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# EFFECT OF SPHINGOSINE AND ITS *N*-METHYL DERIVATIVES ON OXIDATIVE BURST, PHAGOKINETIC ACTIVITY, AND TRANS-ENDOTHELIAL MIGRATION OF HUMAN NEUTROPHILS

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**Abstract**—Neutrophils display three major functions: (i) oxidative burst, (ii) phagokinetic activity, and (iii) trans-endothelial migration. Sphingosine (SPN) is known to inhibit oxidative burst in human neutrophils via inhibition of protein kinase C (PKC). SPN is metabolically converted into *N,N*-dimethylsphingosine (DMS) in some tissues and cell lines. In previous studies, we have demonstrated that the PKC-inhibitory effect of DMS is stronger than that of SPN, and that of the synthetic analogue *N,N,N*-trimethylsphingosine (TMS) is even stronger. Therefore, in the present study, we compared the effects of SPN, DMS, and TMS on the neutrophil functions mentioned above. These three compounds, at 10–20 $\mu$M, showed equal inhibition of phorbol 12-myristate 13-acetate (PMA)-dependent superoxide ($O_2^-$) production and $O_2$ consumption. They and other known PKC inhibitors (H-7, staurosporine, calphostin C), at 1–5 $\mu$M, showed equal inhibition of the phagokinetic activity of neutrophils. On the other hand, trans-endothelial migration of neutrophils was suppressed by SPN, DMS, and TMS at 5–10 $\mu$M, but was relatively unaffected by the other PKC inhibitors. All of these compounds inhibited PMA-induced phosphorylation of major neutrophil proteins with a $M_r$ of 60 and 47 kDa; this effect is ascribable to inhibition of PKC. Despite the similar effects of SPN, DMS, and TMS on neutrophil function, TMS was considerably less cytotoxic to neutrophils under the same experimental conditions. Furthermore, SPN and DMS at 10–20 $\mu$M caused obvious morphological changes of the endothelial cells, but TMS did not. SPN undergoes rapid metabolic conversion to various sphingolipid compounds, but TMS is stable. In view of all these findings, TMS appears to be a superior pharmacological agent, compared to SPN derivatives or other PKC inhibitors, for suppression of neutrophil overfunction associated with inflammatory processes and tissue injury.

Neutrophils (polymorphonuclear leukocytes) display three functional responses upon exposure to a variety of agonists: (i) oxidative burst leading to the production of free radical oxygen (superoxide; $O_2^-$); (ii) chemotactic and phagokinetic activity; and (iii) interaction with activated endothelial cells (ECs†) and platelets, followed by extravascular migration (trans-endothelial migration). These functions collectively provide a basis for bactericidal action, tissue repair, homeostasis, and self-defense mechanisms. While recruitment of neutrophils at the site of infection or inflammation is an essential physiological event, excessive accumulation and extravascular infiltration of neutrophils can result in tissue damage and serious circulatory disturbances. Therefore, a need exists for pharmacological reagents capable of suppressing neutrophil overfunction. Superoxide dismutase (SOD) has been shown to eliminate $O_2^-$ and thereby reduce oxidative tissue damage due to neutrophil overfunction [1, 2]. Sphingosine (SPN), a membrane sphingolipid catabolite known to inhibit protein kinase (PKC) activity, was shown recently to inhibit oxidative burst in neutrophils [3, 4]. The effect of SPN on chemotactic/phagokinetic activity and trans-endothelial migration of neutrophils remained unclear. SPN is metabolically converted to *N,N*-dimethylsphingosine (DMS) [5, 6] or ceramide (Cer) [6], or is degraded via the classic SPN kinase pathway through sphingosine-1-phosphate (SPN-1-P) into ethanolamine phosphate and palmitaldehyde [7–11]. In comparison to SPN and DMS, the analogue *N,N,N*-trimethylsphingosine (TMS), which was chemically synthesized in this laboratory, shows a much stronger inhibitory effect on PKC [12]. Therefore, TMS was considered as a potential pharmacological reagent for the suppression of tumor cell growth and metastatic potential [12, 13]. In the present study, we evaluated the effect of TMS

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† Abbreviations: BSA, bovine serum albumin; Cer, ceramide; DMS, *N,N*-dimethylsphingosine; EC, endothelial cell; FBS, fetal bovine serum; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HUVEC, human umbilical vein endothelial cell; MeOH, methanol; $M_r$, relative molecular weight; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase; SPN, sphingosine; SPN-1-P, sphingosine-1-phosphate; and TMS, *N,N,N*-trimethylsphingosine.

(relative to SPN) on phagokinetic activity and transendothelial migration of human neutrophils.

## EXPERIMENTAL PROCEDURES

### *Materials*

SPN and DMS were synthesized as previously described [14], and TMS was synthesized by exhaustive methylation of O-protected DMS [15]. Cer (type III), dimethyl sulfoxide, cytochrome *c* (type VI), SOD from bovine erythrocytes, dextran (average $M_r$ 162K), trypan blue staining agent, and RPMI were purchased from the Sigma Chemical Co. (St. Louis, MO). Human umbilical vein endothelial cells (HUVECs) and CS-C culture medium (acidic fibroblast growth factor and heparin attached) were purchased from Cell Systems (Kirkland, WA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochem (La Jolla, CA). Protein kinase inhibitor H-7 was from Seikagaku Kogyo (Tokyo, Japan). Staurosporine and calphostin C were from the Kamiya Biochemical Co. (Thousand Oaks, CA). Other chemicals were reagent grade and purchased from Sigma. $^{32}$P-Labeled sodium phosphate was from NEN Products (Boston, MA). [1-$^3$H]SPN was synthesized in this laboratory as previously described [15]. [$^{14}$C]DMS (*N*,*N*-dimethyl-[*N*-$^{14}$C]-SPN) and [$^{14}$C]TMS (*N*,*N*,*N*-trimethyl-[*N*-$^{14}$C]-SPN) were prepared from SPN and DMS, respectively, with $^{14}CH_3I$ and $K_2CO_3$ reacted in methanol (MeOH) solution. *N*-Acetyl-SPN and $C_8$-Cer (*N*-octanoyl-SPN) were synthesized as previously described [16]. SPN-1-P was prepared as previously described [17] from sphingosyl-phosphocholine with bacterial phospholipase D. Stock solutions of PMA (3 mM) and SPN-1-P (2 mM) were prepared in ethanol, formyl-methionyl-leucyl-phenylalanine (4 mM) in dimethyl sulfoxide, and TMS (2 mM), DMS (2 mM), and SPN (2 mM) in 50% ethanol. These stock solutions were stored in the dark at −20°, and sonicated for 30 sec at room temperature before use, with appropriate dilution.

