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Sphingosine Inhibition of NADPH Oxidase Activation in a Cell-Free System[1]

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The effects of sphingoid bases, sphingosine and dihydrosphingosine, which are protein kinase C (PKC) inhibitors, on NADPH oxidase were examined in a cell-free system. The bases inhibited cell-free activation of NADPH oxidase by arachidonic acid at lower concentration than *N*-acetylsphingosine. Thus, positive charge in the molecules may play a critical role in inhibition of the oxidase. Sphingosine did not change the K_m value for NADPH, but shifted the optimum concentration of arachidonic acid for activation of the oxidase. Moreover, sphingosine suppressed the translocation of p47-*phox*, one of the cytosolic components of the oxidase, to the membrane fraction, suggesting that the base inhibits the assembly of the components.

Key words: NADPH oxidase, neutrophil, sphingolipids, sphingosine, superoxide anion.

NADPH oxidase, a microbicidal enzyme complex, of phagocytes is activated by phagocytosis or by treatment with various stimulators, resulting in production of superoxide anion (O_2^-). Active oxygen, such as O_2^- and its metabolites, not only kills bacteria, but also injures adjacent normal cells and tissues. Thus, the host needs to control the activity of the oxidase.

The enzyme is composed of membrane proteins, large and small subunits of cytochrome b_{558} (*1-5*) and cytosolic proteins, p47-*phox* (*6-8*), p67-*phox* (*6, 9*), as well as a small GTP-binding protein Rac (*10, 11*). These cytosolic factors translocate to plasma membranes in the activation process. Protein kinase C (PKC) is involved in the activation by phosphorylating 46 kDa cytosolic protein (equivalent to human p47-*phox*) in guinea pig neutrophils (*12, 13*). On the other hand, anionic amphiphilic agents, such as SDS, activate the enzyme without phosphorylation by PKC (*14*). We have reported a possible role of cytosolic protein phosphatase as a regulator of the oxidase activation mediated by PKC (*15*). But it is unclear whether the PKC-independent activation is regulated by the same mechanism.

Recently, the physiological effects of sphingolipids have attracted much interest. Sphingosine, one of the sphingoid bases, was reported to have inhibitory effects on PKC (*16*) and phosphatidic acid phosphohydrolase (*17, 18*), and a stimulatory effect on 80 kDa diacylglycerol kinase (*19*). The base has also been reported to inhibit the production of O_2^- in human neutrophils through PKC inhibition (*20*). We reevaluated these effects using PMA and SDS as PKC-dependent and PKC-independent activators for NADPH oxidase, respectively. Furthermore, the effects of sphingosine were examined on cell-free activation of NADPH oxidase by arachidonic acid.

MATERIALS AND METHODS

Materials—Ferricytochrome *c* (horse heart), superoxide dismutase (SOD), D-sphingosine, DL-*erythro*-dihydrosphingosine, and *N*-acetyl-D-sphingosine were purchased from Sigma Chemical; SDS (electrophoresis grade) from Wako Pure Chemical; acrylamide (electrophoresis grade) from Nacalai Tesque; phorbol-12-myristate-13-acetate (PMA) and arachidonic acid from Funakoshi Pharmaceutical; NADPH from Kohjin; staurosporine from Boehringer Mannheim; peroxidase-conjugated goat IgG against rabbit IgG Fc from Organon Teknika; molecular weight standards for SDS-PAGE from Bio-Rad Laboratories. All other chemicals were of reagent grade from commercial sources. Anti-mouse p47-*phox* C-terminal peptide antibody was prepared as described previously (*21*).

PMA, staurosporine, arachidonic acid, D-sphingosine, DL-*erythro*-dihydrosphingosine, and *N*-acetyl-D-sphingosine were dissolved in dimethylsulfoxide and the final concentration of dimethylsulfoxide in the assay system was adjusted to 1% throughout.

