Roles of p38 MAPK, PKC and PI3-K in the signaling pathways of NADPH oxidase activation and phagocytosis in bovine polymorphonuclear leukocytes

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Abstract Stimulation of bovine polymorphonuclear leukocytes (PMN) with serum-opsonized zymosan (sOZ) induced the activation of p38 mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3-K) and sOZ-induced O_2^- production was significantly attenuated by their inhibitors (SB203580 for p38 MAPK, GF109203X for PKC and wortmannin for PI3-K). They caused significant attenuation of sOZ-induced phosphorylation of p47phox as well. Flow cytometric analysis, however, revealed that SB203580 and wortmannin attenuated phagocytosis, but GF109203X facilitated it. The results suggest that p38 MAPK and PI3-K participated in both signaling pathways of NADPH oxidase activation (O_2^- production) and phagocytosis, and PKC participated in the signaling pathway of NADPH oxidase activation alone.

Key words: Polymorphonuclear leukocyte; O_2^- production; Phagocytosis; NADPH oxidase; p38 Mitogen-activated protein kinase; Signaling pathway

1. Introduction

Polymorphonuclear leukocytes (PMN) play an important role in host defense against bacterial infections. The bactericidal mechanism consists of phagocytosis of pathogens, generation of reactive oxygen intermediates (ROI), and release of bactericidal proteins to phagosomes. During the bactericidal responses, phagosomes contain a variety of ROI, including OCl^-, •OH and H_2O_2, which are derivatives of O_2^- generated from oxygen by NADPH oxidase [1]. NADPH oxidase is a multicomponent enzyme consisting of at least two membrane proteins (gp91phox and p22phox) and three cytosolic proteins (p47phox, p67phox and Rac). When PMN are exposed to the appropriate stimuli, NADPH oxidase is activated by associating with these components on the plasma membrane followed by O_2^- production [2]. Phagocytosis is the process that recognizes the infectious agents and engulfs them, and thereby separates these organisms from normal tissue. Receptors participating in the NADPH oxidase activation and phagocytosis include the IgG Fc receptor (FcγR) and β2-integrin receptor (CR3, CD11b/CD18), which binds to iC3b [3–5].

FcγR and CR3 act cooperatively to stimulate the intracellular signaling pathways not only to initiate the actin polymerization necessary for phagocytosis but also to activate NADPH oxidase for O_2^- production [6,7]. Recently, some components of the signaling pathways associated with the PMN responses were identified. Cross-linking of FcγRs induces the activation of src family tyrosine kinases followed by the activation of Syk tyrosine kinase [8]. Tyrosine phosphorylation of FcγR by src family tyrosine kinases is an important process for phagocytosis in macrophages and FcγR-transfected cells, and PMN defective in Syk tyrosine kinase exhibits significant decreases in phagocytosis and O_2^- production [9–11]. Cross-linking of FcγRs also stimulates the tyrosine phosphorylation of phospholipase Cγ, which leads to an increase in the concentration of intracellular calcium and the production of diacylglycerol, an activator of protein kinase C (PKC) [12,13]. PKC inhibition prevents O_2^- production in PMN [6] and attenuates phagocytosis in monocytes and PMN [14,15]. However, the phagocytosis in macrophages is not affected by this treatment [11]. Phosphatidylinositol 3-kinase (PI3-K) activation as well as tyrosine kinase activation was reported to occur after PMN stimulation and to be associated with both O_2^- production and phagocytosis in PMN [16,17].

p38 Mitogen-activated protein kinase (MAPK) is a Ser/Thr kinase belonging to the family of MAPKs and its importance in PMN was recently demonstrated. Stimulation of FcγR and CR3 activates the p38 MAPK in macrophages and PMN [18,19]. Inflammatory stimuli, including lipopolysaccharide (LPS) and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), also activate p38 MAPK and a specific inhibitor of p38 MAPK prevents O_2^- production in PMN when exposed to fMLP and LPS [20,21]. Several papers have suggested that p38 MAPK was involved in the signaling pathway of O_2^- production. However, its role in phagocytosis is still uncertain. In this study, we examined the roles of p38 MAPK, PKC and PI3-K in the signaling pathways leading to not only O_2^- production but also phagocytosis using various pharmacological inhibitors.