### *Isolation of human neutrophils*

Human neutrophils were obtained from healthy adult males. Heparinized peripheral blood was mixed with an equal amount of 1% dextran solution in phosphate-buffered saline (PBS) in a 60-mL injection syringe, and left to sit vertically for 60–90 min at room temperature [18]. The upper phase (rich in white blood cells) was placed gently on the same volume of Ficoll–Paque (Pharmacia LKB, Uppsala, Sweden) in Falcon 2095 plastic test tubes (Becton Dickinson Labware, Lincoln Park, NJ). Centrifugation at 450 *g* for 30 min at 4° brought neutrophils to the bottom of the tubes. Upper phase and interface, which contain lymphocytes, were removed carefully by aspiration. Contaminating erythrocytes in the pellet were removed by hypotonic lysis, i.e. the pellet was resuspended in ice-cold distilled water for 30 sec, and then an equal volume of ice-cold 1.8% NaCl solution was added. After centrifugation at 100 g for 10 min, cells were resuspended with buffer or medium as described for each experiment. The final preparation consisted of >98% neutrophils, as determined by Wright-Giemsa staining. Suspensions were stored on ice and used within 3 hr.

### *Inhibition of $O_2^-$ production*

$O_2^-$ production in human neutrophils was examined based upon $O_2^-$-mediated reduction of cytochrome *c* [19]. Freshly purified neutrophils ($1 \times 10^6$/mL) were incubated with 10 μM cytochrome *c* in PBS containing 7.5 mM glucose with or without SPN derivatives (which were dissolved in 50% ethanol solution, and then added to the cell suspension; final concentration of ethanol in the suspension medium was 0.5%) for 5 min at room temperature in a 1-mL cuvette. Next, neutrophils were stimulated by the addition of 1 μM PMA, and $O_2^-$ production was quantified by reduction of cytochrome *c* as monitored by $O.D._{550}$ reading. Viability of neutrophils under these conditions was >95%, as examined separately by trypan blue exclusion assay.

### *$O_2$ Consumption*

$O_2$ consumption was measured using a Clark-type electrode with a Y.S.I. model 5300 biological $O_2$ monitor and micro $O_2$ chamber assembly (Y.S.I. Inc., Yellow Springs, OH). Assays were performed at 37° with $6 \times 10^6$ neutrophils/mL (total volume 600 μL). Cell suspensions in Eppendorf test tubes were warmed up at 37° before being placed in the microchamber. Test compounds were injected carefully into the chamber using a Hamilton syringe, and mixed with a microstirrer (120 rpm) at 37° for 5 min. PMA (final concentration 1.0 μM) was added to the preincubated cell suspension, and $O_2$ concentration in the chamber was monitored.

### *Assay of phagokinetic activity*

*Preparation of gold particle and coating.* Phagokinetic activity was observed by tracing phagokinetic tracks of neutrophils moving on a glass slide covered with gold colloid particles, as previously described [20]. Briefly, 22 × 22 mm glass coverslips (Corning Glass Works, Corning, NY) were dipped for 1 min into a solution of 1% bovine serum albumin (BSA) in distilled water. BSA solution was freshly prepared each day, and filtered using 0.20 μm Nalgene filters (Nalgene Labware Division, Rochester, NY). The coverslip was drained by contact with a Kim-wipe, dipped into 100% ethanol, quickly dried under a hairdryer (85°), and placed in a 3.5-cm Falcon 3001 plastic dish. $AuCl_4H$ solution (5.4 mL) and 36.5 mM $Na_2CO_3$ solution (18 mL) were added to 33 mL of distilled water and heated in a glass flask over a bunsen burner. Immediately after the boiling point was reached, 5.4 mL of 0.1% formaldehyde solution in water was added and mixed. Gold particle suspension (brownish color) was placed into dishes (2 mL each) and left for 2 hr at room temperature. Particle-coated coverslips were washed in PBS, twice in RPMI 1640, and placed in a different Falcon 3001 plastic dish containing 2 mL of RPMI 1640 with glutamate and pyruvate without fetal bovine serum (FBS). Coverslips were stored at 4° and used within 24 hr after preparation.

*Experimental.* Freshly purified human neutrophils ($1 \times 10^4$/plate) were seeded on gold colloid-coated coverslips in RPMI 1640 medium. SPN derivatives

solubilized in 50% ethanol solution were added to the culture (final concentration of ethanol in culture was 0.1%). In the control experiment, medium alone (0.1% ethanol) was added. At this concentration, ethanol has no significant effect on neutrophil activity. To examine cell recovery from the inhibitory effect of SPN derivatives, neutrophils ($2 \times 10^6$/mL) were preincubated for 10 min at 37° with 12 μM SPN, DMS, or TMS, and then added dropwise to plates at 200-fold dilution (final concentration $1 \times 10^4$ cells/plate; 0.06 μM SPN derivative). These dilution methods were designed to avoid aggregation during centrifugation of incubated neutrophils. Incubation was continued at 37° for 2 hr under 5% $CO_2$, and terminated by adding 200 μL of 10% formaldehyde. Microscopic examination of each plate was performed with a Nikon microscope, connected to a Polaroid camera. Phagokinetic activity was measured by tracing the track of each neutrophil, moving around to sweep the gold colloid on the coverslip by phagocytosis. The track was photographed, and phagokinetic activity quantitated by cutting and weighing the swept area in the photograph [20].