Preparation of Guinea Pig Neutrophils—Neutrophils were obtained from the peritoneal cavities of female guinea pigs of Hartley strain, as reported previously (*22*). The content of neutrophils in the preparation was more than 90%.

Measurement of O_2^- Production in Neutrophils—O_2^- production was measured on the basis of superoxide

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Abbreviations: O_2^-, superoxide anion; Pipes, 1,4-piperazine-diethanesulfonic acid; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; SDS, sodium dodecylsulfate; SOD, superoxide dismutase.

dismutase-inhibitable reduction of ferricytochrome *c* by the anion produced (*23*). Neutrophils (1×10^6 cells/ml) were suspended in a Hanks'-Pipes buffer (isotonic medium) containing 8 mM Pipes, 137 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 1.28 mM $CaCl_2$, 0.43 mM Na_2HPO_4, 0.44 mM KH_2PO_4, and 5.5 mM glucose or hypotonic medium prepared by decreasing the Na^+ concentration in an isotonic medium to 50 mM. The suspension was preincubated at 37°C for 5 min in the presence of 100 μM ferricytochrome *c* and PKC inhibitors (staurosporine, sphingosine, or dihydrosphingosine). Then the stimulants were added and the reaction mixture was incubated at 37°C for 5 min. To stop the reaction, the mixture was chilled in an ice water bath and centrifuged at 4°C at $120\times g$ for 10 min to precipitate neutrophils. Reduced cytochrome *c* in the supernatant was measured on the basis of the increase in absorbance at 550 nm. Accumulation of O_2^- production was calculated on the basis of an absorption coefficient of 21.0 $mM^{-1}\cdot cm^{-1}$.

Preparation of Plasma Membranes and Cytosol from Neutrophils—Neutrophils (7.5×10^7 cells/ml) were suspended in a relaxation buffer (pH 7.3) containing 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, and 10 mM Pipes, and disrupted by sonication (2 A, 15 s) twice at 0°C in the presence of protease inhibitors. The lysate was fractionated on a discontinuous Percoll gradient by a reported method with a slight modification (*24*). Plasma membranes were obtained by centrifugation of pooled γ-fraction at $100,000\times g$ at 4°C for 1 h and suspended to a concentration equivalent to 10^8 cells/ml in the same buffer. The plasma membranes and cytosol were divided into portions and stored at −80°C. PMA-activated membranes were prepared from neutrophils stimulated with 100 nM PMA for 3 min at 37°C.

Measurement of NADPH Oxidase Activity in a Cell-Free System and in Activated Membrane Fraction—In the case of the cell-free system, assay mixtures consisting of plasma membranes (30 μg/ml protein), cytosol (100 μg/ml protein), 100 μM ferricytochrome *c*, and arachidonic acid were preincubated for 3 min at 25°C. Cytosol fraction and arachidonic acid were not added in the case of measurement of PMA-activated membranes. Then the reaction was initiated by the addition of 200 μM NADPH. NADPH oxidase activity was measured as superoxide dismutase-sensitive reduction of ferricytochrome *c* at 25°C and expressed as nmol O_2^-/min/mg membrane protein. Inhibitors were added just before preincubation.

Translocation Assay—The reaction mixture (2.5 ml) consisted of plasma membranes (30 μg/ml protein), cytosol (100 μg/ml protein), 38 μM arachidonic acid, and sphingosine (0, 5, or 15 μM); this was incubated for 3 min at 25°C to reconstitute NADPH oxidase. The mixture (2 ml) was carefully layered onto the same volume of relaxation buffer containing 15% (w/v) sucrose, and centrifuged at 4°C and $100,000\times g$ for 2 h according to Park's method with a slight modification (*25*). The supernatant was concentrated to about 50 μl in a Centricon 10 (Amicon).

