01$ compared with corresponding vehicle-treated controls.

A **B**

Fig. 4. Rotenone induced superoxide production in p47^{phox}-deficient macrophages
 (A) Extracellular superoxide release was measured by the SOD-inhibitable reduction of WST-1 after peritoneal macrophages from wild-type and p47^{phox}-deficient mice were treated with vehicle, rotenone (10 nM), or PMA (40 nM). Data were expressed as mean $\pm$ SEM from three independent experiments in triplicate. *, P < 0.05 compared with corresponding vehicle-treated control. (B) Intracellular ROS production was measured by fluorescence probe DCFH-DA as described in Methods. Data were collected at 30 min after the addition of vehicle or rotenone into cells and shown as arbitrary fluorescence units. Results were presented as mean $\pm$ SEM from three independent experiments in triplicate. *, P < 0.05 compared with corresponding vehicle-treated control.

Fig. 5. Rotenone induced membrane translocation of cytosolic subunits in wild-type and p47^{phox}-deficient macrophages

Peritoneal macrophages from wild-type or p47^{phox}-deficient mice were treated with vehicle or rotenone (10 nM) for 15 min. PMA-treated wild-type macrophages were used as positive controls. Distribution of p47^{phox} and p67^{phox} in membrane fractions was detected by western blot assays (A). The ratios of densitometry values of p67^{phox} to values of membrane gp91^{phox} (loading control) were normalized to corresponding controls. *, $P < 0.01$ compared with vehicle-treated control. (B) The expression levels of p67^{phox} and gp91^{phox} in wild-type and p47^{phox}-deficient macrophages were determined by western blotting. The ratios of densitometry values of p67^{phox} or gp91^{phox} relative to loading control GAPDH were normalized to wild-type. All experiments shown in this figure have been performed at least three times. *, $P < 0.05$ compared with corresponding wild-type.

Fig. 6. Rotenone treatment recruited p67^{phox} from cytosol to the membrane where it bound to gp91^{phox}

(A) Immunoprecipitation for NADPH oxidase subunits gp91^{phox} and p67^{phox} was performed by using membrane fractions of macrophages from wild-type and gp91-deficient mice (the latter as a negative control). P67^{phox} was co-immunoprecipitated by the anti-gp91^{phox} antibody. Experiments were performed three times with similar results. (B, C) Cell membrane fractions of peritoneal macrophages prepared from rats, C57BL/6J mice, or p47-knockout mice were incubation with vehicle or rotenone (10 nM) for 30 min at 37 °C; afterwards, membrane fractions were reconstituted with the corresponding cytosol extracts and were incubated at 37°C for another 30 min. SDS (0.01%, a positive control) and GTPγS

(10 μ M) were added as indicated. Occurrence of p67^{phox} and p47^{phox} in membrane fractions was detected by western blot assays. The ratios of densitometry values of p67^{phox} or p47^{phox} relative to gp91^{phox} (used as a membrane marker) were normalized to vehicle-treated control. Experiments have been performed at least four times. *, #, $P < 0.05$ and **, $P < 0.01$ were considered statistically significant.

A **B**

Fig. 7. Rac1 participates in rotenone-induced PHOX activation

(A) In the cell-free system reconstituted from membrane and cytosolic fractions of RAW 264.7 cells, rotenone-induced superoxide production was blocked by DPI. GTP γ S significantly increased rotenone-elicited superoxide production; such superoxide production was also prevented by DPI. (B) Rac1 inhibitor NSC 23766 (100 μ M) significantly attenuated rotenone-induced extracellular superoxide release in primary microglia.