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Abbreviations: CR3, complement receptor 3; FcγR, receptor for the Fc region of IgG; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; p38 MAPK, p38 mitogen-activated protein kinase; PMN, polymorphonuclear leukocytes; sOZ, serum-opsonized zymosan

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2. Materials and methods

2.1. Materials

Zymosan, horseradish peroxidase, adenosine, ATP, phosphatidylinositol, and glutathione (GSH)-agarose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). GF109203X, wortmannin, and SB203580 were from Calbiochem (La Jolla, CA, USA). Protein A-agarose, anti-PKC antibody and anti-phosphotyrosine antibody (PY-20) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-phosphorylated p38 MAPK antibody was from New England Biolabs, Inc. (Beverly, MA, USA). Silica gel 60 F_{254} was from Merck KGaA (Darmstadt, Germany). Luminol was from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). [γ-^{32}P]ATP was from ICN Biomedicals Inc. (Costa Mesa, CA, USA). Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS(−)) and isopropyl thio-β-galactoside (IPTG) were from Gibco BRL (Grand Island, NY, USA). Fluorescent polystyrene latex particles (1.0 μm diameter) were obtained from Polysciences, Inc. (Warrington, PA, USA).

2.2. PMN isolation

Peripheral blood was obtained from clinically healthy cows and suspended in HBSS(−). The suspension was layered on Ficoll–Conray solution and centrifuged at 3000 rpm for 30 min at 20°C. After removal of plasma and monocytes, the pellet was suspended in 0.8% NH_4Cl and incubated for 10 min at 4°C to lyse erythrocytes. Cells were collected by centrifugation at 1200 rpm for 10 min at 4°C and washed with HBSS(−). Isolated cells were suspended in HBSS(−). Viability was always more than 95% by the dye exclusion test with trypan blue.

2.3. Preparation of serum-opsonized zymosan (sOZ)

Zymosan was suspended in fresh autologous serum at a concentration of 10 mg/ml and incubated for 30 min at 37°C. After incubation, the suspension was washed twice with HBSS(−) and resuspended in HBSS(−) at a concentration of 10 mg/ml.

2.4. Immunoblotting for activated p38 MAPK

PMN (5×10^6) were preincubated in HBSS(+) at 37°C for 5 min before addition of sOZ at 1 mg/ml. After stimulation at 37°C for indicated times, reactions were terminated by adding 1 ml of ice-cold HBSS(−) and subsequent centrifugation at 6000 rpm for 30 s at 4°C. Subsequently, cell pellets were suspended with 50 μl of lysis buffer (1% Triton X-100, 20 mM Tris–HCl [pH 8.0], 100 mM NaCl, 10 mM $Na_2P_2O_7$, 2 mM EDTA, 50 mM NaF, 10% (v/v) glycerol, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and sonicated (15 s×2). After centrifugation at 12000 rpm for 15 min at 4°C, 15 μl of 3×Laemmli sample buffer was added to 30 μl of supernatant and boiled for 5 min. Each sample containing 75 μg of cellular protein was subjected to 10% SDS–PAGE and then transferred to a nitrocellulose membrane. The membrane was incubated with anti-phosphorylated p38 MAPK antibody diluted 1/1000 in TBST (10 mM Tris–HCl [pH 7.4], 0.1 M NaCl, 0.1% Tween-20) containing 5% bovine serum albumin, and then HRP-conjugated anti-rabbit IgG. After three washes with TBST, bound antibody was detected with a chemiluminescence detection kit (Boehringer Mannheim GmbH, Mannheim, Germany).

