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# A possible role of sphingosine in induction of apoptosis by tumor necrosis factor-α in human neutrophils

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**Abstract** Treatment of human neutrophils with tumor necrosis factor-α (TNF-α) resulted in an increase in concentration of ceramide and its catabolite, sphingosine. Sphingosine, a potent endogenous protein kinase C (PKC) inhibitor, as well as TNF-α, induced internucleosomal DNA fragmentation and morphological changes characteristic of apoptotic cells. Ceramide and sphingosine-1-phosphate, the initial product of sphingosine catabolism, did not cause apoptosis under our experimental conditions. In addition, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) and *N,N*-dimethylsphingosine (DMS), known as PKC inhibitors, also induced apoptosis, suggesting that induction of apoptosis by sphingosine may be related to inhibition of PKC activity. These results indicate that sphingosine deacylated from ceramide may be an endogenous modulator mediating apoptotic signals by TNF-α in neutrophils.

*Key words:* Apoptosis; Sphingosine; Tumor necrosis factor-α; Neutrophil

## 1. Introduction

Apoptosis is an active, energy-dependent process through which living cells participate in their own destruction [1,2]. Apoptosis provides a mechanism for removal of unwanted cells in a variety of situations including thymus involution, gut crypt epithelial cell turnover, and embryonic tissue remodeling. Aging neutrophils have also been shown to undergo apoptosis spontaneously [3,4]. Apoptotic senescent neutrophils are recognized and phagocytosed by macrophages. This process has been suggested to represent a mechanism in vivo to limit neutrophil-mediated tissue injury in inflamed sites.

Various cytokines released during inflammation regulate the survival of neutrophils in the lesion either by promoting or by inhibiting their death. Inflammatory mediators, such as endotoxic lipopolysaccharide, complement factor 5a, and granulocyte-macrophage colony-stimulating factor, markedly inhibited neutrophil apoptosis [5]. In contrast, TNF-α, a potent neutrophil activator [6–8], has been shown to accelerate the rate of neutrophil apoptosis [9]. TNF-α responsive sphingomyelin hydrolysis and ceramide generation have been reported to be implicated in a signal transduction pathway that mediates induction of apoptosis by TNF-α in U-937 cells [10,11]. Although the mechanism of action of ceramide in mediating apoptotic signals remains poorly understood, we recently found that sphingosine, a catabolite of ceramide, induced apoptosis in a variety of tumor cell lines including human leukemic HL-60 cells (E.A. Sweeney et al., unpublished data). The present studies were undertaken to determine whether sphingolipids such as ceramide and sphingosine are involved in the intracellular signaling of apoptosis by TNF-α in neutrophils.

## 2. Materials and methods

### 2.1. Chemicals

Sphingosine, *N*-octanoylsphingosine ($C_8$-ceramide), sphingosine-1-phosphate and DMS were synthesized as described previously [12–14]. All sphingolipids were dissolved in ethanol. Control experiments were performed with ethanol (< 0.1%) as the vehicle. H7 was obtained from Calbiochem-Novabiochem Co. (San Diego, CA). Natural human TNF-α ($5 \times 10^7$ U/mg) was a gift from Otsuka Cellular Technology Int. (Tokushima, Japan). Ribonuclease-A, and bovine serum albumin (BSA, essentially fatty acid-free) were obtained from Sigma Chemical Co. (St. Louis, MO). [$^3$H]Acetic anhydride was from DuPont- New England Nuclear (Boston, MA). HPLC-grade chloroform and methanol, and thin-layer chromatography (TLC) plates were from EM Separations (Gibbstown, NJ). An autoradiography enhancer spray, Resolution TLC, was from L.M. Corp. (Chestnut Hill, MA).

### 2.2. Isolation of human neutrophils

Heparinized venous blood was obtained from healthy volunteers. Neutrophils were isolated by dextran sedimentation and centrifugation on a Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) cushion as previously described [15]. After contaminating erythrocytes were removed by hypotonic lysis, cells were resuspended with RPMI 1640 containing 0.1% BSA. Such processing yielded > 98% viable neutrophils, as determined by Wright-Giemsa staining.