*Transmigration assay with HUVEC monolayer*

*Endothelial culture.* HUVECs were maintained and cultured as previously described [21], with some modifications. Briefly, HUVECs were maintained in 10-mL tissue culture flasks on a collagen type I bed obtained from rat tail (Upstate Biotechnology, Lake Placid, NY). Cells became confluent within 2–4 days when incubated in 5% $CO_2$ at 37°. For the transmigration assay, thick gels were formed in 24-well tissue culture plates. Preparation of gel solution was carried out at 4°. Eight parts of collagen solution (Vitrogen 100, from bovine tendon containing less than 5 ng/mL endotoxin; Celtrix Laboratories, Palo Alto, CA), one part of 10× RPMI, and one part of alkaline solution (2.2 g $NaHCO_3$ in 100 mL of 0.05 N NaOH and 200 mM HEPES) were mixed and then distributed at 0.7 mL/well [22]. The solution was allowed to gel at 37° for 3 hr; then wells were washed with 1.0 mL of CS-complete medium at least four times, every 3–6 hr, during further incubation at 37° in 5% $CO_2$. HUVECs (passage level<3) were detached from 10-mL tissue culture flasks with 3 mL of trypsin-EDTA. M199 medium (3 mL) with 20% heat-inactivated FBS was added and centrifuged to collect the cells, and the pellet was resuspended in 10 mL of CS-complete medium. Homogeneous cell suspension was placed (0.8 mL each) onto the thick collagen bed, and incubated for 3 hr at 37°. Supernatant (containing dead cells) was aspirated carefully, and then switched to new CS-complete medium. Under these conditions, HUVECs formed confluent monolayers within 48 hr, and retained this property after a day in culture. These results are in agreement with previous studies in which HUVECs grown on collagen gels displayed silver stain at cell borders and development of intracellular junctions as determined by electron microscopy [23]. Light microscopic examination of cross-sections of HUVEC monolayers also confirmed the structure (see Fig. 4).

*Transmigration assay.* HUVEC monolayers in 24-well tissue culture plates were washed with M199 without FBS, and then incubated with 1 mL/well of IL-1β (Boehringer Mannheim Biochemical Products, Indianapolis, IN) dissolved in M199 with 1% heat-inactivated FBS at 10 U/mL. Wells were incubated at 37° in 5% $CO_2$ for 4 hr and then washed with 1 mL of M199. Purified neutrophil suspension ($1.5 \times 10^6$ cells/mL) in M199 with 1% heat-inactivated FBS was preincubated with TMS or other effectors for 10 min at 37°. After aspirating the medium in the wells, neutrophil suspension was added (0.5 mL each) onto the HUVEC monolayers. The tissue culture plate was incubated for 90 min at 37°, then terminated by aspirating the cell suspension, adding 1 mL of 10% formaldehyde in PBS, and stored at 4° overnight for fixation. The edge of the collagen bed was cut with a small spatula along the wall 2 hr before removal from the well. The collagen bed (having HUVEC on its surface and transmigrated neutrophils inside) was embedded in paraffin, followed by staining with hematoxylin-eosin for microscopic examination.

*Protein phosphorylation*

Freshly purified neutrophils ($\approx 8 \times 10^7$) were preincubated at 37° with 2 mCi of $^{32}$P-labeled sodium phosphate (specific activity 8500–9120 Ci/mmol) in a buffer containing 0.1% lipid-free BSA/10 mM HEPES (pH 7.4), 136 mM NaCl, 4.9 mM KCl, 5.6 mM glucose, and 0.33 mM $CaCl_2$ for 60 min in a shaking waterbath. Excess unbound components were removed by centrifugation and pellets were resuspended in the buffer (repeated twice). Cells were divided into seven treatment groups, each containing $\approx 1 \times 10^7$ cells in a volume of 0.4 mL. TMS, SPN, DMS, or an equal volume of 50% ethanol were added to the suspension and incubated at 37° for 10 min, followed by addition of PMA to a final concentration of 1.5 μM. Two minutes later, the reaction was terminated by adding 0.1 mL of Laemmli's sample buffer including 20 mM EDTA, followed by heating at 100° for 5 min. Aliquots were electrophoresed on 10% sodium dodecyl sulfate gel as previously described [24]. $^{32}$P Incorporation was visualized by autoradiography.

*Metabolism of [$^3$H]SPN and [$^{14}$C]TMS in human neutrophils*

Freshly isolated human neutrophils ($2 \times 10^6$) were incubated in 1 mL RPMI medium with [$^3$H]SPN ($1.5 \times 10^4$ cpm) or [$^{14}$C]TMS ($1 \times 10^4$ cpm). After various periods of time, the cell suspension was extracted as previously described [25]. Upper and lower fractions were dissolved in MeOH and $CHCl_3$/MeOH (2:1), respectively, then applied to high performance silica gel TLC plates (EM Science, Gibbstown, NJ) and developed in $CHCl_3$/MeOH/water (50:40:10), $CHCl_3$/MeOH/$NH_4OH$ (45:35:10), and butanol/acetic acid/water (3:1:1). The bands were identifed by staining the control lipids with primulin, and visualized under ultraviolet light. After enhancer (Resolution TLC™, L.M. Corp., Chestnut Hill, MA) treatment of TLC plates, autoradiography was performed with Kodak X-OMAT film at −80° for 1–14 days.

## RESULTS

### *Inhibitory effects of TMS and SPN on $O_2^-$ production and $O_2$ consumption*

$O_2^-$ production in neutrophils as determined by reduction of cytochrome *c* was inhibited by a 10-min TMS pretreatment in a concentration-dependent manner (Fig. 1A). $O_2^-$ production was completely abolished by 20 μM TMS, DMS, or SPN; however, pretreatment with the same concentration of Cer produced a minimal effect (Fig. 1B). When TMS was added halfway through the normal reaction (5 min), inhibition of $O_2^-$ production was apparent immediately. $O_2$ consumption of neutrophils determined by electrical conductivity was enhanced markedly by PMA. This PMA-dependent enhancement of $O_2$ consumption was inhibited strongly by TMS and SPN (Fig. 2). A similar range of concentrations (15–45 μM) of Cer, $C_8$ Cer (*N*-octanoyl-SPN), and *N*-acetyl-SPN did not produce any inhibition of PMA-induced $O_2$ consumption (data not shown).

