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Effects of antioxidants and NO on TNF-α-induced adhesion molecule expression in human pulmonary microvascular endothelial cells

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Summary Pro-inflammatory cytokines initiate the vascular inflammatory response via upregulation of adhesion molecules on the endothelium. Recent observations suggest that reactive oxygen intermediates may play a pivotal role in TNF-α signaling and upregulate gene expression. We therefore evaluated the effects of pyrrolidine dithiocarbamate (PDTC; 0.1 mM) and spermine NONOate (Sper-NO; 1 mM) on adhesion molecule expression and nuclear factor kappa B (NF-κB) activation induced by TNF-α(10 ng/ml) in cultured human pulmonary microvascular endothelial cells (PMVEC). Treatment of cells with TNF-α for 4 h significantly induced the surface expression of E-selectin and ICAM-1. Treatment with TNF-α for 8 h significantly induced the surface expression of E-selectin, ICAM-1 and VCAM-1. The upregulation of these adhesion molecules was suppressed significantly by pretreatment with PDTC or Sper-NO for 1 h. 8-Bromo-cyclic GMP (1 mM) had no such effect, suggesting that the NO donor's effect was non-cGMP-dependent. The mRNA expression of E-selectin, ICAM-1 and VCAM-1, and activation of NF-κB induced by TNF-α for 2 h were decreased significantly by the above two pretreatments. *N*-acetylcysteine (10 mM) and *S*-nitroso-*N*-acetylpenicillamine (1 mM) had little inhibitory effects on the cell surface and mRNA expression of these adhesion molecules stimulated by TNF-α. Treatment with TNF-α for 4 h enhanced HL-60 leukocyte adhesion to human PMVEC, the effect of which was inhibited significantly by pretreatment with PDTC or Sper-NO. These findings indicate that both cell surface and mRNA expression of adhesion molecules in human PMVEC induced by TNF-α are inhibited significantly by

Abbrevations: 8-Br-cGMP, 8-bromo-cyclic GMP; NAC, *N*-acetylcysteine; PBS, phosphate-buffered saline; PDTC, pyrrolidine dithiocarbamate; PMVEC, pulmonary microvascular endothelial cells; ROI, reactive oxygen intermediates; SNAP, *S*-nitroso-*N*-acetylpenicillamine; Sper-NO, spermine NONOate

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pretreatment with PDTC or Sper-NO, possibly in part through blocking the activation of NF-κB. Although our in vitro results cannot be directly extrapolated to the in vivo situation, they suggest a potential therapeutic approach for intervention in cytokine-mediated inflammatory processes in the human lung.

Introduction

One of the proximal events in vascular inflammation is the activation of endothelial cells. The activated endothelium expresses cell adhesion molecules in response to a variety of pro-inflammatory stimuli, such as TNF-α, interleukin-1β and lipopolysaccharide.[1–3] The endothelial–leukocyte adhesion molecules, such as E-selectin, ICAM-1 and VCAM-1, facilitate the attachment of peripheral blood leukocytes to the endothelial cell surface. The regulation of adhesion molecule expression is complex and occurs at multiple levels ranging from gene transcription to post-translational protein modifications.

A key player in the regulation of inflammatory gene expression is the NF-κB family of transcription factors.[2–6] The NF-κB consisting of p50 and p65 subunits is sequestered in the cytoplasm of most cells where they are bound to inhibitory proteins known as IκBα. Treatment of endothelial cells with TNF-α leads to activation of IκBα kinase, which phosphorylates IκBα and targets it for rapid polyubiquitination followed by degradation through the 26S proteasome. This results in release of the NF-κB dimer and its translocation to the nucleus to activate transcription of multiple κB-dependent genes, including cytokines, chemokines, adhesion molecules, cyclooxygenase, NO synthase and IκBα. It has been proposed that reactive oxygen intermediates (ROI) are important and widely used second messengers in NF-κB activation.[3,4,6–13] NO may also exhibit a dual redox function based on its interaction with ROI and regulate the activation of NF-κB.[3,4,6,14–20]