2.5. PKC translocation

PMN (3.5×10^7) were treated as described above. After stimulation, cell pellets were suspended in modified relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM PIPES [pH 7.4], 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and then sonicated at 4°C (20 s×3). Nuclei, granules and unbroken cells were removed by centrifugation at 2500 rpm for 10 min at 4°C. The supernatant was centrifuged at 42000 rpm for 30 min at 4°C to separate cytosol and membrane fractions. The pellet (membrane) was dissolved in 1×Laemmli sample buffer and boiled for 5 min. Each sample containing 30 μg of membrane protein was subjected to 7.5% SDS–PAGE and then transferred to a nitrocellulose membrane. Blocking, treatment with the primary and secondary antibodies and visualization were similar to the procedures used for p38 MAPK.

2.6. Measurement of PI3-K activity

PMN (1×10^7) were treated as described above. After stimulation, cell pellets were lysed for 30 min at 4°C with 1 ml of lysis buffer and centrifuged at 12000 rpm for 30 min at 4°C to remove insoluble debris. The supernatant was precleared by incubation with protein A-agarose for 30 min at 4°C before immunoprecipitation. The measurement of PI3-K activity was performed by the method of Coffer et al. [22]. Briefly, the precleared lysates were incubated with 2 μg of anti-phosphotyrosine monoclonal antibody (PY-20) on a rotating wheel for 1 h at 4°C before the addition of 40 μl of a 50% slurry of protein A-agarose and further incubated for 1 h. The samples were washed three times with lysis buffer and twice with 10 mM Tris–HCl (pH 7.4) containing 1 mM Na_3VO_4. PI3-K activity was measured by adding 100 μg of sonicated phosphatidylinositol and 10 μCi of [γ-^{32}P]ATP in the presence of 200 μM adenosine (to inhibit phosphatidylinositol 4-kinase activity), 30 mM $MgCl_2$ and 35 μM ATP in a total volume of 60 μl. Reactions were performed for 20 min at 25°C and terminated by the addition of 100 μl of 1 M HCl and 200 μl of chloroform/methanol (1:1, v/v). After centrifugation at 3000 rpm for 5 min and removal of the upper layer, 80 μl of methanol/HCl (1:1) was added. After further centrifugation, lipids were separated on TLC plates (silica gel 60 F_{254}) with a solvent system of chloroform/methanol/H_2O/NH_4OH (45:35:7.5:2.5, v/v). The radioactivities on the TLC plates were visualized and analyzed with an imaging analyzer (Fujix BAS1000; Fuji Photo Film Co., Ltd., Tokyo, Japan).

2.7. Assay of O_2^- production

O_2^- production was measured by chemiluminescence with luminol. HBSS with 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$ (HBSS[+]) containing 3×10^6 PMN, 10 μM luminol and 50 μg/ml of horseradish peroxidase was prepared in each well of a 96-well microplate. The suspension, with a volume of 315 μl, was incubated for 5 min at 37°C. After incubation, PMN were activated by adding 35 μl of sOZ (10 mg/ml) and then chemiluminescence from each well was measured by luminometer (Luminescencer-JNR; ATTO Co., Tokyo, Japan) for 0.5 s at 37°C.

2.8. Recombinant p47phox fusion protein and kinase assay

Recombinant p47phox fusion protein as a substrate for kinases was prepared with the procedure described by Park and Babior [23]. Briefly, *Escherichia coli* transformed with pGEX-1λT containing an insert of p47phox cDNA were grown in the presence of 1 mM IPTG and lysed. The glutathione *S*-transferase (GST)–p47phox fusion protein in the cell lysate was purified on GSH-agarose. The purity and identity of the fusion protein were assayed by 10% SDS–PAGE and immunoblotting.

