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Effects of Antioxidant and Nitric Oxide on Chemokine Production in TNF-α-stimulated Human Dermal Microvascular Endothelial Cells

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Chemokines have been implicated convincingly in the driving of leukocyte emigration in different inflammatory reactions. Multiple signaling mechanisms are reported to be involved in intracellular activation of chemokine expression in vascular endothelial cells by various stimuli. Nevertheless, redox-regulated mechanisms of chemokine expression in human dermal microvascular endothelial cells (HDMEC) remain unclear. This study examined the effects of pyrrolidine dithiocarbamate (PDTC, 0.1 mM) and spermine NONOate (Sper-NO, 1 mM) on the secretion and gene expression of chemokines, interleukin (IL)-8, monocyte chemotactic protein (MCP)-1, regulated upon activation normal T cell expressed and secreted (RANTES), and eotaxin. This study also addresses PDTC and Sper-NO effects on activation of nuclear factor kappa B (NF-κB) induced by TNF-α (10 ng/ml). Treatment with TNF-α for 8 h significantly increased secretion of IL-8, MCP-1, and RANTES, but not of eotaxin, in cultured HDMEC. Up-regulation of these chemokines was suppressed significantly by pretreatment with PDTC or Sper-NO for 1 h, but not by 1 mM 8-bromo-cyclic GMP. The mRNA accumulation of IL-8, MCP-1, RANTES, and eotaxin, and activation of NF-κB were induced by TNF-α for 2 h; all were suppressed significantly by the above two pretreatments. These findings indicate that both secretion and mRNA accumulation of IL-8, MCP-1, and RANTES in HDMEC induced by TNF-α are inhibited significantly by pretreatment with PDTC or Sper-NO, possibly via blocking redox-regulated NF-κB activation. These results suggest that restoration of the redox balance using antioxidant agents or nitric oxide pathway modulators may offer new opportunities for therapeutic interventions in inflammatory skin diseases.

Keywords: Antioxidants; Chemokines; Endothelial activation; Nitric oxide; Nuclear factor kappa B; Reactive oxygen species

INTRODUCTION

Chemokines are small heparin-binding proteins that play a fundamental role in the recruitment of leukocytes.[1] They are divided into the CXC, CC, C, and CX3C subfamilies according to the positions of four conserved cysteine residues at the N-terminus of the molecule. The CXC and CC chemokine subfamilies are the most important. These two families of chemokines exhibit functional diversity: CXC chemokines are chemotactic for neutrophils and lymphocytes, whereas CC chemokines act on monocytes, activated T-cells, eosinophils, and basophils.[1–5]

Based on their ability to induce leukocyte chemotaxis and adhesion to endothelial cells, chemokines have been implicated in the driving of inflammatory leukocyte emigration. The chemokine genes are induced in vascular endothelial cells either by proinflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor alpha (TNF-α) or by interaction with microbial pathogens.[6] As demonstrated by Goebeler *et al.* and other investigators, human dermal microvascular endothelial cells (HDMEC) are capable of synthesizing CXC and CC chemokines such as IL-8, monocyte chemotactic protein (MCP)-1, regulated upon activation normal T cell expressed and secreted (RANTES), and eotaxin upon cytokine stimulation. Therefore, they may contribute to the establishment of a gradient of chemokine concentration.[2,6–8]

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For these reasons, it is essential to clarify regulatory mechanisms of chemokine production in inflammatory skin diseases. A transcription factor nuclear factor family, kappa B (NF-κB)/Rel, plays a central role in regulation of a variety of genes involved in host innate immunity, including the chemokines. Promoters of many chemokine genes, including IL-8, MCP-1, RANTES, and eotaxin, contain binding sites for NF-κB.[6,9] Several NF-κB activation inhibitors, such as antioxidants and proteasome inhibitors, have been used to examine whether activation of NF-κB is involved in the transcriptional activation of inflammatory genes.[6,10–12] Pyrrolidine dithiocarbamate (PDTC), an antioxidant, inhibits the phosphorylation of inhibitor kappa B alpha (IκBα), a prerequisite for its subsequent proteolytic degradation.[11,12] It has been reported that PDTC inhibits the production of IL-8, MCP-1, and RANTES in human umbilical vein endothelial cells (HUVEC), intestinal epithelial cells, and pancreatic periacinar myofibroblasts through a blockade of NF-κB activation;[11,13–15] it also suppresses ultraviolet B-induced NF-κB activation in keratinocytes.[16] On the other hand, nitric oxide (NO) actively participates in the modulation of inflammatory reactions and the trafficking of leukocytes. NO inhibits expression of adhesion molecules on endothelial cells, such as E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1.[17,18] Moreover, NO suppresses production of chemokines such as IL-8, MCP-1, and RANTES in human keratinocyte cell line HaCaT and in keratinocyte primary cultures established from healthy controls and patients with psoriasis,[3,19,20] and in HUVEC.[21]