The precipitated "membranes" and the concentrated "cytosol" were subjected to SDS-PAGE on 10% polyacrylamide gels using the Laemmli buffer system (*26*). The separated proteins were electrophoretically transferred onto a nitrocellulose sheet, probed with a 1:1,000-fold dilution of anti-mouse p47-*phox* antibody and then probed with a 1:3,000-fold dilution of horseradish peroxidase-conjugated goat anti-rabbit Fc antibodies, and finally detected by enhanced chemiluminescence with Du Pont's Western blotting detection system. Increase in p47-*phox* in the membrane fraction and decrease in the component in the cytosol fraction indicates the translocation of p47-*phox* from cytosol to membranes.

RESULTS

Effects of Staurosporine and Sphingoid Bases on O_2^- Production in Neutrophils—We examined the inhibitory effects of two types of PKC inhibitors, staurosporine and sphingoid bases (sphingosine and dihydrosphingosine), on O_2^- production in guinea pig neutrophils. As reported previously (*14*), O_2^- production by PMA was inhibited by staurosporine, but that by SDS in hypotonic medium was little affected (Fig. 1a). In contrast, treatment with sphingosine (Fig. 1b) and dihydrosphingosine (Fig. 1c) concentration-dependently inhibited the O_2^- production by both activators. These data indicate that inhibition of O_2^- production by sphingoid bases cannot be explained only by

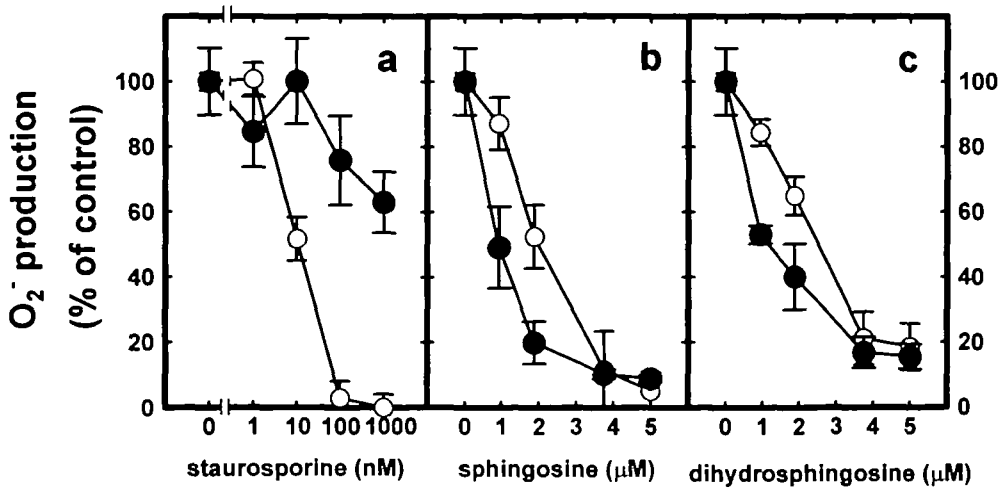

Fig. 1. **Effects of PKC inhibitors on NADPH oxidase activation in intact neutrophils induced by PMA or SDS.** Neutrophils were preincubated in the presence of PKC inhibitors (a, staurosporine; b, sphingosine; and c, dihydrosphingosine), and then stimulated by 50 μM SDS in hypotonic medium (●) or 100 nM PMA in isotonic medium (○). Other experimental conditions are described in "MATERIALS AND METHODS." The O_2^- production is expressed as a percentage of the control (without inhibitors): 27.2±2.8 (SDS in hypotonic medium) and 24.2±0.7 nmol O_2^-/5 min/10^6 cells (PMA). Each value is the mean±SE of at least three experiments.

the effect on PKC inhibition. The above-mentioned inhibitory effect was not due to cytotoxicity of sphingoid bases, as judged from the results of the trypan blue exclusion test (data not shown).