The human lung contains approximately 600 million alveoli surrounded by a dense meshwork of capillaries ~1500 miles long when placed end to end. Functional impairment of the endothelial barrier is a serious pathological condition underlying inflammatory lung injury.[21] Expression of cell adhesion molecules on human pulmonary microvascular endothelial cells (PMVEC) is thought to play an important role in the recruitment of leukocytes in pulmonary inflammation. TNF-α induces expression of E-selectin, ICAM-1 and VCAM-1 on the surface of human PMVEC.[22,23] It has been reported that inhibitors of phosphodiesterase 3 and 4 isoenzymes attenuate TNF-α-induced expression of E-selectin and VCAM-1 and leukocyte adhesion to human PMVEC,[24] and that tyrosine kinase inhibitors alter TNF-α-induced expression of ICAM-1 and leukocyte adhesion to these cells.[25] Another in vitro study suggested that the presence of a co-stimulus such as lipopolysaccharide is necessary for interleukin-4 to induce effective VCAM-1 expression in human PMVEC.[22] Furthermore, in vivo studies have demonstrated that NF-κB activation in the lung is suppressed by antioxidants or anti-inflammatory cytokines.[7,8,26]

In humans, an imbalance between the production of ROI and antioxidant capacity can lead to a state of "oxidative stress" that contributes to the pathogenesis of a number of pulmonary diseases, such as acute respiratory distress syndrome, interstitial pulmonary diseases, emphysema, bronchial asthma and brochopulmonary dysplasia.[4,21,27] Previous studies have reported high levels of expression of NF-κB in airway cells of asthmatic patients.[28,29] Thus, increased NF-κB expression and DNA binding may underlie the increased expression of several inflammatory proteins in the asthmatic airway.

Much of the extensive work, to date, examining the redox mechanisms by which cytokines or lipopolysaccharide induce cell adhesion molecules and factors that control these processes has been carried out using endothelial cells derived from large vessels.[9,11–14,18,19] There is evidence, however, for heterogeneity of endothelial cells between different vascular sites.[1] Thus, it may be necessary to use endothelial cells derived from the microvasculature of the lung in in vitro studies that seek to examine the signaling mechanisms of inflammatory conditions within this organ. The purpose of the present study was to test the hypothesis that TNF-α induces cell adhesion molecule expression through a redox-sensitive pathway in human PMVEC. We examined the effects of antioxidants and exogenous NO on the induction of adhesion molecules, including E-selectin, ICAM-1 and VCAM-1, and the consequent adhesion of leukocytes, after TNF-α-stimulation of human PMVEC in vitro.

Methods

Endothelial cell line

Human PMVEC (Applied Cell Biology Research Institute, Kirkland, WA) were cultured in M199 medium (Sigma, St. Louis, MO) containing 20% fetal bovine serum (Sigma), 30 μg/ml endothelial cell growth factor (Biomedical Technologies, Stoughton, MA) and 100 μg/ml heparin (Aventis Pharma, Tokyo, Japan) using T-25 flasks (Becton Dickinson, Franklin Lakes, NJ) precoated with 10 μg/ml human fibronectin (Sigma) 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, acetylated low-density lipoprotein and diaminofluorescein-2 (Daiichikagaku, Tokyo), as described previously.[30]

Human promyelocytic leukemic cell line (HL-60)

HL-60 cells (Applied Cell Biology Research Institute) were grown in RPMI 1640 medium (Sigma) containing 5% fetal bovine serum using T-25 flasks and maintained in log phase growth (about 5×10^5 cells/ml).