### 2.3. Measurement of ceramide and sphingosine concentration

Purified neutrophils ($5 \times 10^6$/ml) were treated with human TNF-α (3,000 U/ml) for the indicated times, and then lipids were extracted from cells. For ceramide measurement, lipid extracts were incubated with *Escherichia coli* diacylglycerol kinase as previously reported [16]. Ceramide phosphate was then isolated by TLC and the ceramide mass was quantitated. Sphingosine concentrations were measured by conversion to *N*-[$^3$H]acetylated sphingosine by acylation with [$^3$H]acetic anhydride [17]. After lipid extracts were treated by 0.1 N NaOH for 1 h, dried samples were dissolved in 40 μl of 0.008N NaOH in methanol/10 mM solution of [$^3$H]acetic anhydride (1:1). Acylation proceeded for 1 h at 37°C. Following treatment of samples with NaOH, *N*-[$^3$H]acetylated sphingosine was isolated by TLC and the sphingosine mass was quantitated.

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*Abbreviations:* TNF-α, tumor necrosis factor-α; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; PKC, protein kinase C; $C_8$-ceramide, *N*-octanoylsphingosine; DMS, *N,N*-dimethylsphingosine; BSA, bovine serum albumin; TLC, thin-layer chromatography; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine.


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*2.4. Analysis of DNA fragmentation*

DNA fragmentation was analyzed by using agarose gel electrophoresis [18]. $5 \times 10^6$ neutrophils were harvested, washed, and incubated in 0.5 ml of 50 mM Tris-HCl, pH 8.0, containing 1mM EDTA, 0.25% Nonidet P-40 (Sigma) and 0.1% ribonuclease-A at 37°C for 30 min. 50 μl of 10 mg/ml proteinase-K (Boehringer, Mannheim, Germany) was then added and the incubation continued for an additional 30 min. After incubation, 0.1 ml of loading buffer (0.25% Bromophenol blue-0.25% xylene cyanol FF-30% glycerol) was added. 25 μl of the tube content was loaded into each well of the 2% agarose gels, and electrophoresis was carried out at 10 V/cm. A *Hae*III digest of øX174 DNA (New England Biolabs Inc., Beverly, MA) was applied to each gel to provide size markers of 1,353, 1,078, 872, 603, 310 bp, respectively. The DNA in gels was visualized under ultraviolet light after staining with ethidium bromide (Sigma).

*2.5. Assessment of neutrophil apoptosis by cytology*

For morphological assessments, neutrophils ($5 \times 10^6$/ml) were treated with different agents. Cytocentrifuge slides were then prepared and stained with Wright-Giemsa stain. Cells were examined under oil immersion light microscopy, and apoptotic neutrophils were defined as cells containing one or more darkly stained pyknotic nuclei [3,5]. For assessment of the percentage of cells showing morphology of apoptosis, 500 cells/ slide were counted. Viability by Trypan blue exclusion was also assessed.

## 3. Results

Fig. 1 demonstrates the effect of TNF-α on ceramide and sphingosine content in neutrophils. The cellular concentrations of ceramide increased by 68% (from 189 ± 42 to 317 ± 15 pmol/$10^6$ cells) at 5 min after the addition of TNF-α (3,000 U/ml) (Fig. 1A). Ceramide concentrations in neutrophils incubated at 37°C in the absence of TNF-α were unchanged. Under the same conditions, TNF-α induced an increase in sphingosine content. Sphingosine concentrations increased by 95% (from 9.1 ± 0.2 to 17.7 ± 3.4 pmol/$10^6$ cells) at 1 h after the addition of TNF-α (Fig. 1B). The increase occurred continuously over 1 h. Sphingosine concentrations in neutrophils incubated at 37°C for 1 h in the absence of TNF-α increased by only 29% (from 9.1 ± 0.2 to 11.8 ± 1.7 pmol/$10^6$ cells). Thus, treatment of neutrophils with TNF-α caused an increase of both ceramide and sphingosine concentrations.