Effects of Sphingolipids on Activation of NADPH Oxidase in a Cell-Free System—The effects of sphingolipids (sphingosine, dihydrosphingosine, and *N*-acetylsphingosine) on activation of NADPH oxidase by arachidonic acid were examined in a cell-free system to investigate whether sphingoid bases directly affected the activation. As shown in Fig. 2, sphingolipids concentration-dependently inhibited the activation of the oxidase. The IC_{50} values were 6 and 7 μM for sphingosine and dihydrosphingosine, respectively, but a higher concentration was required in the case of *N*-acetylsphingosine ($IC_{50}=23$ μM). It is generally accepted that activation of NADPH oxidase in a cell-free system is independent of PKC. We also confirmed that staurosporine (up to 100 nM) had little effect on the cell-free activation (data not shown). Thus, the inhibitory effect of sphingolipid seems not to be due to PKC inhibition.

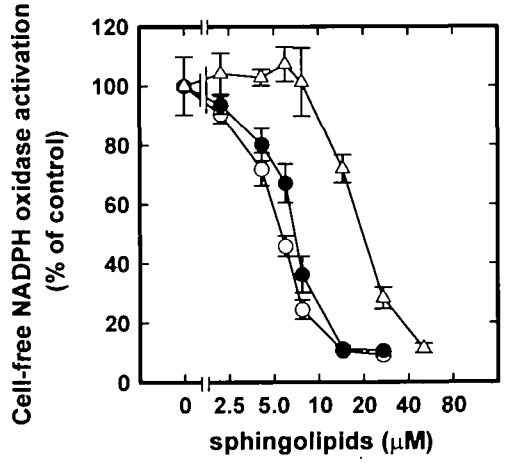

Fig. 2. **Effects of sphingolipids on activation of NADPH oxidase in a cell-free system.** NADPH oxidase was activated in a cell-free system by arachidonic acid in the presence of sphingosine (○), dihydrosphingosine (●), or *N*-acetylsphingosine (△). Other experimental conditions are described in "MATERIALS AND METHODS." Values are expressed as percentages of the control (without sphingolipids, producing 97.1±9.6 nmol O_2^-/min/mg protein of membranes) and each value is the mean±SE of at least three experiments.

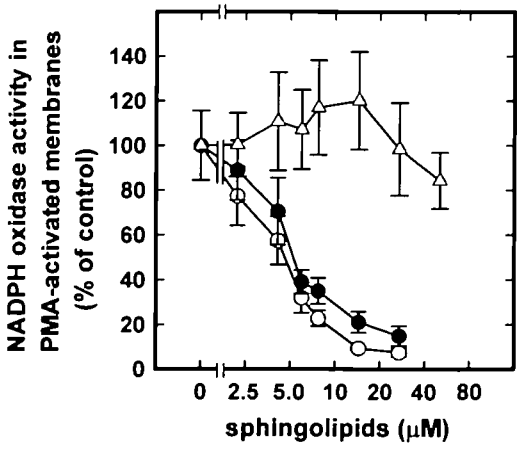

Fig. 3. **Effects of sphingolipids on NADPH oxidase in PMA-activated membrane.** Neutrophils were stimulated with 100 nM PMA, then the membrane fraction was prepared. NADPH oxidase activity in the fraction was measured in the presence of sphingosine (○), dihydrosphingosine (●), or *N*-acetylsphingosine (△). See "MATERIALS AND METHODS" for other experimental conditions. Values are expressed as percentages of the control (without sphingolipids, producing 18.0±2.8 nmol O_2^-/min/mg protein of membranes) and each value is the mean±SE of at least three experiments.

It was previously reported that alkylamine suppressed O_2^- production of the membrane fraction prepared from neutrophils activated by PMA (*27*). Thus, the effects of sphingolipids were examined on NADPH oxidase activity of the membrane fraction of neutrophils stimulated with PMA. Sphingosine and dihydrosphingosine suppressed the activities at the same concentration in the case of cell-free activation, but *N*-acetylsphingosine did not (Fig. 3).

The former two bases inhibited not only the enzyme activation, but also the activated oxidase, differing from *N*-acetylsphingosine. These data suggest that positive charge in these molecules may be significantly involved in the inhibitory effect.