Flow cytometry analysis

Indirect immunofluorescence using flow cytometry analyzed cell surface expression of adhesion molecules in human PMVEC. After cells reached confluence in T-25 flasks, they were incubated for 4 h in M199 medium containing 0.5% human albumin (Nacalai, Kyoto, Japan) alone or together with 10 ng/ml TNF-α (Sigma). In other flasks, human PMVEC were pretreated for 1 h in M199 medium containing 0.5% human albumin with 0.1 mM pyrrolidine dithiocarbamate (PDTC), 10 mM *N*-acetylcysteine (NAC) (Sigma), 1 mM spermine NONOate (Sper-NO), 1 mM *S*-nitroso-*N*-acetylpenicillamine (SNAP) (Cayman) or 1 mM 8-bromo-cyclic GMP (8-Br-cGMP) (Sigma); then they were incubated in the same medium alone or with 10 ng/ml TNF-α for 4 h. In other independent experiments, we also performed eight-hour-treatment with TNF-α and examined the effects of PDTC or Sper-NO. Viability of endothelial cells was unaffected during the time course of these experiments, as assessed by the trypan blue test. After completion of the treatment, cells were washed twice with phosphate-buffered saline (PBS), detached by incubation with 0.02% EDTA, fixed with 3.7% formaldehyde in PBS for 10 min at 4 °C and washed twice with PBS containing 0.2% human albumin. Cells were incubated for 45 min at 4 °C with 2 μg/ml mouse anti-human monoclonal antibodies against E-selectin, ICAM-1, VCAM-1 or IgG_1 isotype control (PharMingen, San Diego, CA) in PBS containing 1 mg/ml human IgG (Mitsubishi Pharma, Osaka, Japan), washed twice with PBS containing 0.2% albumin and then incubated with a 1:200 dilution of goat anti-mouse IgG conjugated with fluorescein isothiocyanate (Tagoimmunologicals, Camarillo, CA) for 30 min at 4 °C. Following two final washes in PBS containing 0.2% albumin, samples were assayed using the Coulter Epics XL flow cytometer (Miami, FL). At least 5000 events were collected for each sample; the results were expressed as mean fluorescence intensity as calculated on a logarithmic scale.

Quantitative real-time RT-PCR analysis

The mRNA expression of adhesion molecules was studied with quantitative real-time RT-PCR. After cells reached confluence in T-25 flasks, they were incubated for 2 h in M199 medium containing 0.5% human albumin alone or together 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, 10 mM NAC, 1 mM Sper-NO or 1 mM SNAP; 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 the RNeasy Kit (Qiagen, 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, Osaka) and 1 mM of each dNTP. The reaction was performed for 50 min at 42 °C, and then for 5 min at 99 °C to inactivate the reverse transcriptase. Real-time PCR experiments were conducted in the 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, which was always included) and 0.5 μM (final concentration) of each primer. The LightCycler quantified β-actin mRNA as an internal control for normalization of adhesion molecule mRNA values. Table 1 lists the primers for E-selectin, ICAM-1, VCAM-1 and β-actin (Nihon Gene Research Laboratories, Miyagi, Japan).

Table 1 List of oligonucleotides used for E-selectin, ICAM-1, VCAM-1 and β-actin cDNA amplification.

Target gene	Sequence	Length	Position (bp)	Annealing	Temperature (°C)
E-selectin	S	TGTGAAGCTCCCACTGAGT	19-mer	1755–2042 (307)	54.1
	AS	TCTGGCATAGTAGGCAAGAA	20-mer		
ICAM-1	S	TAGAGACCCCGTTGCCTAA	19-mer	191–389 (217)	55.0
	AS	GTAAGGTTCTTGCCCACTG	19-mer		
VCAM-1	S	GGGAACGAACACTCTTACCT	20-mer	262–482 (240)	52.9
	AS	ATCCTCCAGAAATTCCTGAC	20-mer		
β-actin	S	ACATCCGCAAAGACCTGT	18-mer	904–1313 (427)	54.9
	AS	CCTTCACCGTTCCAGTTT	18-mer		

S=sense, AS=antisense.