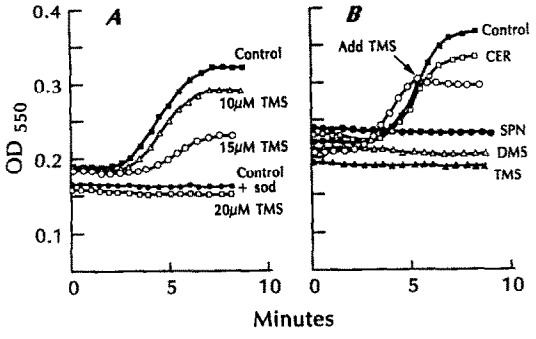


Fig. 1. Inhibition of $O_2^-$ production in neutrophils by SPN derivatives. Freshly purified neutrophils ($1 \times 10^6$/mL) were incubated with 10 μM cytochrome *c* in PBS containing 7.5 mM glucose with or without SPN derivatives (which were dissolved in 50% ethanol solution, and then added to the cell suspension; final concentration of ethanol in the suspension medium (0.5%) for 5 min at room temperature in a 1-mL cuvette. Next, neutrophils were stimulated by the addition of 1 μM PMA, and $O_2^-$ production was quantified by the reduction of cytochrome *c* (SOD-sensitive) as monitored by $O.D._{550}$ reading. (A) Concentration-dependent effect of TMS. In the "Control + SOD" experiment, SOD (30 μg/mL) was added during incubation. (B) Effects of various SPN derivatives at a 20 μM concentration. In one experiment, 20 μM TMS was added at the midpoint of incubation (indicated by arrow).

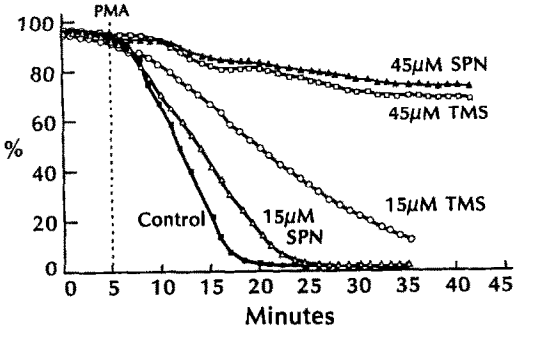


Fig. 2. Inhibition of $O_2$ consumption by SPN and TMS. $O_2$ consumption of human neutrophils ($6 \times 10^6$/mL; total volume 600 μL) was measured as described in the text. Partial pressure of oxygen ($pO_2$) in culture solution before the reaction (air-saturated) is defined as 100%, and relative reduction in $pO_2$ is shown as a percentage. PMA (final concentration 1 μM) was added to the cell suspension preincubated with SPN or TMS in 0.5% ethanol solution for 5 min. In the control experiment, medium alone (0.5% ethanol) was used.

### *Phagokinetic activity of neutrophils on gold sol-coated plates, and its suppression by various SPN derivatives and PKC inhibitors*

Phagokinetic tracks produced by neutrophils, evaluated as described in Experimental Procedures, are shown in Fig. 3. Phagokinetic activity was markedly suppressed by as little as 1.5 μM TMS, and completely suppressed by 4.5 μM TMS (Fig. 3, B and C). The activity was also strongly inhibited SPN, DMS, and various PKC inhibitors (H-7, staurosporine, calphostin C) (Table 1). However, the TMS-dependent inhibitory effect was reversed completely when culture medium was replaced with TMS-depleted medium by the dilution method, whereas the inhibitory effects of SPN and DMS persisted even when these compounds were eliminated from the culture medium. Uptake and release rates of [$^{14}$C]TMS, [$^{14}$C]DMS, and [$^3$H]SPN by neutrophils under these treatments were roughly the same. These findings suggest that the inhibitory effects of SPN and DMS on phagokinetic activity are at least partially dependent on cytotoxicity, whereas that of TMS is not.

### *Inhibitory effects of SPN derivatives on trans-endothelial migration of neutrophils*

One remarkable phenotypic characteristic of neutrophils is their ability to adhere to activated ECs and migrate through the EC monolayer into the vascular or extravascular matrix. The experimental system described in Experimental Procedures allowed us to study the effects of SPN, DMS, and TMS on neutrophil interactions with ECs, and their subsequent trans-endothelial migration. When ECs were activated with IL-1β in M199 medium for 4 hr, neutrophils migrated into the collagenous matrix within 90 min (Fig. 4, A and B). However, this type of migration was strongly inhibited by pretreatment of neutrophils with 8 μM TMS or SPN (Fig. 4, C and E), and completely inhibited by pretreatment with 25 μM TMS (Fig. 4D). In contrast, Cer showed no inhibitory effect whatsoever. The effects of TMS, DMS, SPN, and PKC inhibitors on migration of neutrophils are summarized in Table 2. It should be noted that 16 μM TMS greatly reduced the number of cells migrating through the EC monolayer, whereas viability of cells remained the same as controls. H-7 (5 μM), Calphostin C and 2 staurosporine (2 μM) produced similar cytotoxic effects on neutrophils, while staurosporine and calphostin C produced striking morphological damage to ECs; the cells