PMN (2×10^7) in HBSS(+) were incubated for 5 min at 37°C and then stimulated with 1 mg/ml of sOZ for the indicated lengths of time at 37°C. The reaction was terminated by centrifugation at 3000 rpm for 5 min at 4°C. The supernatant was removed and cells were suspended in 50 μl of ice-cold WCE 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 dithiothreitol (DTT), 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 μg/ml of leupeptin, 100 μg/ml of PMSF). Cells were then sonicated and the extracts were obtained by centrifugation at 12000 rpm for 10 min at 4°C. The extracts were mixed with 75 μl of GSH-agarose suspension to which 15 μg of GST–p47phox was bound. The mixture was rotated for 3 h at 4°C and pelleted by centrifugation at 10000 rpm for 20 s. After the agarose 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, 0.05% Triton X-100), they were resuspended in 30 μl of kinase buffer (20 mM HEPES [pH 7.6], 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM DTT) containing 20 μM ATP and 10 μCi of [γ-^{32}P]ATP. After incubation for 20 min at 30°C, the reaction was terminated by washing with HEPES binding buffer. Laemmli sample buffer (1×, 45 μl) was added to the beads, which were boiled to separate p47phox–GST from them. The separated p47phox–GST was subjected to 10% SDS–PAGE. After drying the gel, radioactivities of phosphorylated p47phox–GST were measured with an imaging analyzer.

2.9. Measurement of phagocytic activity

Phagocytosis of fluorescent latex particles by PMN was measured by the following flow cytometric technique. To prepare serum-opsonized fluorescent particles, fluorescent particles (1.0 μm diameter) were suspended in fresh autologous serum and incubated for 30 min at 37°C. After incubation, the suspension was washed twice with

HBSS(−) and resuspended in HBSS(−) at a concentration of 1×10^8 particles/ml. 200 μl of HBSS(+) containing 2×10^6 PMN was preincubated for 5 min at 37°C. Then, 100 μl of suspension containing opsonized particles was added to this preincubated cell suspension (cell:particle = 1:5) and the mixture was further incubated for 30 min at 4°C or 37°C. After centrifugation at 1200 rpm for 10 min at 4°C, the cells were washed and suspended in HBSS(−). The fluorescence of the particles in PMN was analyzed by flow cytometry (EPICS 752; Coulter Electronics, Hialeah, FL, USA). The PMN that ingested the particles were gated by excluding the counts of free particles.

3. Results

3.1. Activation of p38 MAPK, PKC and PI3-K of PMN induced by sOZ stimulation

To determine whether sOZ could activate p38 MAPK, PKC and PI3-K of PMN, phosphorylation of p38 MAPK, PKC translocation from cytosol to membrane, and PI3-K activity were assessed. The time course for the activation of these three kinases after sOZ stimulation is shown in Fig. 1. A slight increase of p38 MAPK phosphorylation was found at 3 min, reaching the maximum at 6 min after stimulation. This phosphorylation was gradually decreased toward 12 min (Fig. 1A). After stimulation, PKC translocation became apparent at 3 min and remained elevated up to 12 min (Fig. 1B). PI3-K activation was clearly observed at 3 min and peaked at 6 min (Fig. 1C). These results indicated that the sOZ stimulation induced the activation of p38 MAPK, PKC and PI3-K of PMN.