Real-time PCR was performed in glass capillaries with an initial denaturation step by heating at 20 °C/s (temperature transition) to 95 °C for 10 min, followed by 40 cycles of three steps, consisting of heating at 20 °C/s to 95 °C with a 15-s hold, cooling at 20 °C/s to 55 °C with a 5-s hold, and heating at 20 °C/s to 72 °C with a 18-s hold. After amplification, a melting curve was obtained by heating at 20 °C/s to 95 °C with a 0-s hold, cooling at 20 °C/s to 60 °C with a 15-s hold, and slowly heating at 0.1 °C/s to 95 °C with a 0-s hold for fluorescence collection. Melting peaks were used to determine the specificity of the PCR reaction. 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 data analysis, the fit-point method was used to set the threshold between background and significant fluorescence. A standard curve was generated from plotting external standard concentrations against threshold cycle. The specificity of the PCR reaction was verified by agarose gel electrophoresis and ethidium bromide staining. The results were obtained from four independent experiments.

Quantification of NF-κB activity in the nucleus and cytoplasm by immunofluorescence

Human PMVEC were grown on glass bottom culture dishes (MatTek Corporation, Ashland, MA). Cells were treated as described in *Quantitative real-time RT-PCR analysis*, in which the experiments with NAC and SNAP were abridged. Thereafter, the endothelial cells were washed three times with PBS and 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, 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, Santa Cruz, CA) for 1 h. After three washes with PBS, 1.0 μg/ml biotinylated goat anti-rabbit antibody (Vector Laboratories) was applied to the cells for 45 min. Following three washes with PBS, 1.0 μg/ml streptavidin–fluorescein isothiocyanate (Vector Laboratories) was added for 30 min. After washing three times with PBS, immunofluorescence was visualized using an IX70 inverted microscope (Olympus, Tokyo). Cell images were captured and quantified using FISH imaging software (Hamamatsu Photonics, Shizuoka, Japan) and Lumina Vision fluorescence imaging analysis software (Mitani, Tokyo). The fluorescence intensity for NF-κB p65 in the nucleus and cytoplasm was measured in 20 cells that were randomly chosen for each experiment. Subsequently, the ratio of nuclear/cytoplasmic NF-κB p65 fluorescence intensity was calculated. The results were based on three independent experimental conditions.

Adhesion assay

Two-hundred microlitres of endothelial cell suspension (5×10^4 cells/well) were seeded on 48-well culture plates (Becton Dickinson) and allowed to grow to confluence. Cells were treated as described in *Flow cytometry analysis*, in which the experiment with 8-bromo-cyclic GMP was abridged. HL-60 cells were labeled with 3 μM 5-chloromethylfluorescein diacetate (Molecular Probes, Eugene, OR) for 45 min at 37 °C in calcium-free Hanks' buffered saline solution (Sigma) and subsequently washed twice with the same buffer. Fluorescence-labeled cells (3×10^5 cells/ml) were resuspended in

Krebs–Ringer buffer containing 0.5% human albumin. Human PMVEC were washed three times with the same buffer, and 200 μl of labeled HL-60 cells (6×10^4/well) were plated on the endothelial monolayers. After incubation for 15 min at 37 °C, non-adherent HL-60 cells were collected by aspiration of the HL-60 suspension buffer and subsequent draining with 200 μl of the same buffer (in a final volume of 400 μl). Thereafter, 400 μl of Krebs–Ringer buffer containing 0.5% albumin were placed into each well. Both adherent and non-adherent HL-60 cells were lysed with the addition of 200 μl of 6% Triton X-100 (Nacalai) in PBS for 2 h at 37 °C. Fluorescence was measured using a spectrofluorometer (Fluoroskan II, Thermo Labsystems, Vantaa, Finland) with excitation at 485 nm and emission at 538 nm. The percentage of HL-60 adhesion was calculated by dividing the fluorescence for adherent cells by that for both adherent and non-adherent cells. The results were based on two experimental conditions, each consisting of six replicates.