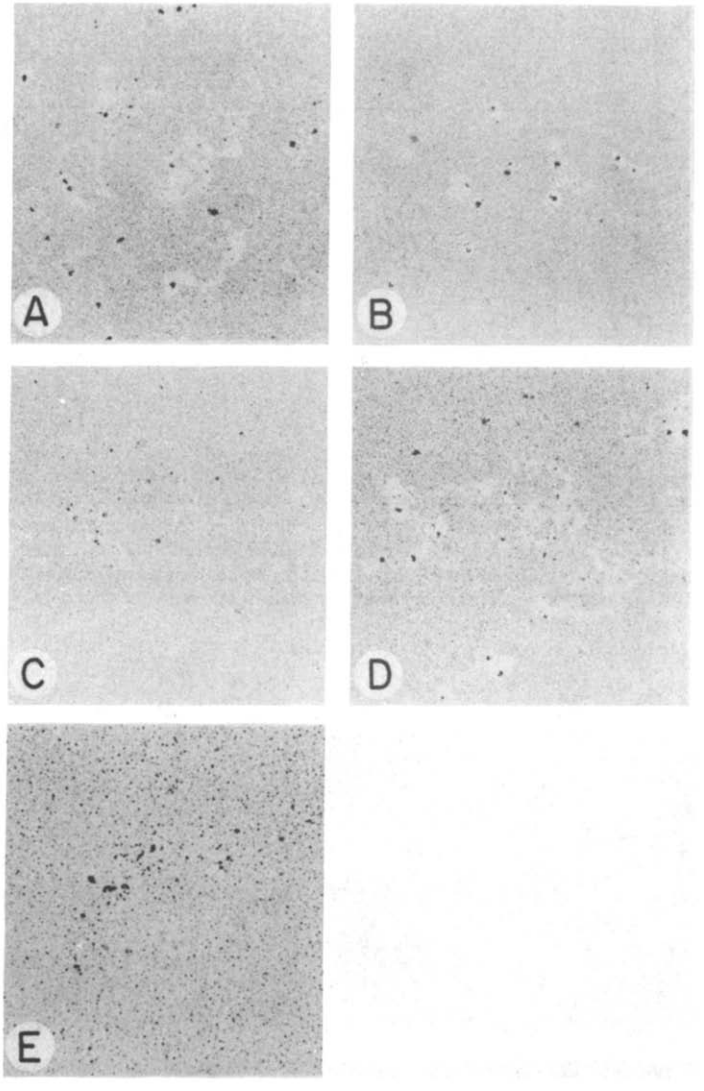


Fig. 3. Effect of TMS on phagokinetic activity of neutrophils. See Table 1 footnote for experimental details. (A) control (0.1% ethanol), (B) 1.5 μM TMS, (C) 4.5 μM TMS, (D) 4.5 μM Cer, and (E) 4.5 μM SPN. Magnification 100×.

shrank to form round shapes which could allow leaking of the intercellular junction. In contrast, no morphological change or damage was observed following treatment with TMS at an effective concentration. At a concentration of 16 μM, SPN and DMS induced similar morphological damage to ECs.

*Cytotoxic effects of SPN, TMS, and Cer on neutrophils*

In view of the striking effects of SPN and its derivatives on neutrophil transendothelial migration and oxidative burst, it is important to evaluate their possible cytotoxicity. Cytotoxicity of SPN was reported to be dependent on neutrophil concentration, as well as on the SPN delivery method in the incubation medium [26]. Under the experimental conditions of this study, using $1 \times 10^6$ neutrophils/mL and an ethanol delivery system, 15 μM TMS showed no significant cytotoxicity, whereas 15 μM SPN or DMS showed weak (12–13%) cytotoxicity. At a concentration of 25 μM, SPN or DMS produced major cytotoxic damage to neutrophils (viability of cells 53 ± 18% and 29 ± 5%, respectively), whereas the effect of TMS was much less (viability of cells 83 ± 6%). At a density of $6 \times 10^6$ cells/mL, there was no significant difference in the cytotoxic effect by TMS vs SPN or DMS; viability of cells ranged from 80 to 90% at 45 μM, and 88 to 94% at 15 μM.

When these compounds were added as an equimolar BSA (fatty acid-free) complex, no cytotoxicity was observed up to 100 μM at both cell densities, in agreement with a previous report [26]. When TMS was added as BSA complex, the inhibitory effect on short-term (5- to 10-min incubation) $O_2^-$ production and $O_2$ consumption was reduced by more than 50%. However, the effect on phagokinetic activity and trans-endothelial migration

Table 1. Inhibition of phagokinetic activity by SPN derivatives and other PKC inhibitors

| Compound | Concentration ($\mu$M) | Area swept ($\mu m^2 \times 10^3$) | % of Control | N |
|---|---|---|---|---|
| Control (0.1% ethanol) | | 4.5 ± 1.1* | 100 | 178 |
| SPN | 0.5 | 2.7 ± 1.0 | 60 | 124 |
| | 1.5 | 0.8 ± 0.3 | 17 | 69 |
| | 4.5 | 0.5 ± 0.3 | 14 | 72 |
| TMS | 0.5 | 3.6 ± 0.9 | 79 | 83 |
| | 1.5 | 1.8 ± 0.6 | 39 | 82 |
| | 4.5 | 0.7 ± 0.7 | 16 | 93 |
| DMS | 4.5 | 1.2 ± 1.1 | 26 | 132 |
| Cer | 4.5 | 4.9 ± 1.1 | 109 | 62 |
| H-7 | 200 | 1.2 ± 0.3 | 27 | 75 |
| Staurosporine | 0.2 | 0.6 ± 0.5 | 13 | 68 |
| Calphostin C | 0.5 | 1.8 ± 0.5 | 39 | 65 |

Freshly purified neutrophils ($1 \times 10^4$) were plated on colloid-coated coverslips in RPMI 1640 medium. Test compounds were solubilized in 50% ethanol and added to the medium (final concentration of vehicle 0.1%). After 2 hr, incubation was terminated by the addition of 10% formaldehyde. The area swept by neutrophils was estimated by taking Polaroid photographs, which were then cut and weighed.

* Mean ± SD. N = number of cells examined.