TABLE I. **Kinetic parameters of NADPH oxidase for NADPH.** Neutrophil membranes (30 μg/ml protein), cytosol (100 μg/ml protein), and arachidonic acid (38 μM) were preincubated in the presence of 5 μM sphingosine, 5 μM dihydrosphingosine, or 15 μM *N*-acetylsphingosine. Then, various concentrations of NADPH were added. The activity of NADPH oxidase was assayed as described in "MATERIALS AND METHODS" and kinetic parameters were calculated from the Lineweaver-Burk plots. Values are the mean±SE of at least five experiments.

Sphingolipids	K_m (μM)	V_{max} (nmol/min/mg)
None	36±5.2	133±19
Sphingosine (5 μM)	38±5.7	71±8.7
Dihydrosphingosine (5 μM)	32±6.4	83±15
N-Acetylsphingosine (15 μM)	33±8.4	112±20

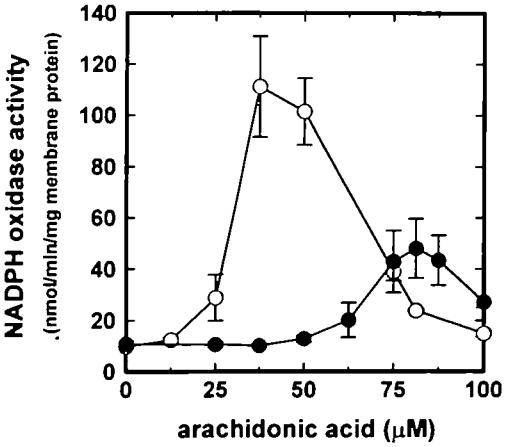

Fig. 4. **Effect of sphingosine on optimum concentration of arachidonic acid to activate NADPH oxidase in a cell-free system.** NADPH oxidase was activated in a cell-free system by various concentrations of arachidonic acid in the absence (○) or presence (●) of 15 μM sphingosine. See "MATERIALS AND METHODS" for other experimental conditions. Values are expressed as O_2^- production and each value is the mean±SE of at least three experiments.

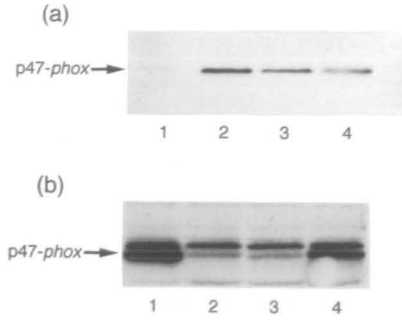

Fig. 5. **Translocation of p47-*phox* by arachidonic acid.** Neutrophil membranes (30 μg/ml protein), cytosol (100 μg/ml protein) and arachidonic acid (lane 1, 0 μM; lanes 2, 3, and 4, 38 μM) were incubated in the presence (lane 3, 5 μM; lane 4, 15 μM) or absence (lanes 1 and 2) of sphingosine. Then the mixture was re-separated to membranes and cytosol as described in "MATERIALS AND METHODS." Each fraction was examined by Western analysis using anti-mouse p47-*phox* antibody. Panels (a) and (b) indicate the results for membranes and cytosol, respectively.

Mechanisms of the Inhibitory Effects of Sphingosine on Activation of NADPH Oxidase—To investigate the inhibitory mechanism of sphingoid bases, the kinetic parameters (K_m and V_{max}) were determined. Sphingolipids (5 μM sphingosine and dihydrosphingosine and 15 μM *N*-acetylsphingosine) did not influence the K_m value for NADPH, but sphingosine and dihydrosphingosine made the V_{max} lower (Table I). These results indicate that sphingolipid bases did not affect the affinity for NADPH. It is well known that anionic amphiphiles such as arachidonic acid and SDS activate NADPH oxidase in a cell-free system. For arachidonic acid, 38 μM gave the maximum activation under our experimental conditions. We examined the effect of sphingosine on the concentration of arachidonic acid for maximum activation. Sphingosine neutralized the effect of arachidonic acid, because the base shifted the optimum concentration from 38 to 81 μM (Fig. 4).