Adhesion blockade by specific monoclonal antibodies was performed to determine the contribution of E-selectin, ICAM-1 and VCAM-1 to the HL-60 cell adhesion to TNF-α-treated human PMVEC. After stimulation of endothelial cells with 10 ng/ml TNF-α for 4 h and three washes with Krebs–Ringer buffer containing 0.5% albumin, saturating amounts of mouse monoclonal antibodies directed against the adhesion molecules were applied to the cells for 1 h. The blocking concentration was 40 μg/ml for anti-human E-selectin antibody, 20 μg/ml for anti-human ICAM-1 antibody and 60 μg/ml for anti-human VCAM-1 antibody (R&D systems, Minneapolis, MN). Mouse IgG_1 isotype control (R&D systems) was applied to the cells in other wells; the concentration was adjusted in accordance with that for different blocking antibodies. After aspirating the medium, the fluorescence-labeled HL-60 cells were plated on the endothelial cells, incubated for 15 min, and then the percentage of HL-60 adhesion was calculated, as outlined above. The results were based on two experimental conditions, each consisting of six replicates.

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 < 0.05 were considered statistically significant.

Results

Effects of antioxidants, NO donors and a cyclic GMP analogue on cell surface expression of adhesion molecules induced by TNF-α

As shown in Fig. 1, treatment of human PMVEC with TNF-α for 4 h resulted in a significant increase in the surface expression of E-selectin and ICAM-1, but not of VCAM-1, compared with the non-stimulated cells. Pretreatment for 1 h with PDTC or Sper-NO significantly inhibited the TNF-α-induced expression of E-selectin (mean fluorescence intensity: by 28%, 39%, respectively) and ICAM-1 (by 44%, 55%, respectively) on endothelial cell surfaces (Fig. 1A and C). NAC or SNAP tended to inhibit the TNF-α-induced expression of ICAM-1 (by 27%, 22%, respectively), albeit not statistically significant (Fig. 1B and D). 8-Bromo-cGMP had no effect on the TNF-α-induced expression of any adhesion molecules (Fig. 1E). Eight-hour-treatment with TNF-α caused a significant increase in the surface expression of E-selectin, ICAM-1 and VCAM-1 (Fig. 2). Their respective effects were inhibited significantly by pretreatment with PDTC (Fig. 2A) or Sper-NO (Fig. 2B) for 1 h. Treatment of endothelial cells with PDTC, NAC, Sper-NO, SNAP or 8-Br-cGMP alone did not alter the expression of any adhesion molecules.

Effects of antioxidants and NO donors on mRNA expression of adhesion molecules induced by TNF-α

As shown in Fig. 3, treatment of human PMVEC with TNF-α for 2 h caused a significant increase in the mRNA level of E-selectin, ICAM-1 and VCAM-1, as compared to the non-stimulated cells. Pretreatment for 1 h with PDTC (Fig. 3A) or Sper-NO (Fig. 3C) significantly inhibited the TNF-α-induced mRNA expression of E-selectin (mean level: by 83%, 80%, respectively), ICAM-1 (by 70%, 76%, respectively) and VCAM-1 (by 90%, 89%, respectively). NAC tended to inhibit the TNF-α-induced mRNA expression of E-selectin (by 39%) and VCAM-1 (by 42%), but the differences were not statistically significant (Fig. 3B). SNAP also tended to inhibit the TNF-α-induced mRNA expression of E-selectin (by 27%), ICAM-1 (by 27%) and VCAM-1 (by 48%), but the difference reached statistical significance only for VCAM-1 (Fig. 3D). Treatment with PDTC, NAC, Sper-NO or SNAP alone did not alter the mRNA level of any adhesion molecules.