As previously reported [9], agarose gel electrophoresis of DNA from neutrophils treated for 6 h with TNF-α (3,000 U/ml) showed DNA fragmentation with a pattern characteristic of internucleosomal fragmentation (Fig. 2). DNA from neutrophils treated with an ethanol vehicle, cell-permeable $C_8$-ceramide (15 μM), or sphingosine-1-phosphate (15 μM) was unfragmented. However, sphingosine (15 μM), as well as H7 (50 μM) and DMS (15 μM), caused internucleosomal DNA fragmentation. The effects of sphingosine were first detected after 2 h and increased with longer treatment (data not shown). Induction of apoptosis by TNF-α had previously been confirmed by ultrastructural examination [9]. Light microscopy of neutrophils treated for 6 h with TNF-α (3,000 U/ml) or sphingosine (15 μM) showed that many neutrophils exhibited morphologic features of apoptosis, such as one or more darkly stained pyknotic nuclei and cytoplasm vacuolation (Fig. 3). There was no evidence of significant necrotic cell death (assessed by the ability of neutrophils to exclude Trypan blue) in culture. Isolated neutrophils have been known to undergo apoptosis spontaneously [3–5]. The percentage of apoptotic cells in neutrophils treated with an ethanol vehicle increased from 0 ± 0 to 1.1 ± 0.5% at 6 h. The percentages of apoptosis in neutrophils treated for 6 h with TNF-α (3,000 U/ml), sphingosine (15 μM), DMS (15 μM), and H7 (50 μM) were 31.1 ± 3.3, 27.7 ± 3.0, 33.1 ± 4.1, and 27.6 ± 3.0%, respectively (Fig. 4A). Treatment with sphingosine induced apoptosis in a dose-dependent manner. The time course study showed that the proportion of neutrophils demonstrating morphological features of apoptosis increased progressively with time after addition of TNF-α (3,000 U/ml) or sphingosine (15 μM) (Fig. 4B).

## 4. Discussion

TNF-α had the capacity to induce or accelerate apoptosis of neutrophils as previously reported [9]. One physiological function of TNF-α, which primes many neutrophil functions including the respiratory burst [6–8], may be the acceleration of apoptosis of primed neutrophils to limit neutrophil-mediated tissue injury. Previous studies showed that TNF-α signaling involved

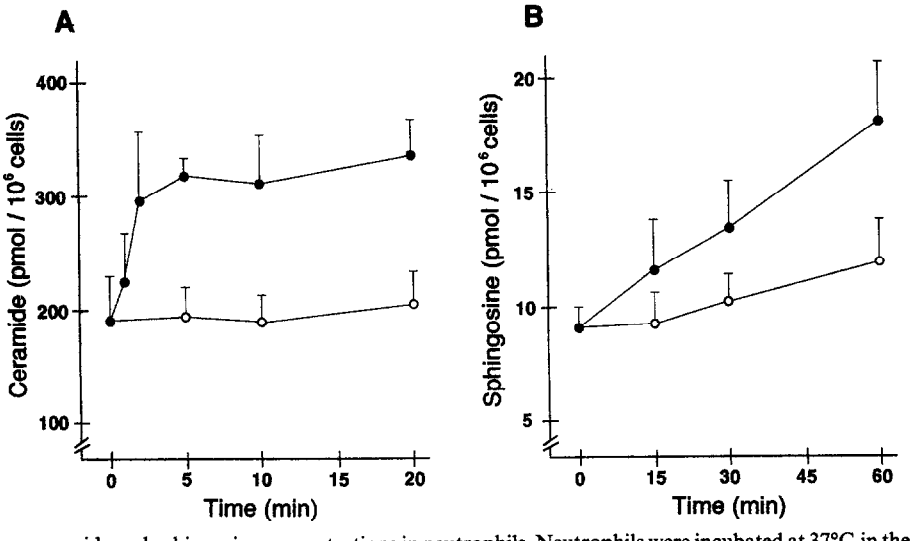


Fig. 1. Effects of TNF-α on ceramide and sphingosine concentrations in neutrophils. Neutrophils were incubated at 37°C in the presence (●) or absence (○) of 3,000 U/ml TNF-α. Cellular lipids were extracted at the indicated times and concentrations of ceramide (A) and sphingosine (B) were measured as described in section 2.