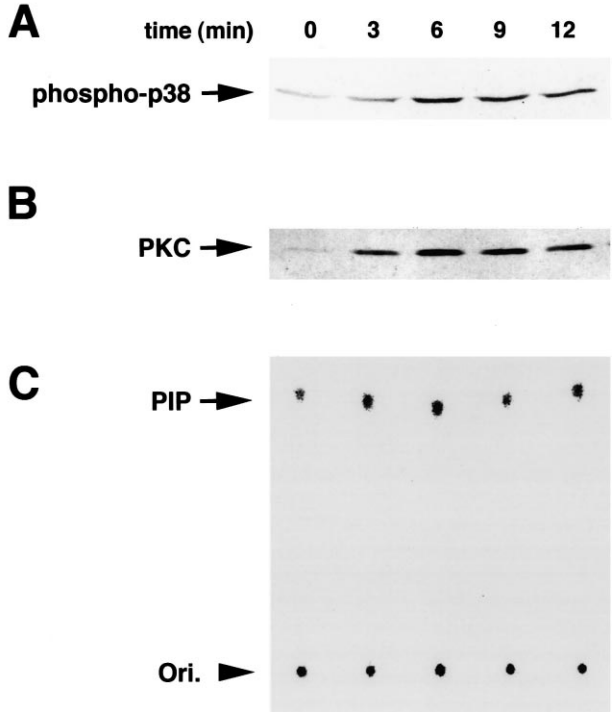

Fig. 1. Time course of activation of p38 MAPK, PKC and PI3-K in sOZ-stimulated PMN. PMN were incubated for 5 min at 37°C prior to stimulation with 1 mg/ml of sOZ. After stimulation for the indicated times at 37°C, activation of each kinase was evaluated as described in the text. A: Time course of p38 MAPK phosphorylation. B: Time course of PKC translocation. C: Time course of PI3-K activation. The positions of phosphorylated phosphatidylinositol and origin are marked by PIP and Ori., respectively.

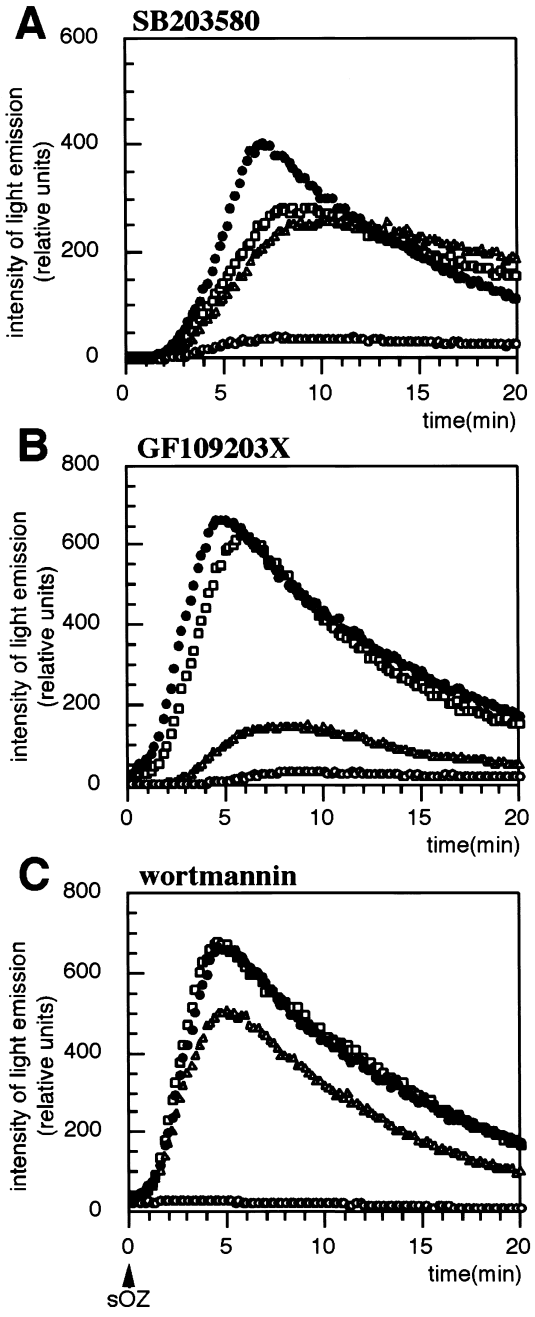

Fig. 2. Time course of O_2^- production and the effects of three inhibitors on it. O_2^- production was measured by chemiluminescence as described in the text. PMN were incubated with an inhibitor for 5 min at 37°C prior to stimulation with 1 mg/ml of sOZ at 37°C. Each point in the time course represents the mean of three experiments. The concentrations of inhibitors were as follows: (A) SB203580, 0 (●), 1 (□), 10 (△), 100 μM (○); (B) GF109203X, 0 (●), 1 (□), 4 (△), 8 μM (○); (C) wortmannin, 0 (●), 0.5 (□), 5 (△), 50 nM (○).