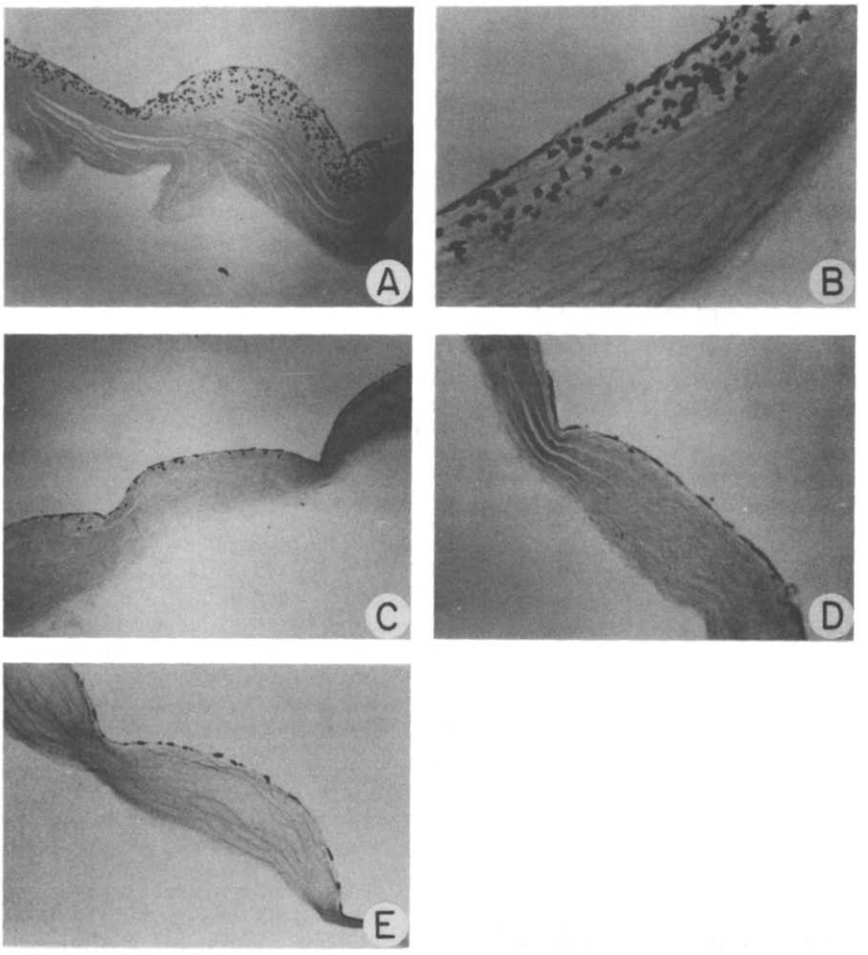


Fig. 4. Effect of TMS on trans-endothelial migration of neutrophils. See Table 2 footnote for experimental details. (A) control (0.5% ethanol), (B) control (0.5% ethanol), (C) 8 $\mu$M TMS, (D) 25 $\mu$M TMS, and (E) 8 $\mu$M SPN. Magnification: for panels A, C, D and E, 100×; for panel B, 400×.

Table 2. Inhibition of neutrophil trans-endothelial migration by SPN derivatives and other PKC inhibitors

| Compound | Concentration ($\mu$M) | Number of cells/view* | % of Control | Cell viability |
|---|---|---|---|---|
| Control (0.5% ethanol) | | 19.8 ± 6.3 | 100 | 94 ± 2 |
| SPN | 4 | 7.6 ± 3.3† | 38 | |
| | 8 | 3.3 ± 2.4† | 16 | |
| | 16 | 0.5 ± 0.8† | 3‡ | 87 ± 5 |
| TMS | 4 | 10.5 ± 4.6† | 53 | |
| | 8 | 1.7 ± 1.4† | 9 | |
| | 16 | 0.6 ± 0.7† | 3 | 97 ± 3 |
| DMS | 4 | 7.4 ± 2.8† | 37 | |
| | 8 | 1.2 ± 1.2† | 6 | |
| | 16 | 0.4 ± 0.5† | 5‡ | 82 ± 16 |
| Cer | 5 | 25.0 ± 6.4§ | 126 | |
| | 15 | 23.6 ± 7.4 | 119 | 95 ± 2 |
| H-7 | 200 | 7.5 ± 2.4† | 38 | 79 ± 7 |
| Staurosporine | 2 | 10.3 ± 5.0† | 52‡ | 83 ± 4 |
| Calphostin C | 5 | 3.8 ± 1.7† | 19‡ | 47 ± 6 |

Human neutrophils ($1.6 \times 10^6$/mL) in M199 medium with 1% heat-inactivated FBS were preincubated with test compounds (solubilized in 50% ethanol, final ethanol concentration 0.5%) for 10 min at 37°. Neutrophil suspension was then added to IL-1$\beta$-stimulated HUVEC monolayer and incubated for 4 hr. Culture plates were incubated for 90 min at 37°, fixed with 10% formaldehyde, paraffin-embedded, and stained with hematoxylin-eosin. Viability of neutrophils under the same conditions was examined separately by trypan blue exclusion assay.

* Values are means ± SD. The number of views per experiment was 25.

† Significantly different from the control value at $P < 0.001$ (*t*-test).

‡ A significant morphological change of HUVEC monolayers was observed during incubation.

§ Significantly different from the control value at $P < 0.005$ (*t*-test).

(90- to 120-min incubation) was essentially unchanged. These findings reflect the fact that free TMS is taken up by cells much more rapidly than TMS–BSA complex.

### *Effects of SPN and TMS on neutrophil protein phosphorylation*

When neutrophils were metabolically labeled with [$^{32}$P]sodium phosphate for 1 hr to enrich intracellular [$\gamma$-$^{32}$P]ATP, followed by washing of cells and stimulation with PMA, two protein bands showed greatly enhanced phosphorylation. These bands had a $M_r$ of 47 and 60 kDa (Fig. 5, lanes 1 and 2). When neutrophils were preincubated with 14–15 $\mu$M SPN or TMS, and then stimulated with PMA, phosphorylation of both bands was diminished markedly (Fig. 5, lanes 3 through 7). The inhibitory effect on phosphorylation was observed within 2 min of incubation, and appeared to be a very rapid response. Phosphorylation of the 47 and 60 kDa proteins appeared to be a substrate of PKC, since PMA-dependent response is largely ascribable to direct or indirect PKC activity. Recent studies [24, 27] indicate that phosphorylation of the 47 kDa protein (a component of the NADPH oxidase complex) is involved in NADPH reduction leading to oxidative burst of neutrophils. The 60 kDa phosphoprotein described in this study could be identical to the $M_r$ 59 kDa protein phosphorylated on neutrophil stimulation, as previously reported [28]. Thus, the inhibitory effects of SPN and TMS may occur via an inhibitory effect on PKC (see Discussion).

