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Protein kinase C-independent pathway for NADPH oxidase activation in guinea pig peritoneal polymorphonuclear leukocytes by cytochalasin D

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Abstract

Cytochalasin D (CD) induced production of the superoxide radical (O_2^-) in guinea pig polymorphonuclear leukocytes (PMNs). The protein kinase C (PKC) inhibitor GF109203X (GFX) was rarely without effect on CD-induced O_2^- production. CD as well as PMA induced the translocation of $p47^{phox}$ to the membrane fraction, and this translocation was slightly decreased by GFX. Moreover, the inhibitory effect of a PKCζ antagonist with sequences based on the endogenous PKCζ pseudosubstrate region was weaker than the inhibitory effect on *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced O_2^- production. On the other hand, the production of O_2^- induced by CD was more strongly suppressed by the PLD inhibitor ethanol and phosphatidylinositol 3-kinase (PI3-K) inhibitor wortmannin than that induced by fMLP, and the activation of phospholipase D (PLD) by CD was restrained by wortmannin. These findings suggest that NADPH oxidase is activated by CD through a PKC-independent signaling pathway in PMNs, and this pathway involves the activation of PLD through PI3-K.
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Keywords: Polymorphonuclear leukocyte; NADPH oxidase; Protein kinase C; Cytochalasin D; Phosphatidylinositol 3-kinase; Phosholipase D

Polymorphonuclear leukocytes (PMNs)[1] generate reactive oxygen species using an NADPH oxidase, which is composed of a membrane-bound cytochrome b_{558} and four cytosolic proteins, $p47^{phox}$ (phox: phagocyte oxidase factor), $p67^{phox}$, $p40^{phox}$, and Rac [1–4]. When the enzyme is activated, the phosphorylation of $p47^{phox}$ by protein kinase C (PKC) promotes the transfer of at least some of the cytosolic components to the membrane [1,5,6]. PKC mainly contributes to the activation of NADPH oxidase. This view is based on the following findings: (1) NADPH oxidase is strongly activated by PKC activators such as PMA [7], (2) components of NADPH oxidase are strongly phosphorylated by PKC [8–10], (3) the activation of NADPH oxidase is inhibited by specific PKC inhibitors [11,12], (4) in a cell-free system using cytosol and membrane fractions, activation of NADPH oxidase was caused by PKC [13,14] and (5) in HL60 cells, PKCβ antisense oligonucleotides inhibited the activation of NADPH oxidase [15]. The PKC family is classified into three subgroups based on molecular structure [16,17], and the presence of PKCα, βI, βII, δ, and ζ in neutrophils is recognized [18–20]. Generally, the PKC-dependent pathway for the activation of NADPH oxidase is thought to be as follows. PLC is activated when a receptor on

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[1] *Abbreviations used:* PMNs, polymorphonuclear leukocytes; PKC, protein kinase C; SOD, superoxide dismutase; HRP, horseradish peroxidase; PEt, phosphatidylethanol; HBSS, Hanks' balanced salt solution; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

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neutrophils is stimulated with certain agents such as fMLP, and inositol 1,4,5-triphosphate (IP_3) and DAG are generated. IP_3 releases Ca^{2+} from the endoplasmic reticulum and the Ca^{2+} and DAG activate PKCα, βI and βII. At the same time, PKCζ is activated through the generation of PIP_3 caused by the activation of PI3-K and PA produced by the activation of PLD and PLC [1–6]. However, a PKC-independent pathway has also been demonstrated [12,21–25]. It was reported that the production of O_2^- by neutrophils stimulated by certain agents such as fMLP, opsonized zymosan, and vanadate was not inhibited completely by PKC inhibitors [12,21,24,25]. Further, we have obtained the result that production of O_2^- in cytochalasin B (CB)-pretreated neutrophils activated by PMA was not depressed completely by a PKC inhibitor [22]. Using an activator of O_2^- production such as fMLP, opsonized zymosan, vanadate or CB plus PMA to study the PKC-independent pathway is not suitable because the PKC-dependent pathway is also activated. On the other hand, cytochalasin D (CD), which is a cytochalasin like CB, alone caused O_2^- production in both human neutrophils and guinea pig peritoneal PMNs [26]. The details of the pathway activated by CD are unclear, but our study using CB suggests that it is possible to induce O_2^- production only through the PKC-independent pathway [22].