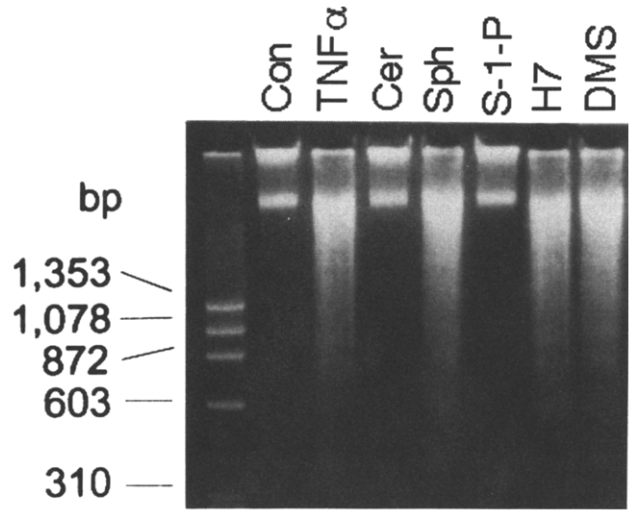


Fig. 2. Agarose gel electrophoresis of DNA from neutrophils treated with different agents. Neutrophils were treated for 6 h with an ethanol vehicle (Con), 3,000 U/ml TNF-α, 15 μM $C_8$-ceramide (Cer), 15 μM sphingosine (Sph), 15 μM sphingosine-1-phosphate (S-1-P), 50 μM H7, and 15 μM DMS. DNA was isolated from neutrophils and analyzed by 2.0% agarose gel electrophoresis as described in section 2. The first lane is a *Hae*III digest of øX174 DNA.

sphingomyelin hydrolysis to ceramide by activation of sphingomyelinase [19,20]. In addition, treatment with exogenously added sphingomyelinase has been shown to cause an increase in concentration of sphingosine deacylated from ceramide, suggesting that sphingomyelinase and ceramidase functioned cooperatively [21]. In this study we found that treatment of neutrophils with TNF-α resulted in an increase in cellular concentration of both ceramide and sphingosine. The increase of ceramide content was more rapid than that of sphingosine content. These findings suggest that the increase of sphingosine content results from degradation of formed ceramide after TNF-α treatment.

Ceramide has been reported to initiate apoptosis [10,11]. Although ceramide-activated protein kinase and phosphatase have been described in some cells [22–24], the mechanism of action of ceramide in mediating apoptotic signaling remains obscure. In neutrophils, sphingosine (5–15 μM), as well as

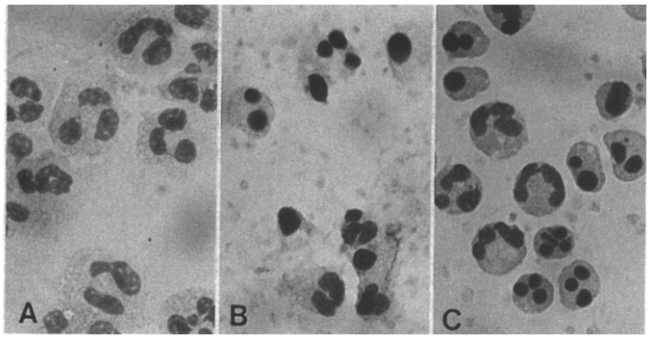


Fig. 3. Morphologic appearance of neutrophils treated for 6 h with an ethanol vehicle (A), 3,000 U/ml TNF-α (B), and 15 μM sphingosine (C). The cells were stained by Wright-Giemsa stain as described in section 2. Treatment with TNF-α or sphingosine induced apoptotic changes such as one or more darkly stained pyknotic nuclei and cytoplasm vacuolation. Original magnification: × 700.