Effect of Sphingosine on Translocation of Cytosolic Component(s)—Cytosolic components of NADPH oxidase translocate to plasma membranes in the course of the activation. The effect of sphingosine on the translocation of p47-*phox* (one of the cytosolic components) was investigated with Western blotting, using anti-mouse p47-*phox* C-terminal peptide antibody. Arachidonic acid promoted translocation of p47-*phox* from cytosol to membranes (Fig. 5, lanes 1 and 2), while sphingosine inhibited the translocation in a concentration-dependent manner (Fig. 5, lanes 2, 3, and 4). These results indicate that sphingosine suppresses the assembly of the components of NADPH oxidase by arachidonic acid for the activation.

DISCUSSION

Sphingoid bases (sphingosine and dihydrosphingosine) have been reported to inhibit O_2^- production *via* PKC inhibition in intact neutrophils (*20*). But the bases inhibited the production induced by SDS under a hypotonic condition, which was resistant to staurosporine (Fig. 1). So, in this study the possibility was examined that the bases affect the PKC-independent cell-free activation of the NADPH oxidase. The bases inhibited both the activation process (Fig. 2) and the activity of the activated oxidase when the system was stimulated by PMA (Fig. 3). *N*-Acetylsphingosine had little effect on the former (Fig. 2) or the latter (Fig. 3). Thus, positive charge in the molecules seemed to be important for the inhibition.

In recent studies, various cationic agents, *e.g.* polyaminoacids (*28*) and spermine (*29*), were found to inhibit cell-free activation of the oxidase. The effect of basic polyaminoacids, not neutral ones, was due to their positive charge. Similarly, positive charge was suggested to be important in the inhibitory effect of sphingosine on NADPH oxidase, because the *N*-acetylated derivative had a weak effect (Figs. 2 and 3). The effect of polyaminoacids and spermine may not be similar to that of sphingosine, because only sphingosine shifted the optimum concentration of arachidonic acid to activate the oxidase (Fig. 4). Sphingoid bases seem to neutralize the effect of arachidonic acid.

Though the exact mechanism by which sphingosine inhibits the cell-free activation of the oxidase is not clear at present, sphingosine probably inhibits constitution of the NADPH oxidase complex, because it suppresses the translocation of one of the NADPH oxidase components, p47-*phox* (Fig. 5).

Amphiphilic agents, *e.g.* arachidonic acid and SDS, affect membrane motility, including fluidity, and may modify lipid-protein interaction. Moreover, arachidonic acid alters protein-protein interaction in the cytosol. For example, Rac-GDI complexes are dissociated by arachidonic acid, resulting in NADPH oxidase activation (*30-32*). Arachidonic acid also enhances the association of p47-*phox* with p22-*phox* or p67-*phox*, which is mediated by the interaction between the SH3 region and a proline-rich region in the components (*33*). Sphingosine may modify the effect of arachidonic acid on lipid-protein or protein-protein interaction.

Active oxygen generated by phagocytes is important for host defense, but injures normal cells and tissues. Thus, control of the oxidase activity would be important. We have previously reported that cytosolic protein phosphatase may be one of the regulators for the oxidase (*15*). Now, we suggest that sphingosine may be another regulator. Sphingosine is reported to exist in intact neutrophils and to increase during incubation (*34*). It was also reported that tumor necrosis factor α (TNF-α) activated sphingomyelinase in HL-60 cells (*35*), stimulated sphingomyelinase, and elevated ceramide and sphingosine levels, resulting in apoptosis in human neutrophils (*36*). It was calculated that several micromolar sphingosine exists in neutrophils (*34, 36*). It is plausible that sphingosine generated by these processes may prevent mis- or over-activation of NADPH oxidase.

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