### *Metabolism of radiolabeled SPN and TMS in neutrophils*

To access the metabolic changes of exogenous SPN and TMS incubated with human neutrophils, we synthesized and used radiolabeled [1-$^3$H]SPN and [$^{14}$C-*N-methyl*]-TMS. Both these radiolabeled compounds were rapidly and equally incorporated into cells (70% of radioactivity incorporated within 30 min). SPN was rapidly converted into SPN-1-P (peak at 10 min), Cer (peak at 30–60 min), and later phosphatidylethanolamine and sphingomyelin (Fig. 6, A and B). SPN-1-P, which was observed only in the upper phase, was identified by co-migration with standard sample on TLC using three different solvent systems. In contrast, [$^{14}$C]TMS underwent no metabolic conversion during incubation of up to 20 hr (Fig. 6C). [$^{14}$C]DMS was also unchanged (data not shown).

## DISCUSSION

There has been considerable recent pharmacological interest in glycosphingolipids and their derivatives, as agents for correcting cellular functions by modulation of transmembrane signaling through their effects on various key regulatory units such as

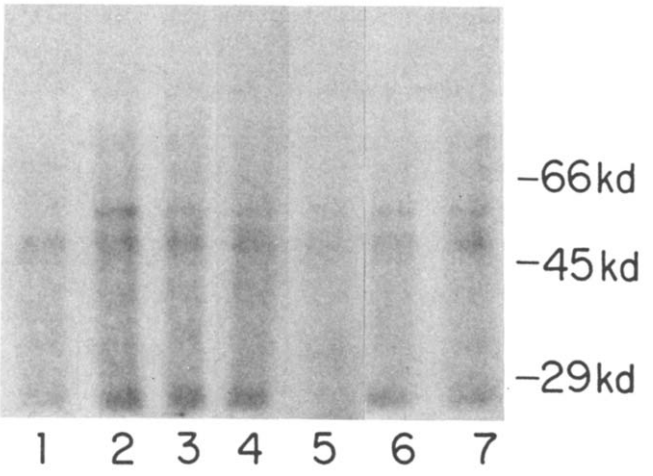


Fig. 5. Effects of SPN derivatives on PMA-induced protein phosphorylation in neutrophils. Freshly purified neutrophils ($8 \times 10^7$/mL) prelabeled with 2 mCi [$^{32}$P]phosphate for 60 min were incubated with or without test compound (dissolved in 50% ethanol; final concentration of ethanol 0.5%) for 10 min at 37°. PMA (1.5 $\mu$M) was added, and incubation was terminated after 2 min by addition of Laemmli's sample buffer. SDS-PAGE electrophoresis was performed, and $^{32}$P-incorporation was visualized by autoradiography. Key: (1) control (0.5% ethanol), (2) PMA alone, (3) PMA + 5 $\mu$M TMS, (4) PMA + 15 $\mu$M TMS, (5) PMA + 45 $\mu$M TMS, (6) PMA + 15 $\mu$M SPN, and (7) PMA + 15 $\mu$M DMS.

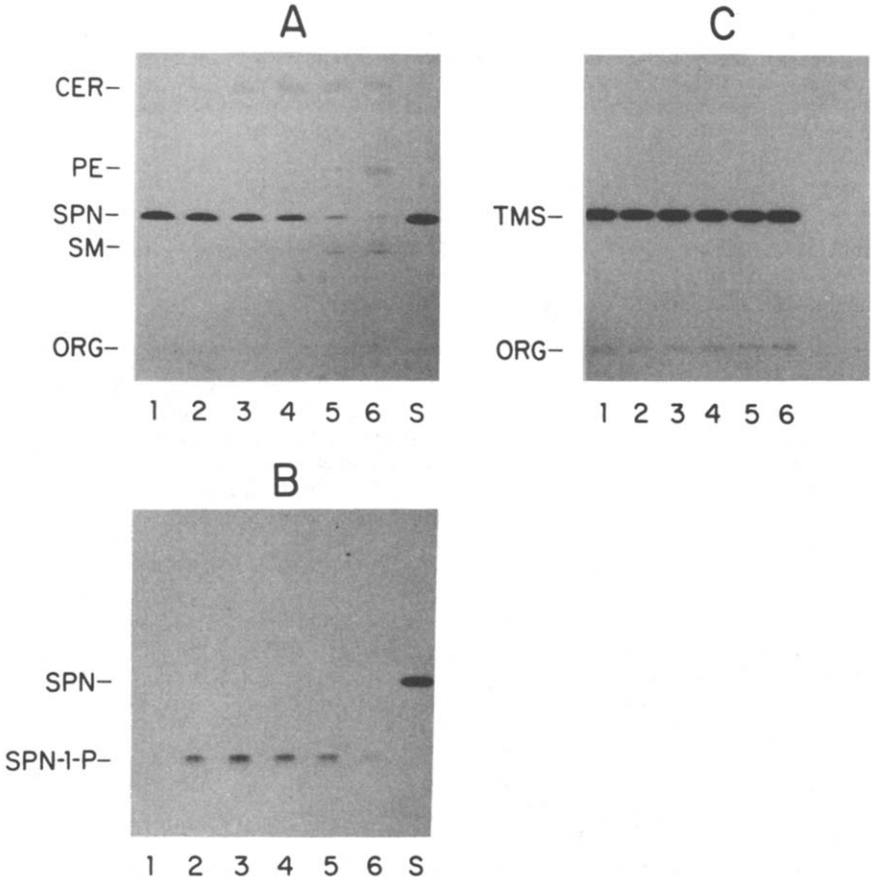


Fig. 6. Metabolic changes of [$^3$H]SPN and [$^{14}$C]TMS in human neutrophils. Freshly isolated human neutrophils ($2 \times 10^6$/mL) were incubated with [$^3$H]SPN (panels A and B) or [$^{14}$C]TMS (panel C) for the indicated times. Lower phase (A and C) and upper phase (B) of the extracts were applied to TLC and developed in $CHCl_3$/MeOH/water (50:40:10) (A) or butanol/acetic acid/water (3:1:1) (B and C). Lane 1, 0 min; 2, 10 min; 3, 30 min; 4, 60 min; 5, 180 min; 6, overnight; S, standard SPN. PE = phosphatidylethanolamine, and SM = sphingomyelin.

receptor-associated protein kinases and PKC. SPN was reported previously to inhibit oxidative burst of human neutrophils via inhibition of PKC [3, 4], and exogenous SPN appears to be converted to DMS, Cer, and other sphingolipids in cells [5, 29]. This approach is based on the physiological modulatory function of sphingolipids and glycosphingolipids in transmembrane signaling in general [for reviews see Refs. 30–34].