3.2. Effects of inhibitors on O_2^- production

Fig. 2 shows O_2^- production from sOZ-stimulated PMN with and without inhibitors. Stimulation of PMN without inhibitors elicited a time-dependent increase of O_2^- production, which peaked at 6 min followed by a gradual decrease (closed circles in Fig. 2). To investigate the involvement of p38 MAPK, PKC and PI3-K to the activation of NADPH oxi-

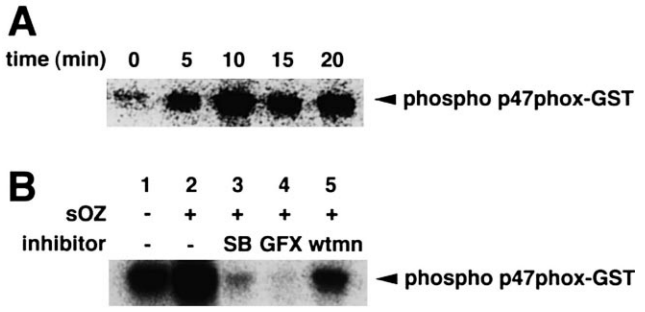

Fig. 3. In vitro phosphorylation of p47phox–GST induced by the cytosolic fraction of sOZ-stimulated PMN. Phosphorylation of p47phox–GST was measured as described in the text. A: Time course of phosphorylation of p47phox–GST. B: The effects of three inhibitors on p47phox phosphorylation. PMN were incubated with an inhibitor for 5 min at 37°C prior to stimulation with 1 mg/ml of sOZ for 7 min at 37°C. (1) No stimulation with sOZ, (2) stimulated with sOZ in the absence of an inhibitor, (3) stimulated with sOZ in the presence of 100 μM SB203580 (SB), (4) stimulated with sOZ in the presence of 8 μM GF109203X (GFX), (5) stimulated with sOZ in the presence of 50 nM wortmannin (wtmn). Autoradiographs of phosphorylated p47phox–GST are shown.

dase, we examined the effects of pharmacological inhibitors, an inhibitor of p38 MAPK (SB203580), an inhibitor of PKC (GF109203X) and an inhibitor of PI3-K (wortmannin), on O_2^- production. All inhibitors dose-dependently reduced the O_2^- production (Fig. 2). Thus, we concluded that NADPH oxidase activation by sOZ requires activation of p38 MAPK, PKC and PI3-K.

3.3. Effects of inhibitors on p47phox phosphorylation

O_2^- production requires NADPH oxidase activation which further requires the phosphorylation of p47phox, one of the cytosolic oxidase components. This phosphorylation is considered to be a trigger for the assembly of cytosolic components to the membrane components [2,24–26]. In fact, as shown in Fig. 3A, sOZ stimulation of PMN time-dependently induced the phosphorylation of p47phox–GST. The phosphorylating activity of the cytosolic fraction of PMN was slightly increased at 5 min and then reached a maximum at 10 min after stimulation. We next examined the ability of kinases in the cytosol fraction with or without various inhibitors. Lanes 1 and 2 in Fig. 3B show autoradiographs of p47phox–GST phosphorylated by cytosol fractions obtained from untreated PMN and PMN stimulated with sOZ for 7 min, respectively. A significant increase of phosphorylated p47phox was observed in sOZ-stimulated PMN. Furthermore, the phosphorylation of p47phox was inhibited by all inhibitors. However, the inhibitory effects were different among them. 100 μM SB203580 (lane 3) and 8 μM GF109203X (lane 4) largely inhibited the phosphorylation of p47phox, whereas 50 nM

