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N,N,N-Trimethylsphingosine inhibits interleukin-1β-induced NF-κB activation and consequent E-selectin expression in human umbilical vein endothelial cells

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Abstract **We examined the effect of *N,N,N*-trimethylsphingosine (TMS) on the interleukin-1β (IL-1β)-induced E-selectin expression in human umbilical vein endothelial cells (HUVEC). Incubation of HUVEC with TMS (0.1–10 μM) resulted in a concentration-dependent inhibition of IL-1β-induced E-selectin expression. Sphingosine or *N,N*-dimethylsphingosine had no effects on the expression. Electrophoretic mobility shift assay revealed that TMS inhibited IL-1β-induced NF-κB activation, which is essential for E-selectin expression. This inhibitory effect of TMS on IL-1β-dependent endothelial cell activation may partly explain the known anti-inflammatory or anti-metastatic effect of TMS in vivo.**

Key words: E-selectin; Sphingosine; NF-κB; Interleukin-1β

1. Introduction

E-Selectin, a member of the selectin family of cell surface lectins is expressed exclusively on endothelial cells stimulated with interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and bacterial lipopolysaccharide (LPS) [1,2]; its lectin domain is known to recognize sialosyl Le^x(SLe^x) [3,4] or its analogs [5] particularly under dynamic flow condition [6]. Exposure of endothelial cells to these stimuli results in rapid transcriptional induction of the gene encoding E-selectin, followed by de novo synthesis of E-selectin. Activation of a transcription factor, nuclear factor κB (NF-κB) precedes E-selectin expression in human umbilical vein endothelial cells (HUVEC). NF-κB was first identified as a protein specific to mature B lymphocytes that interacted with the B site of the κ light chain gene enhancer [7]. NF-κB has been implicated in control of transcription of a number of cellular genes involved in immune and inflammatory responses, growth, and adhesion (reviewed in [8]). The activation of NF-κB has been shown to be essential, but not sufficient for E-selectin gene transcription [9]. E-selectin, as well as P-selectin mediate adhesion of blood leukocytes to endothelium, a critical early event in a wide variety of inflammatory processes [1,2]. E-Selectin may also mediate adhesion of cancer cells to endothelium, leading to cancer spread and metastasis [5], in the presence of platelets [10]. In the context of anti-adhesion therapy, reagents inhibiting the expression of this adhesion molecule may be good candidates for controlling inflammation and cancer metastasis [11].

Sphingolipids have been recognized as playing important roles in cell-to-cell interaction, modulation of cell growth, and apoptosis through regulation of transmembrane signaling (see [12] for review). Biological functions of sphingoid bases such as sphingosine, ceramide, sphingosine-1-phosphate, and *N,N*-dimethylsphingosine (DMS) have been extensively investigated, and they have been implicated as important signaling molecules in regulation of numerous cellular functions [13,14]. *N,N,N*-Trimethylsphingosine (TMS), a synthetic sphingosine derivative, is a potent protein kinase C (PKC) inhibitor [15,16] and has been shown to inhibit P-selectin expression in human platelets [17], and neutrophil migration [18]. In vivo studies, TMS significantly suppressed human tumor cell growth in nude mice [15] and metastasis of B16 melanoma cell in syngeneic C57BL mice [19,20]. The molecular mechanism of these suppression, however, remains to be studied.

In this study, we examined the effect of TMS on E-selectin expression in HUVEC. E-selectin expression induced by IL-1β was significantly suppressed by addition of TMS to the culture media. IL-1β-dependent NF-κB activation was also inhibited by TMS, suggesting that E-selectin expression is down-regulated in part through inhibition of NF-κB.