However, chemokine production regulation by antioxidants and NO in HDMEC has been poorly elucidated. The present study was intended to evaluate effects of antioxidants, PDTC, and NO donor, spermine NONOate (Sper-NO), on mRNA expression and secretion of IL-8, MCP-1, RANTES, and eotaxin in HDMEC after TNF-α-stimulation.

METHODS

Endothelial Cell Line

HDMEC (Applied Cell Biology Research Institute, Kirkland, WA) were cultured in M199 medium (Sigma-Aldrich Corporation, St. Louis, MO) containing 20% fetal bovine serum (Sigma-Aldrich Corporation), 30 μg/ml endothelial cell growth factor (Biomedical Technologies Incorporation, Stoughton, MA) and 100 μg/ml heparin (Aventis Pharma Limited, Tokyo, Japan) using T-25 flasks (Becton, Dickinson and Company, Franklin Lakes, NJ) in a humidified atmosphere of 5% CO_2 at 37°C. The cells were confirmed to be endothelial in origin based on distinct cobblestone morphological features and positive immunofluorescent labeling with factor VIII and acetylated low-density lipoprotein. All experiments were conducted with endothelial cells at passages 3–6.

Determination of Chemokines

We seeded 500 μl of endothelial cell suspension (5×10^4 cells per well) on 24-well culture clusters (Costar; Corning Incorporation, Corning, NY). After cells reached confluence, HDMEC were incubated for 8 h in M199 medium containing 0.5% human albumin alone or with 10 ng/ml TNF-α in a final volume of 300 μl. In other wells, cells were pretreated for 1 h in M199 medium containing 0.5% human albumin with 0.1 mM PDTC, 1 mM Sper-NO or 1 mM 8-bromo-cyclic GMP (8-Br-cGMP) (Sigma-Aldrich Corporation); they were then incubated in the same medium alone or with 10 ng/ml TNF-α for 8 h. Quadruplicate supernatants for each condition were collected and centrifuged by 10,000 rpm for 5 min at 4°C. Cell-free supernatants were transferred to clean tubes and stored at −20°C prior to use. Preliminary experiments were done by treatment with TNF-α for 0, 4, 6, 8, 10, 12, 16, and 24 h. Viability of endothelial cells was unaffected by incubation with TNF-α up to for 8 h, as assessed by the trypan blue test.

Cell-free supernatants from resting or stimulated HDMEC cultures were tested for IL-8, MCP-1, RANTES, and eotaxin content by two-site sandwich enzyme-linked immunosorbent assay (ELISA) kits (Biosource International Incorporation, CA) according to the manufacturer's protocol. The plates were analyzed in duplicate in a microplate spectrophotometer (Spectra Max 250; Molecular Devices Corporation, Sunnyvale, CA) at 450 nm. Results were obtained from six independent experiments.

Quantitative Real-Time RT-PCR Analysis

After cells reached confluence in T-25 flasks, they were incubated for 2 h in M199 medium containing 0.5% human albumin alone or with 10 ng/ml TNF-α. In other flasks, cells were pretreated for 1 h in M199 medium containing 0.5% human albumin with 0.1 mM PDTC or 1 mM Sper-NO; they were then incubated in the same medium alone or with 10 ng/ml TNF-α for 2 h. After completion of the treatment, total RNA was extracted from cells using an RNeasy Kit (Qiagen Incorporation, Tokyo). First-strand cDNA synthesis was performed in a 20-μl reaction volume containing 1 μg of total RNA, 0.5 μM oligo$(dT)_{18}$, 20 U RNase inhibitor, 100 U murine leukemia virus reverse transcriptase (Toyobo Company Limited, Osaka), and 1 mM of each dNTP. The reaction was performed for 50 min