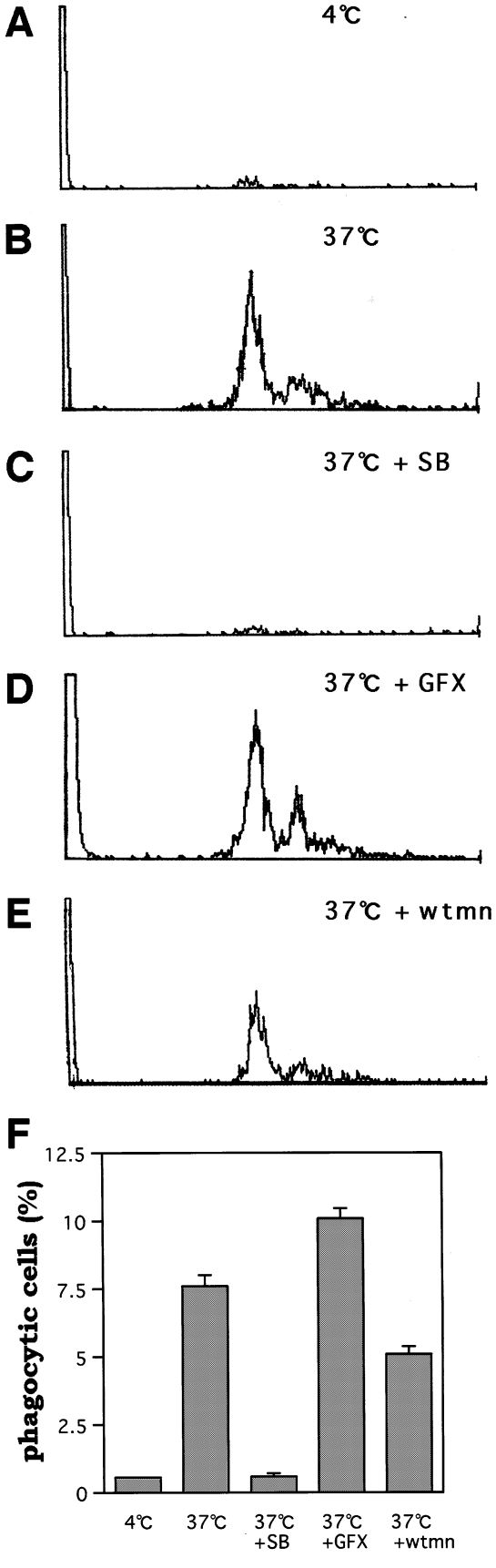

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Fig. 4. The effects of three inhibitors on phagocytosis. A–E: Representative flow cytometric profiles. PMN were incubated with serum-opsonized fluorescent particles for 30 min (A) at 4°C without inhibitor, (B) at 37°C without inhibitor, and (C–E) at 37°C with inhibitor. In these pharmacological experiments, PMN were incubated in the presence of the inhibitor for 5 min at 37°C prior to addition of particles. C: 100 μM SB203580 (SB); D: 8 μM GF109203X (GFX); E: 50 nM wortmannin (wtmn). F: Summary of phagocytosis expressed as the percentage of PMN containing fluorescent particles against total cells. The results are expressed as the mean ± S.D. of three experiments.

wortmannin moderately reduced it (lane 5), though this dose of wortmannin completely inhibited O_2^- production as the others did. These data indicate that the reduction of O_2^- production by p38 MAPK and PKC inhibitors is explained by their inhibitory activity to p47phox, whereas the reduction of O_2^- production by the PI3-K inhibitor is not fully explained by its inhibitory activity to p47phox. PI3-K may participate in other signaling pathways leading to the activation of NADPH oxidase.