2. Experimental

2.1. Materials

TMS, DMS and sphingosine were prepared as previously described [15,16,21], dissolved and stocked at 2 mM in ethanol/water (50:50 v/v). Bovine serum albumin (BSA), sodium azide, mouse IgG, hydrogen peroxide, aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and Nonidet P-40 were purchased from Sigma (St. Louis, MO). Dithiothreitol (DTT) was from Calbiochem (San Diego, CA). Fetal bovine serum (FBS) was from HyClone (Logan, UT). Recombinant IL-1β was purchased from Boehringer Mannheim-Biochemicals (Indianapolis, IN). [γ-^{32}P]ATP was from DuPont-New England Nuclear (Wilmington, DE). Anti-E-selectin mouse monoclonal antibody 3B7 (5 μg mouse IgG per ml culture supernatant) [22] was kindly provided by Otsuka Pharmaceutical Co. (Tokyo, Japan). Oligonucleotide probe for the NF-κB consensus sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was from Promega (Madison, MI).

2.2. Cells

HUVEC, their optimized culture medium containing endothelial cell growth factor (ECGF), and ECGF were purchased from Cell Systems (Kirkland, WA). HUVEC were cultured according to the manufacturer's instruction and cells between passage three and five were used for all experiments. One day prior to experiment, HUVEC were seeded on gelatin-coated 96-well flat-bottom plates (Costar, Cambridge, MA) for enzyme-linked immunosorbent assay (ELISA), or on T-25 flasks (Corning, Corning, NY) for flow cytometry.

2.3. Treatment of HUVEC

HUVEC were treated with indicated reagents for 30 min at 37°C, in RPMI 1640 supplemented with ECGF, 2% L-glutamine and 1% FBS.

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IL-1β was then added to the culture medium to a final concentration of 50 IU/ml. Following 4-h incubation with IL-1β, E-selectin expression was measured.

2.4. ELISA

ELISA was performed as previously described as slight modification [10]. All manipulations were performed at room temperature and all washes were with phosphate-buffered saline (PBS) except as specifically indicated. Cells were washed twice and fixed with 2% paraformaldehyde in PBS for 30 min. Following two washes with 20 mM glycine in PBS, cells were blocked with 2% BSA and 0.1% sodium azide in PBS for 1 h. Cells were incubated with 100 μl of 3B7 or with 5 μg/ml mouse IgG as a control for 1 h. After three washes, anti-mouse IgG antibody conjugated with peroxidase (Southern Biotechnology Assoc., Birmingham, AL) was added and incubated for 1 h. After four washes, soluble substrate 1,2-phenylenediamine (Dako, Glostrup, Denmark) in 0.1 M citric acid–phosphate buffer (pH 5.0), and hydrogen peroxide (final concentration 0.03%) were added according to the manufacturer's instruction. E-Selectin expression was determined by difference in absorbance at wavelength 490 vs. 630 nm.

2.5. Flow cytometry

Flow cytometry was performed as previously described with slight modification [19]. After incubation with reagents, cells were harvested with 0.02% EDTA solution and washed with washing buffer (20 mM HEPES, 2% FCS, 0.1% sodium azide in PBS). Following a wash, 3B7 or mouse IgG was added and incubated for 1 h on ice. Cells were then washed four times and incubated with fluorescin-conjugated goat anti-mouse IgG (TAGO, Burlingham, CA) for 1 h on ice. Cells were washed thoroughly and evaluated on EPICS PROFILE (Coulter Corp., Hialeah, FL).

2.6. Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from 1×10^7 cells using the method described by Dignam et al. [23] and Osborn et al. [24] with slight modification. Following the incubation, cells were harvested with 0.02% EDTA, washed twice with ice-cold PBS and resuspended in 400 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF, 5 μg/ml aprotinin and 5 μg/ml leupeptin) containing 0.1% Nonidet P-40 for 15 min on ice, vortexed vigorously for 15 s and centrifuged at 14,000 rpm for 30 s. The pelleted nuclei were resuspended in 40 μl of buffer C (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5 μg/ml aprotinin and 5 μg/ml leupeptin). After 20 min on ice, lysates were centrifuged and supernatants containing the nuclear proteins were transferred to new vials. Protein concentration was determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Oligonucleotide probe for the NF-κB consensus sequence was end-labeled with [γ-^{32}P]ATP by incubation with T4 polynucleotide kinase (Promega) at 37°C for 10 min. The labeled probe was separated from unincorporated nucleotides using Quick Spin G-25 column (Boehringer-Mannheim).