Previous studies in this laboratory indicated that: (i) TMS (a totally synthetic compound), relative to SPN and DMS, has a much stronger inhibitory effect on PKC activity of tumor cells, and inhibits tumor cell growth *in vitro* and *in vivo* [12]; (ii) TMS is a stronger inhibitor than DMS or SPN of platelet activation and tumor metastatic potential [13]; (iii) the expression of GMP-140 (P-selectin) on activated platelets is strongly inhibited by TMS and DMS, but not by SPN, H-7, or calphostin C [35]. In contrast to these previous findings, the present study indicated that SPN, DMS, and TMS have roughly equal effects on the three functions of neutrophils as described at the beginning of the paper. Also, TMS and DMS had inhibitory effects comparable to that of SPN on phosphorylation of two proteins ($M_r$ 47 and 60 kDa) in neutrophils. Therefore, SPN and its methylated derivatives may influence neutrophil function equally well through PKC and other transmembrane signal transduction mechanisms. Although the nature of the multiple PMA-induced phosphoprotein bands in neutrophils [3] (particularly those with $M_r$ 47 and 60 kDa) remains to be elucidated, we assume that both phosphoproteins are cooperatively involved in activation of NADPH to produce $O_2^-$ [24].

The inhibitory effects of TMS and SPN on IL-1$\beta$-stimulated trans-endothelial migration of neutrophils are of particular interest, since this process is mediated by expression of ELAM-1 [21], which recognizes sialosyl-$Le^x$ present on neutrophils [36]. Initial selectin-mediated adhesion to ECs is followed by migration into subendothelial matrix, and this process is inhibited by SPN and TMS. Since sialosyl-$Le^x$ expression by neutrophils appears to be unaffected by TMS, the inhibitory effect of TMS on trans-endothelial migration may be related to its inhibitory effect on phagokinetic activity, i.e. to its effect on membrane-associated structural units controlling cell motility [37].

PKC activity and cell growth in a variety of tumor cell lines were inhibited more strongly by TMS than by SPN or DMS. Likewise, *in vivo* growth and metastatic potential of tumor cells were inhibited more strongly by TMS, weakly by DMS, and not at all by SPN. Despite these findings in tumor cells, SPN, DMS, and TMS showed equal inhibitory effects on three major functions of neutrophils: oxidative burst, phagokinesis, and trans-endothelial migration. The three compounds also had similar inhibitory effects on protein phosphorylation in neutrophils. In some tumor cell lines, TMS has shown an inhibitory effect on PKC activity which is clearly stronger than that of DMS or SPN [12]. However, this does not account for the greater inhibitory effect of TMS on phagokinetic activity and trans-endothelial migration of neutrophils. Some transmembrane signaling mechanism may well be involved. We recently observed that a low concentration (5 $\mu$M) of TMS induces rapid $Ca^{2+}$ influx, whereas larger concentrations (up to 20–30 $\mu$M) of SPN or DMS did not induce the same effect, as determined by laser beam cytofluorometer (Meridian ACAS 470) (Sadahira Y, Hakomori S and Igarashi Y, unpublished observations). Further studies along this line are in progress. At present, however, the molecular basis for the differential effects of TMS, DMS, and SPN on the function of neutrophils and human tumor cell lines remains unclear.

Nevertheless, TMS is clearly distinguished from SPN and DMS in several important respects: (i) it gives a stable, clear aqueous solution, in contrast to the unstable, opaque aqueous solution given by SPN and DMS; (ii) it is considerably less cytotoxic to neutrophils or ECs than are SPN and DMS; and (iii) it is metabolically stable, whereas SPN undergoes rapid metabolic conversion to Cer, glycosphingolipids, and sphingomyelin, as well as to phosphatidylethanolamine, which is derived from the SPN-1-P degradation pathway (via phosphoethanolamine). In view of all these factors, TMS appears to be clearly superior to SPN, DMS, or other known PKC inhibitors as a pharmacological agent for blocking transmembrane signaling mechanisms in neutrophils, and for suppression of neutrophil overfunction during inflammatory reactions (see below).

Neutrophils function physiologically as part of the body's natural defense system, by migrating to areas of infection and destroying the infectious microorganisms through phagocytosis and oxidative burst. The first demonstration that active $O_2$ is involved in inflammatory processes was based on *in vitro* experiments [38]. Since then, numerous studies have addressed this topic, and it is now well-established that overaccumulation and overfunction of neutrophils can result in deleterious, unnecessary tissue damage, mostly through excessive $O_2^-$ production. Furthermore, overaccumulation of neutrophils and monocytes, together with platelet aggregation, results in capillary embolism and serious circulatory disturbances. For example, in the case of myocardial infarction, irreversible tissue damage and necrosis result not only from anoxia due to thrombosis, but also from subsequent infiltration and overfunction of neutrophils. Even if circulation is recovered by lysis of clots, severe myocardial damage still results from $O_2^-$ production by infiltrating neutrophils. Thus, inhibition of neutrophil overfunction could provide a primary therapeutic modality for the reperfusion injury of myocardial infarction. Based on the present study, SPN derivatives, especially TMS, appear to be suitable reagents for this purpose, and investigations of this possibility are underway.

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