TABLE I List of oligonucleotides used for IL-8, MCP-1, RANTES, and β-actin cDNA amplification

Target gene		Sequence	Length (mer)	Position (bp)	Annealing temperature (°C)
IL-8	S	GATTTCTGCAGCTCTGTGTG	20	144–381 (257)	52.8
	AS	ATGAATTCTCAGCCCTCTTC	20		
MCP-1	S	CTGTGCCTGCTGCTCATA	18	75–258 (201)	54.6
	AS	GATCTCCTTGGCCACAAT	18		
RANTES	S	GTGCCCACATCAAGGAGTAT	20	157–374 (237)	54.2
	AS	GTAGGATAGTGAGGGGAAGC	20		
β-actin	S	ACATCCGCAAAGACCTGT	18	904–1313 (427)	54.9
	AS	CCTTCACCGTTCCAGTTT	18		

S = sense, AS = antisense.

at 42°C, and then for 5 min at 99°C to inactivate the reverse transcriptase.

Real-time PCR experiments were conducted in a LightCycler system (Roche Diagnostics, Idaho Falls, ID). Amplification was performed in duplicate in a 20-μl reaction volume containing 2 μl of 10 × LightCycler DNA Master SYBR Green I (containing 10 × PCR buffer, dNTP mixture, 10 mM $MgCl_2$, SYBR Green I dye, and Taq DNA polymerase), 1.6 μl of 25 mM $MgCl_2$ (final concentration 3 mM), 2 μl of cDNA (or water as negative control), and 0.5 μM (final concentration) of each primer. The LightCycler quantified β-actin mRNA as an internal control for normalization of chemokine mRNA values. Table I lists the primers for IL-8, MCP-1, RANTES, and β-actin (Nihon Gene Research Laboratories Incorporation, Miyagi, Japan). Real-time PCR was performed in glass capillaries with an initial denaturation step by heating at 95°C for 10 min, followed by 40 cycles of three steps: heating at 95°C for 15 s, cooling at 55°C for 5 s, and heating at 72°C for 18 s. After amplification, a melting curve was obtained for fluorescence collection. Melting peaks were used to determine the PCR reaction specificity. Standard curves were generated using 10-fold serial dilutions (10^{-3}–10^{1} ng/20 μl) of total RNA extracted from TNF-α-treated cells. For eotaxin mRNA measurement, real-time PCR was performed according to the manufacturer's protocol using the LightCycler primer set (Roche Diagnostics). For data analysis, the fit point method was used to set the threshold between background and significant fluorescence. The specificity of the PCR reaction was verified by agarose gel electrophoresis and ethidium bromide staining. Results were obtained from four independent experiments.

Quantification of NF-κB Activity in the Nucleus and Cytoplasm by Immunofluorescence

HDMEC were grown on glass-bottom culture dishes (MatTek Corporation, Ashland, MA). Cells were treated as described in Quantitative Real-Time RT-PCR Analysis. Thereafter, the endothelial cells were washed three times with phosphate-buffered saline (PBS), then fixed and permeabilized with −20°C acetone for 2 min. They were washed three times with PBS, treated with 10% normal goat serum (Vector Laboratories Incorporation, Burlingame, CA) for 20 min and then incubated with 0.2 μg/ml rabbit polyclonal antibody directed against the NF-κB subunit p65 (Santa Cruz Biotechnology Incorporation, Santa Cruz, CA) for 1 h. After three washes with PBS, 1.0 μg/ml biotinylated goat anti-rabbit antibody (Vector Laboratories Incorporation) was applied to the cells for 45 min. Following three washes with PBS, 1.0 μg/ml streptavidin-fluorescein isothiocyanate (Vector Laboratories Incorporation) was added for 30 min. After washing three times with PBS, immunofluorescence was visualized using an IX70 inverted microscope (Olympus Optical Company Limited, Tokyo). Cell images were captured and quantified using FISH imaging software (Hamamatsu Photonics K.K., Shizuoka, Japan) and Lumina Vision software (Mitani Corporation, Tokyo). The fluorescence intensities for NF-κB p65 in the nucleus and cytoplasm were measured in 20 cells that were chosen randomly for each experiment. Subsequently, the ratio of nuclear/cytoplasmic NF-κB p65 fluorescence intensity was calculated. Three independent experimental conditions were performed.