EMSA was performed as follows. Binding reaction mixtures (20 μl) containing 10 μg nuclear protein, 1.5 μg poly(dI-dC)·poly(dI-dC) (Pharmacia, Piscataway, NJ), ^{32}P-end-labeled NF-κB oligonucleotide probe, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$,, 5% (v/v) glycerol and 10 mM Tris-HCl (pH 7.5) were incubated for 20 min at 22°C, and electrophoresed through a native 6% polyacrylamide gel in a running buffer of 0.5 × TBE, followed by autoradiography.

3. Results

3.1. IL-1β-induced E-selectin expression was inhibited by TMS

We first examined the effect of TMS on E-selectin expression in HUVEC by ELISA. Without cytokine stimulation, no E-selectin expression was detected by ELISA, and TMS itself did not induce E-selectin expression. When HUVEC were stimulated with IL-1β (50 IU/ml) for 4 h, they expressed E-selectin, and addition of TMS to cultured medium inhibited E-selectin expression in a dose-dependent manner as shown in Fig. 1.

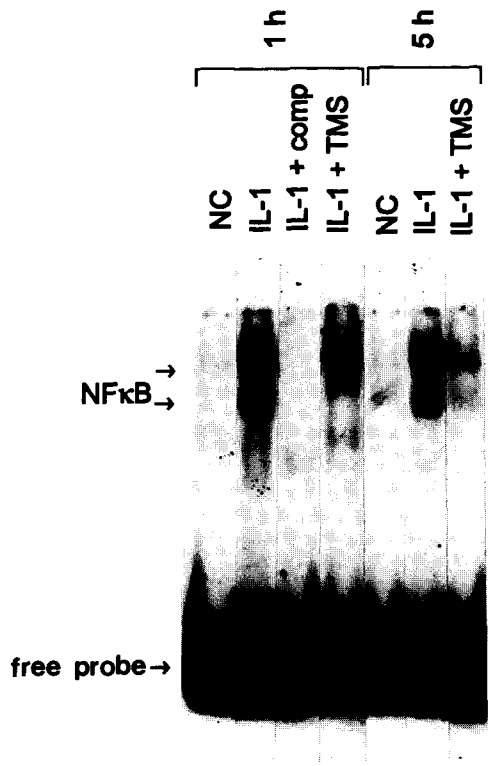

Fig. 1. Concentration-dependent inhibition by TMS of E-selectin expression on HUVEC. E-Selectin expression on HUVEC was measured by ELISA. HUVEC were treated with TMS for 30 min. Incubation was continued in the presence (■) or absence (▲) of IL-1 at a final concentration of 50 IU/ml. Cells were treated with IL-1 and sphingosine (Sph) in a similar manner (●). The data represent mean ± standard error of triplicate determinations from three independent experiments.

Maximal effect was observed at 10 μM, where the expression was approximately 15% of the control value. In these studies, TMS was added to the culture medium using ethanol as a vehicle, and the final concentration of ethanol (0.25%) did not affect cell viability or morphology. Viability under these conditions (10 μM TMS in 0.25% ethanol) was >95% as determined separately by Trypan-blue exclusion assay. However, when HUVEC were treated with TMS at concentrations above 20 μM, cells became round-shaped, detached from the bottom and floated in the medium during the incubation. To confirm that the decreased expression of E-selectin was not due to decreased number of cells by TMS, we also assessed the effect by flow cytometry (Fig. 2). When 5 μM TMS was added, E-selectin expression was reduced to approximately 25% of control value, roughly consistent with the results with ELISA. In contrast, neither unsubstituted sphingosine (Figs. 1, 2) nor DMS (data not shown) up to 40 μM produced effects detectable by ELISA or flow cytometry.