Therefore, in this study, using guinea pig peritoneal PMNs, we examined in detail the pathway by which NADPH oxidase is activated in neutrophils, that is, the PKC-independent pathway stimulated by CD.

Materials and methods

Materials

Casein sodium was purchased from Tokyo-Kasei, cytochrome *c* (typeIII), catalase, PMA, superoxide dismutase (SOD, from bovine liver), fMLP, CD (from Zygosporium mansoni), and PA were from Sigma (MO, USA), GF109203X (GFX) was from Tocris Cookson (MO, USA), PKCζ antagonist (myristoylated peptide inhibitor, Myr-SIYRRGARRWRKL) was from Calbiochem–Novabiochem (Schwalbach, Germany), and wortmannin was from Wako Pure Chemicals (Osaka, Japan). Horseradish peroxidase (HRP) and ECL were obtained from Amersham Biosciences (NJ, USA), RPMI 1640 medium was obtained from Invitrogen (CA, USA), [9,10 (N) ^{3}H]myristic acid (40 Ci/mmol) from American Radiolabeled Chemicals (MO, USA), phosphatidylethanol (PEt) from Biomolecular Research Laboratories (PA, USA), and Silica gel 60 TLC plates were from Merk (NJ, USA). Anti-p47^{phox} was as described previously [27]. All other chemicals used were of reagent grade and obtained commercially.

Preparation of PMNs

Peritoneal PMNs were obtained from casein-induced peritoneal exudates from female Hartley strain guinea pigs as described previously [27].

Stimulation of PMNs and determination of O_2^-

The production of O_2^- by PMNs was measured on the basis of the reduction of ferricytochrome *c* by the anion produced. In brief, PMNs (10^6 cells/ml) were incubated in the absence or presence of various reagents in Hanks' balanced salt solution (HBSS) (pH 7.4) with catalase (50 μM) for 5 min at 37 °C. The reference sample also contained SOD (10 μg/ml). O_2^- production by PMNs pretreated as described above was started by adding cytochrome *c* (50 mM) and CD (20 μM), PMA (0.32 μM) or fMLP (23 μM). Reduced cytochrome *c* was measured on the basis of the increase in absorption at 550 nm using a Hitachi U-3210 spectrophotometer. The amount of O_2^- produced was calculated using an absorption coefficient of 19.1 mM^{-1} cm^{-1} at 550 nm [28].

Preparation of membrane fractions from PMNs

PMNs (10^6 cells/ml) were pretreated with or without GFX (0.5 μM) for 5 min in HBSS at 37 °C, and then CD (20 μM) or PMA (0.32 μM) was added. After incubation for 5 min, the cell suspensions were centrifuged (100*g*, 5 min) and the precipitated cells were resuspended in a relaxation buffer (10 mM Pipes, pH 7.3, containing 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 20 μg/ml leupeptin, 20 μg/ml pepstatin A, 3 mM PMSF, 1 mM Na_3VO_4, 10 μg/ml catalase, 10 mM NaF, and 0.34 M sucrose) and then sonicated. The supernatant was separated by centrifugation (120,000*g*, 20 min) and the precipitate, the plasma membrane fraction, was suspended in a relaxation buffer, and analyzed immunochemically for p47^{phox}. The protein concentration was measured by the method of Bradford [29].

Immunochemical detection of p47phox

p47^{phox} was detected using anti-p47^{phox} antibodies as described previously [27]. In brief, the above membrane fractions were mixed (1:1) with lysis buffer [2% sodium dodecyl sulfate (SDS), 30% glycerol, 10% 2-mercaptoethanol, and 0.01% bromophenol blue in 0.25 M Tris–HCl, pH 6.8] [30], heated at 100 °C for 3 min, subjected to electrophoresis on 4–20% SDS–polyacrylamide gels, electrotransferred to Immobilon (Millipore), blocked for 1 h with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (TPBS) plus 5% dried milk, and then incubated with anti-p47^{phox} (1:5000) rabbit polyclonal antibody in TPBS containing 5% dried milk

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