Statistical Analysis

Values were expressed as the mean ± SEM. Differences between groups were examined for statistical significance using the paired *t*-test or one-way analysis of variance followed by Scheffe's test, as appropriate. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Effects of PDTC, Sper-NO, and 8-Br-cGMP on the Secretion of IL-8, MCP-1, RANTES, and Eotaxin in the Supernatant of HDMEC Induced by TNF-α

Figure 1 shows that treatment of HDMEC with TNF-α for 8 h resulted in a significant increase in the secretion of IL-8, MCP-1, and RANTES, as compared

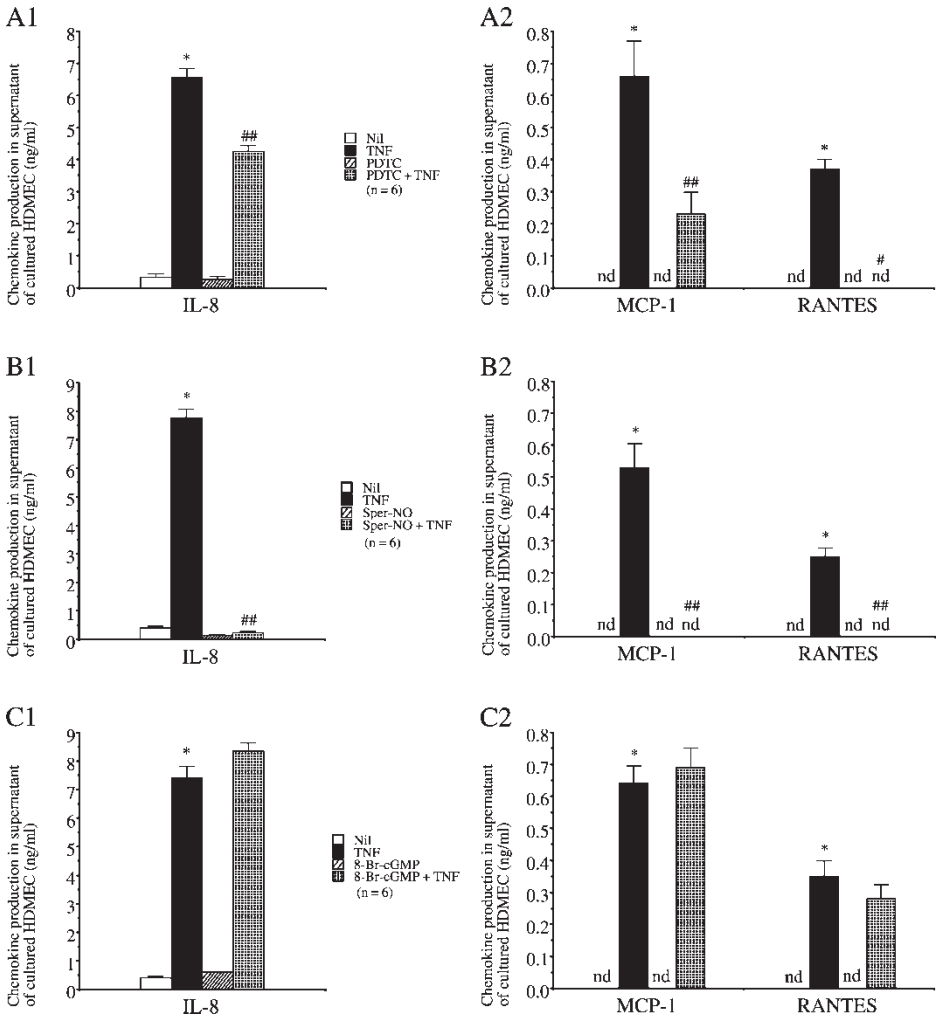

FIGURE 1 *Effects of PDTC, Sper-NO, and 8-Br-cGMP on the secretion of IL-8, MCP-1, and RANTES in TNF-α-stimulated HDMEC*. Cells were without pretreatment or pretreated with 0.1 mM PDTC (A), 1 mM Sper-NO (B) or 1 mM 8-Br-cGMP (C) for 1 h; thereafter, they were not stimulated or stimulated with TNF-α (10 ng/ml) for 8 h. The Nil groups consisted of cells incubated with medium alone. Quadruplicate supernatants for each condition were harvested, and ELISA measured the levels of IL-8, MCP-1, and RANTES. Results are expressed as the mean ± SEM of six independent experiments. $^{*}P < 0.001$ vs. Nil; $^{\#}P < 0.005$, $^{\#\#}P < 0.001$ vs. TNF-α alone. nd: not detectable.