3.2. TMS inhibits IL-1β-induced NF-κB activation

We next studied the mechanisms by which TMS inhibits the E-selectin expression in HUVEC. The signaling pathways leading to E-selectin expression are not fully understood. It is believed that expression is controlled at the transcriptional level, and is mediated by a transcription factor, NF-κB [8]. We examined the effect of TMS on NF-κB activation by EMSA. Incuba-

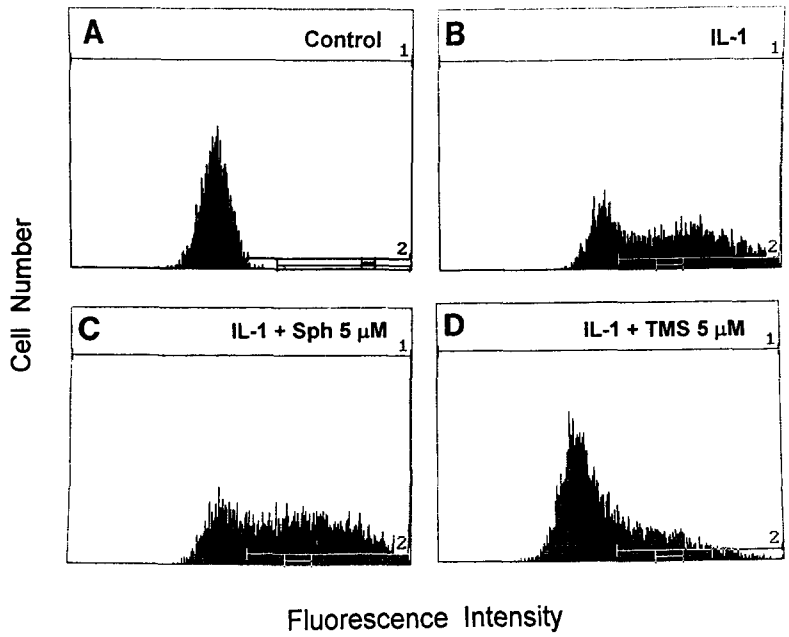

Fig. 2. Confirmation of inhibitory effect of TMS on E-selectin expression by flow cytometry. HUVEC were treated with 5 μM of sphingosine (panel C) or TMS (panel D) for 30 min followed by the 4-h incubation in the presence of IL-1 (50 IU/ml). As control experiments, incubations without any treatment (panel A) and with IL-1 only (panel B) were performed in a similar manner. The mean fluorescence intensity of each panel is as follows: (A) 5.22, (B) 48.72, (C) 45.15, and (D) 10.14.

tion of HUVEC with 50 IU/ml IL-1β resulted in appearance of two NF-κB specific DNA–protein complexes, upper and lower complexes [25]. TMS (10 μM) decreased the abundance of NF-κB binding activity (Fig. 3). When induction of NF-κB binding activity reaches the maximal level after 1 h incubation [26], TMS significantly decreased the abundance of lower complex. After 5 h, both complexes were almost eliminated by TMS treatment. Sphingosine and DMS showed no inhibitory effect at 10 μM (data not shown). These findings suggest that TMS down-regulates IL-1β-induced E-selectin expression in HUVEC, at least in part through inhibition of NF-κB activation.