3.4. Roles of p38 MAPK, PKC and PI3-K in phagocytosis

To investigate the roles of these kinases in phagocytosis, we examined the effects of inhibitors on phagocytosis in PMN. Phagocytic activity was evaluated by PMN that ingested serum-opsonized fluorescent latex particles with flow cytometry. Fig. 4A,B show the flow cytometric profiles obtained from PMN incubated in the presence of fluorescent particles for 30 min at 4°C and 37°C, respectively. A highly fluorescent cell population, indicating phagocytic PMN, appeared in the flow cytometric profile at 37°C without any inhibitor as shown in Fig. 4B. These phagocytic cells disappeared completely after treatment with 100 μM SB203580 (Fig. 4C,F) and were suppressed about 33% by 50 nM wortmannin (Fig. 4E,F). By contrast, treatment of PMN with 8 μM GF109203X facilitated the phagocytic activity (Fig. 4D,F). These results led us to conclude that p38 MAPK and PI3-K are deeply involved in the signaling pathway of phagocytosis and that PKC is inversely involved in it.

4. Discussion

Zymosan and latex particles used as stimuli in this study were opsonized by serum. These particles bound with IgG and iC3b were considered to be able to bind FcγR and CR3, respectively, followed by the induction of physiological responses. It was reported that cross-linking of FcγRs induced activation of PI3-K accompanied by activation of tyrosine kinases in U937 cells and PMN [8,16]. Several papers have shown that the inhibition of PI3-K attenuated NADPH oxidase activity, phosphorylation of p47phox in PMN stimulated by fMLP, and phagocytosis of IgG-coated erythrocytes in PMN [16,17]. In the present study we confirmed that PI3-K was involved in both signaling pathways leading to NADPH oxidase activation and phagocytosis induced by stimulation with FcγR and CR3.

The importance of PKC on PMN responses is already well known. Phorbol myristate acetate, a direct activator of PKC, promotes the phosphorylation and translocation of p47phox as the initial step for NADPH oxidase activation [2,24–26]. In this study, we showed that PKC was an important factor in NADPH oxidase activation and that its inhibition facilitated phagocytosis. These different responses of PMN to PKC activation indicated that PKC played different roles in the signaling pathways of NADPH oxidase activation and phagocytosis in bovine PMN. However, PKC inhibition has been reported to prevent phagocytosis in monocytes and PMN but not in macrophages in humans [6,11,14]. Therefore, the role of PKC in phagocytosis may be dependent on the cell type and/or species. Further research is required to solve this discrepancy.

Because FcγR and CR3 stimulation with sOZ induced p38 MAPK activation and both O_2^- production and p47phox phosphorylation were inhibited by SB203580, it is obvious that p38 MAPK is involved in the p47phox phosphorylation or upstream of it.

Concerning the mechanism of p38 MAPK-mediated phagocytosis, one possible target of p38 MAPK can be inferred to be MAPK-activating protein kinase-2, a Ser/Thr kinase that possesses the ability to phosphorylate small heat shock protein HSP27 [28] since HSP27 was reported to modulate actin microfilament dynamics [29] and the actin polymerization is known to be essential for phagocytosis [30]. Therefore, it seems possible to speculate that the inhibition of phagocytosis by SB203580 is due to impairment of p38 MAPK-mediated phosphorylation of HSP27. Additionally, it was reported that wortmannin prevented p38 MAPK activation in PMN and differentiated HL-60 cells stimulated by fMLP [27,28]. These data suggest that PI3-K exists upstream of p38 MAPK.

In conclusion, we showed the importance and the different roles of p38 MAPK, PKC and PI3-K in the signaling pathways of O_2^- production and phagocytosis in PMN. p38 MAPK and PI3-K participated in both signaling pathways of O_2^- production and phagocytosis stimulated with FcγR and CR3, and PKC participated in the signaling pathway of O_2^- production alone. The distinct role of each enzyme in O_2^- production and phagocytosis is important to understand the signaling pathways of bactericidal responses in PMN.

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