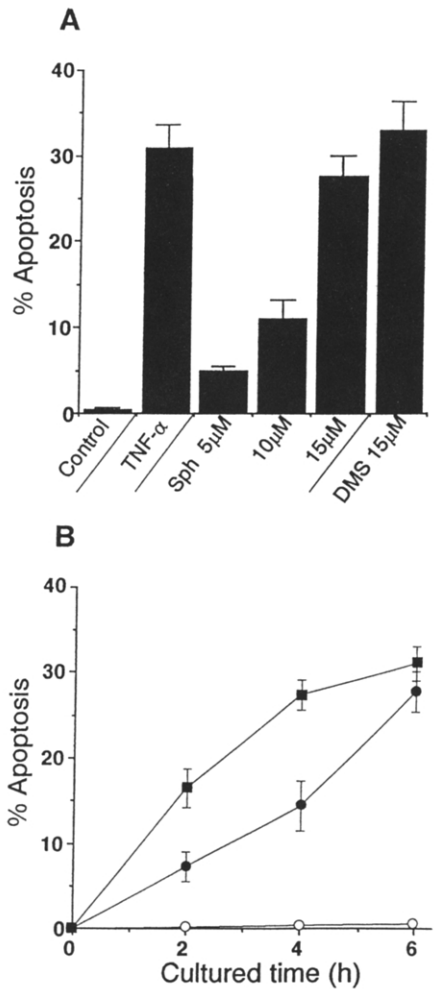


Fig. 4. (A) The percentage of apoptotic cells (assessed morphologically) of neutrophils treated for 6 h with an ethanol vehicle (Con), 3,000 U/ml TNF-α, 5, 10 and 15 μM sphingosine (Sph) and 15 μM DMS. (B) Time course of apoptosis of neutrophils incubated in the presence of an ethanol vehicle (○), 3,000 U/ml TNF-α (■), or 15 μM sphingosine (●). The values represent the average of three separate determinations (± S.E.M.).

TNF-α, was capable of inducing apoptosis. However, the same concentrations of ceramide or sphingosine-1-phosphate did not induce apoptosis. Sphingosine has been shown to inhibit PKC in vitro and in cells [25–27]. In addition, H7 and DMS, known as PKC inhibitors, also induced apoptosis. Induction of apoptosis by sphingosine may be related to inhibition of PKC activity (unpublished results by H. Ohta et al). On the other hand, sphingosine has been shown to activate protein kinases, which were distinct from PKC, cyclic nucleotide-activated kinases, and calcium-dependent kinases, with high specificity for D-erythro-sphingosine [28], although the involvement of sphingosine-dependent protein kinase in processes of apoptosis remains to be examined. An increase (8.6 pmol/$10^6$ cells) of sphingosine at 1 h after treatment with TNF-α was similar in magnitude to the transient increase in diacylglycerol that occurred upon *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) stimulation of neutrophils (about 6 pmol/$10^6$ cells) [29]. Moreover, this increase was estimated to correspond to that by exposure to about 10–20 μM sphingosine sufficient to induce

apoptosis. On the other hand, ceramide concentration is very high (about 200 $\mu$M) in the unstimulated human neutrophils and the addition of exogenous cell permeable ceramide (C-8 ceramide,up to 20 $\mu$M) did not induce apoptosis of neutrophils incubated in medium containing 0.1% BSA. These findings suggest that sphingosine deacylated from ceramide, not ceramide itself, may function as an endogenous modulator mediating induction of apoptosis by TNF-$\alpha$ in neutrophils.

Sphingosine and its methylated derivative, DMS, showed inhibitory effects on in vitro as well as in vivo tumor cell growth [30,31]. We recently found that induction of apoptosis by sphingosine and DMS was found in many tumor cell lines including human leukemic HL-60 cells, Colo 205 colon tumor cells, and A431 epidermoid carcinoma cells (E.A. Sweeney et al., unpublished data). In most tumor cell types, DMS was a stronger inducer of apoptosis than sphingosine. DMS has been detected in A431 epidermoid carcinoma cells [32] and murine IL2-dependent T lymphocyte CTLL cells [33]. However, changes in the levels of DMS and its participation in signaling pathways have not been evaluated, because of the difficulty of quantification of its mass levels in cells.

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