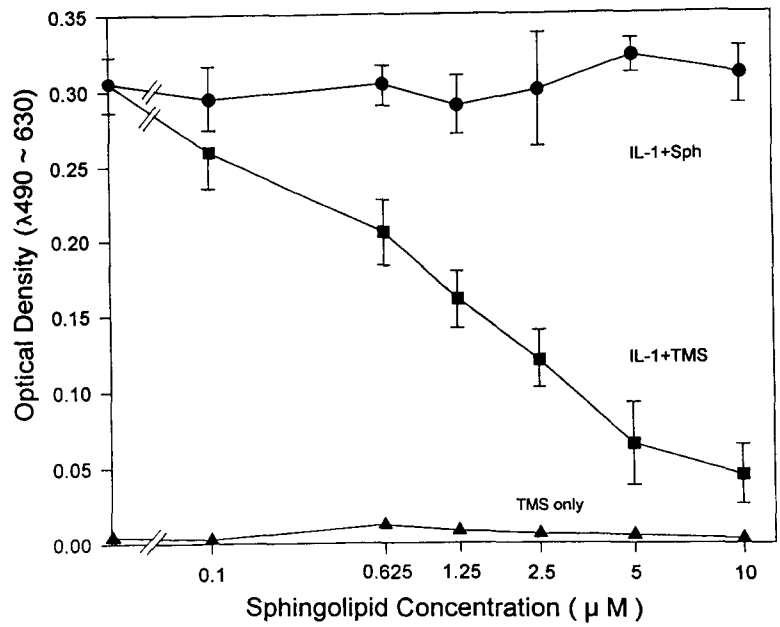

Fig. 3. Inhibition by TMS of IL-1β-induced NF-κB activation. HUVEC were treated with 10 μM TMS for 30 min, then IL-1 was added and incubation was continued another 1 h (lanes 1–4) or 5 h (lanes 5–7). Nuclear extracts were prepared as described in the text and subjected to EMSA. As a competition experiment, 50-fold molar excess of unlabeled NF-κB was added to the binding reaction mixture. NC = negative control without IL-1 stimulation; comp = unlabeled competitor nucleotide.

4. Discussion

We have demonstrated that a synthetic sphingosine derivative, TMS, down-regulates NF-κB activation and the consequent IL-1β-induced E-selectin expression in HUVEC. Sphingosine-related compounds have been extensively studied during the past 5 years and shown to regulate a wide variety of cellular processes, including cellular differentiation, proliferation [13,14], and apoptosis [27]. Among them, ceramide, which is produced by lymphokine-induced sphingomyelin cycle, is shown to induce NF-κB activation in human leukemia HL60 cells [28].

Sphingosine, DMS, TMS are known to inhibit PKC activity in the cells [15,16]. This suggests a possible role of PKC in mediating activation of HUVEC by cytokines. PKC activators, such as phorbol esters induce expression of E-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [29]. Unlike with expression of ICAM-1 and VCAM-1, however, PKC inhibitors such as staurosporine, calphostin C, Ro31-7549 fail to diminish cytokine-induced E-selectin expression [30]. We have observed that another PKC inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), does not affect IL-1β-dependent E-selectin expression (data not shown). Therefore, it seems unlikely that TMS down-regulates the expression through PKC inhibition. This assumption is supported by the present findings that other sphingolipid PKC inhibitors, sphingosine and DMS had no effect on E-selectin expression in HUVEC.

The mechanism by which TMS inhibits NF-κB activation remains unclear. Activation of NF-κB is a post-translational event [31]. Phosphorylation and degradation of the inhibitory protein IκB-α, and subsequent dissociation of this protein from NF-κB are thought to be necessary for the activation [31–33]. Several protease inhibitors and phosphatase inhibitors have been shown to inhibit the activation [31–33]. However, we have not observed such effects by TMS. Involvement of PKC in NF-κB activation is unlikely, because cytokine-induced NF-κB binding activity is not prevented by PKC inhibitors [30]. One possible mechanism is suggested by the findings of Menon et al., who demonstrated the importance of oxidation state for NF-κB activation, especially in non-transformed primary cells including HUVEC [34]. They showed that free radical-dependent oxidation and protein phosphorylation are both required for the activation. A similar mechanism was suggested for the inhibitory effects of vitamin E derivatives on TNF-α induced NF-κB activation in Jurkat T cells [35]. We previously reported that TMS inhibited superoxide production in PMA-induced human neutrophils [18]. Alternation of oxidation state by TMS in HUVEC may, therefore, partly explain the inhibition of NF-κB activation and the consequent E-selectin expression.

P- or E-selectin expression on endothelial cells or activated platelets plays a crucial role in leukocyte-dependent tissue injury associated with acute or chronic inflammation [36]. Therefore, there has been considerable interest in possible prevention of this type of injury by administration of SLe^x oligosaccharides or their analogs [37,38]. We previously demonstrated effective inhibition of P-selectin expression by TMS in vitro [17] and in vivo [39]. TMS blocked PKC-dependent secretory response of endothelial cells or platelets. We [39] and others [40] observed that TMS has a protective effect on reperfusion injury following myocardial ischemia. We believe that TMS is superior to SLe^x oligosaccharides or their analogs in term of anti-inflammatory drug efficiency, because of its metabolic stability and lower cost of production.

In summary, we have now demonstrated a further inhibitory effects of TMS on NF-κB activation and E-selectin expression in HUVEC. The present observation provides an additional strong rationale for anti-inflammatory and anti-metastatic effects of TMS.

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