to the non-stimulated cells. Eotaxin could not be detected after 8 h treatment with TNF-α. Pretreatment for 1 h with PDTC or Sper-NO significantly inhibited the secretion of IL-8, MCP-1, and RANTES induced by TNF-α (Fig. 1A and B). Pretreatment for 1 h with 8-Br-cGMP did not attenuate the secretion of IL-8, MCP-1, or RANTES induced by TNF-α (Fig. 1C). Treatment of endothelial cells with PDTC, Sper-NO, or 8-Br-cGMP alone did not alter production of any chemokines.

Effects of PDTC and Sper-NO on mRNA Expression of IL-8, MCP-1, RANTES, and Eotaxin in HDMEC Induced by TNF-α

Figure 2 shows that treatment of HDMEC with TNF-α for 2 h caused a significant increase in the mRNA level of IL-8, MCP-1, RANTES, and eotaxin, in comparison with non-stimulated cells. Pretreatment for 1 h with PDTC (Fig. 2A) or Sper-NO (Fig. 2B) significantly inhibited the TNF-α-induced mRNA accumulation of IL-8, MCP-1, RANTES, and eotaxin. Treatment with PDTC or Sper-NO alone did not alter the mRNA level of any chemokines.

Effects of PDTC and Sper-NO on the TNF-α-Induced Nuclear Translocation of NF-κB p65

Figure 3 shows that non-stimulated HDMEC revealed a diffuse distribution of immunoreactive NF-κB p65 within the cytoplasm (Fig. 3A). In contrast, treatment with TNF-α for 2 h caused dense accumulation of immunoreactive NF-κB p65 within the nucleus (Fig. 3B), the effect of which was inhibited by pretreatment with PDTC (Fig. 3D) or Sper-NO (Fig. 3F) for 1 h. Pretreatment with PDTC (Fig. 3C) or Sper-NO (Fig. 3E) alone had no effect. Accordingly, the TNF-α-induced high ratio of

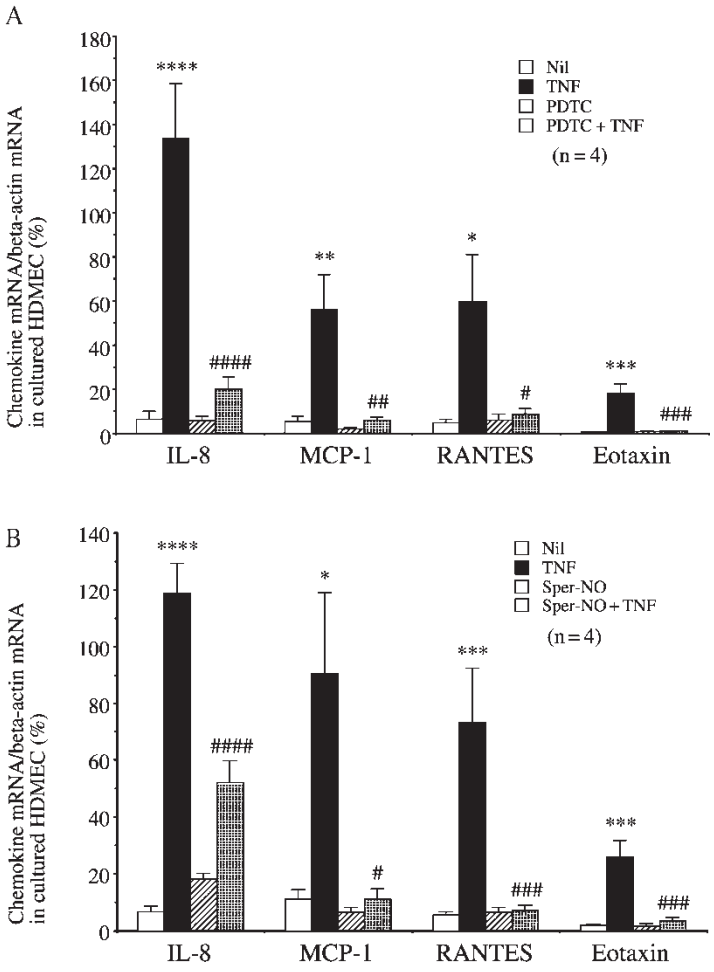

FIGURE 2 *Effects of PDTC and Sper-NO on the mRNA expression of IL-8, MCP-1, RANTES, and eotaxin in TNF-α-stimulated HDMEC*. Cells were without pretreatment or pretreated with PDTC (0.1 mM) (A) or Sper-NO (1 mM) (B) for 1 h; thereafter, they were not stimulated or stimulated with TNF-α (10 ng/ml) for 2 h. The Nil groups consisted of cells incubated with medium alone. The mRNA levels of chemokines were determined by quantitative real-time PCR analysis. Data indicate specific PCR products expressed as a percentage relative to β-actin mRNA and represent the mean ± SEM of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.001$ vs. Nil; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.005$, $^{\#\#\#\#}P < 0.001$ vs. TNF-α alone.

nuclear/cytoplasmic NF-κB p65 fluorescence was decreased significantly in endothelial cells pretreated with these chemicals in comparison with non-pretreated cells (Fig. 4).

DISCUSSION

The vascular endothelium plays a critical role in initiation and modulation of tissue inflammation through expression of surface proteins and secretion of soluble mediators. In particular, the chemokines and adhesion molecules expressed in endothelial cells play an important role in regulating recruitment of leukocytes to inflammation sites.[2,6–8] IL-8 is a CXC chemokine, whereas MCP-1, RANTES, and eotaxin are CC chemokines.[1] Although enhanced production of chemokines has been described for inflammatory diseases of the skin, including atopic dermatitis, allergic contact dermatitis, and psoriasis,[3–5,20,22,23] the effects of antioxidant and NO on chemokine production in HDMEC remain unclear.

This study demonstrated that the secretion of IL-8, MCP-1, and RANTES, but not of eotaxin, in cultured HDMEC was increased significantly after stimulation with TNF-α for 8 h. Our preliminary experiments using cultured HDMEC indicated that the secretion of IL-8 was increased significantly at 4 h after the start of treatment with TNF-α; it peaked at 12 h. Significant MCP-1 secretion was found at 8 h; its maximal level was attained at 10 h. Similarly, TNF-α-stimulated RANTES secretion reached a significant increase at 6 h and peaked at 12 h. Eotaxin remained undetectable, even after treatment with TNF-α for 24 h. Therefore, it appears that time-dependent kinetics of chemokine expression are diverse in TNF-α-stimulated human HDVEC. Furthermore, we found that treatment of HDMEC with TNF-α for 2 h induced a significant increase in the mRNA level of IL-8, MCP-1, RANTES, and eotaxin. The mRNA level of eotaxin appeared lower than that for the other

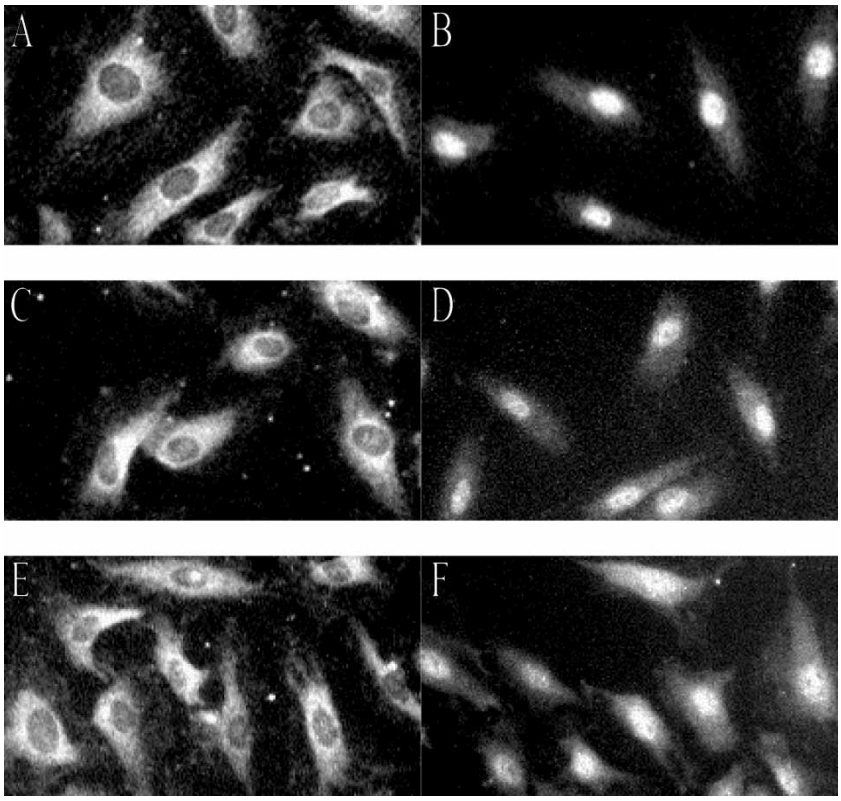

FIGURE 3 *Effects of PDTC and Sper-NO on nuclear translocation of NF-κB p65 in TNF-α-stimulated HDMEC*. Cells were without pretreatment or pretreated with PDTC (0.1 mM) or Sper-NO (1 mM) for 1 h and then not stimulated or stimulated with TNF-α (10 ng/ml) for 2 h. The NF-κB subunit p65 was immunostained with the specific antibody and visualized using an IX70 inverted microscope. Representative immunofluorescence studies are shown: (A) non-stimulated cells; (B) TNF-α-stimulated cells; (C) PDTC alone; (D) cells pretreated with PDTC and then stimulated by TNF-α; (E) Sper-NO alone; (F) cells pretreated with Sper-NO and then stimulated by TNF-α.

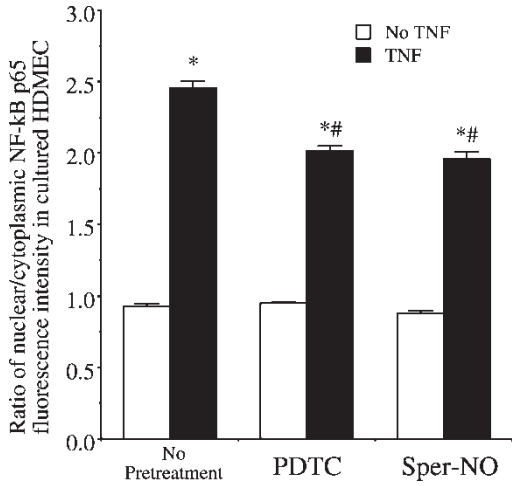

FIGURE 4 *Effects of PDTC and Sper-NO on the TNF-α-induced high ratio of nuclear/cytoplasmic NF-κB p65 fluorescence in HDMEC*. Cells were without pretreatment or pretreated with PDTC (0.1 mM) or Sper-NO (1 mM) for 1 h and then not stimulated or stimulated with TNF-α (10 ng/ml) for 2 h. The NF-κB subunit p65 was immunostained with the specific antibody and the fluorescence intensity was quantified. Results are expressed as the mean ± SEM of representative experiment (20 cells for each treatment). $^{*}P < 0.001$ vs. no TNF-α; $^{\#}P < 0.001$ vs. TNF-α alone. The experiment was repeated twice with similar results.

chemokines. These results suggest that TNF-α is not a major stimulator of eotaxin in HDMEC.

The NF-κB system is the most critical regulator of immediate transcriptional responses in inflammatory situations. Analysis of promoter elements of chemokine genes has revealed that IL-8, MCP-1, RANTES, and eotaxin contain binding sites for NF-κB.[6,9] The NF-κB consisting of p50 and p65 subunits is sequestered in the cytoplasm of most cells, and upon stimulation by various agents, it translocates rapidly into the nucleus, where it activates its target genes, including cytokines, adhesion molecules, chemokines, and NO synthase.[24–26]

Our experiments revealed that treatment with TNF-α for 2 h caused a significant translocation of NF-κB p65 to the nucleus in HDMEC. TNF-α stimulates production of reactive oxygen species (ROS), including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH) in a variety of cell types.[27,28] ROS may function as second messengers in mediating TNF-α- and IL-1β-activated signal transduction pathways that regulate the NF-κB system.[17,25]

Based on the above considerations, we evaluated the impact of antioxidant, PDTC, and NO donor, Sper-NO, on TNF-α-induced gene expression and secretion of chemokines in HDMEC. PDTC has a radical-scavenging, antioxidant property in addition to a metal-chelating property that decreases oxidative stress by preventing formation of hydroxyl radicals from hydrogen peroxide via the Fenton reaction.[24,29] A series of studies have demonstrated that TNF-α results in ROS production and that pretreatment with PDTC inhibits TNF-α-induced generation of ROS, thereby inhibiting NF-κB activation.[12,18,27,28] Activation of NF-κB DNA binding activity is also inhibited by treatment with NO donors in studies using vascular endothelial cells. Khan *et al.*[30] showed that in HUVEC and HDMEC, an NO donor (diethylamine-NO) reduced NF-κB activation induced by TNF-α. Impaired activation of NF-κB and signal transducer and activator of transcription 1 is observed when keratinocytes are stimulated in the presence of NO donors.[19]

Present experiments showed that pretreatment with PDTC or Sper-NO significantly decreased the level of NF-κB p65 present in the nucleus of TNF-α-stimulated HDMEC compared to cells treated with TNF-α alone. Our results indicate the inhibitory effect of these agents on TNF-α-induced NF-κB activation in HDMEC. We also assessed the inhibitory effect of PDTC or Sper-NO on TNF-α-induced chemokine gene expression in HDMEC. PDTC and Sper-NO significantly decreased the mRNA levels of IL-8, MCP-1, RANTES, and eotaxin that were induced by treatment with TNF-α for 2 h. They also significantly suppressed the secretion of IL-8, MCP-1, and RANTES induced by treatment with TNF-α for a period of 8 h.

NO may offer similar mechanisms to those of antioxidants in terms of suppressing ROS production and inhibiting NF-κB because NO can bind superoxide radical with extremely high affinity and thereby decrease its dismutated product, hydrogen peroxide.[17,30] NO donors may also inhibit NF-κB primarily through induction of its inhibitory protein, IκBα.[18] Inhibitory effects of NO donor, Sper-NO, on the production of chemokines were not mediated by cGMP-dependent mechanisms in our experiments because a cGMP analog, 8-Br-cGMP, did not affect TNF-α-induced secretion of IL-8, MCP-1, or RANTES. One attractive speculation is that inducible-type NO synthase expression by dermal endothelial cells, keratinocytes, or infiltrating cells in inflammatory conditions would produce relatively large amounts of NO. Such production would implicate an autoprotective regulatory mechanism because the induction of NO synthase in these cells requires activation of NF-κB.[26,31]

In preliminary experiments, we evaluated the effect of another NO donor, S-nitroso-N-acetylpenicillamine (SNAP, 1 mM), on the secretion and gene expression of IL-8, MCP-1, and RANTES in TNF-α-stimulated HDMVE. SNAP significantly inhibited the TNF-α-induced secretion of RANTES alone. SNAP tended to inhibit the TNF-α-induced mRNA expression of RANTES, but the difference was not statistically significant (data not shown). The more pronounced effect of Sper-NO than SNAP could be the result of the higher dose of NO released from Sper-NO than SNAP during the experiments because their reported half-lives at pH 7.4 are 39 min and a few hours, respectively.[32–34]

In summary, our studies demonstrate that PDTC and Sper-NO are each capable of suppressing the secretion and mRNA accumulation of chemokines, IL-8, MCP-1, and RANTES in HDMEC stimulated by TNF-α, possibly through blocking the activation of NF-κB. Supplemental antioxidants or NO donors that provide strategic targeting to affected cellular sites may offer protection from oxidative damage. Thereby, they may aid the treatment of inflammatory skin diseases.

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