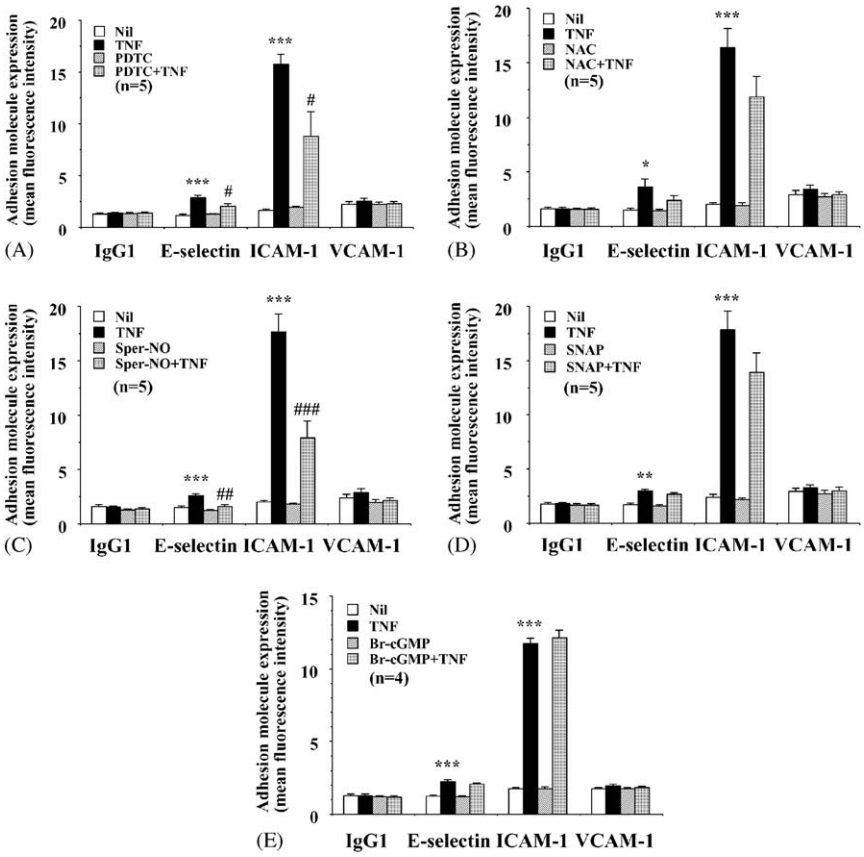

Figure 1 Effects of antioxidants, NO donors and a cyclic GMP analogue on the cell surface expression of E-selectin, ICAM-1 and VCAM-1 in human PMVEC stimulated by TNF-α for 4 h. Cells were without pretreatment or pretreated with antioxidants (0.1 mM PDTC, 10 mM NAC), NO donors (1 mM Sper-NO, 1 mM SNAP) or a cyclic GMP analogue (1 mM 8-Br-cGMP) for 1 h and then not stimulated or stimulated with TNF-α (10 ng/ml) for 4 h. The Nil group consisted of cells incubated with medium alone. These cells were immunostained with specific antibodies against adhesion molecules and analyzed by flow cytometry. Immunostaining with isotype-matched IgG_1 served as internal control. Results are expressed as the mean ± SEM of four or five independent experiments. $^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.001$ vs. Nil; $^{\#}P<0.05$, $^{\#\#}P<0.005$, $^{\#\#\#}P<0.001$ vs. TNF-α alone.

Effects of antioxidants and NO donors on the TNF-α-induced nuclear translocation of NF-κB

As shown in Fig. 4, non-stimulated human PMVEC revealed a diffuse distribution of immunoreactive NF-κB p65 within the cytoplasm (Fig. 4A). In contrast, treatment with TNF-α for 2 h resulted in dense accumulation of immunoreactive NF-κB p65 within the nucleus (Fig. 4B). This effect was inhibited by pretreatment with PDTC (Fig. 4C) or Sper-NO for 1 h (Fig. 4D). Accordingly, the TNF-α-induced high ratio of nuclear/cytoplasmic NF-κB p65 fluorescence was decreased significantly in endothelial cells pretreated with these chemicals than in non-pretreated cells (Fig. 5). Pretreatment with PDTC or Sper-NO alone had no effect (data not shown).

Effects of antioxidants and NO donors on HL-60 cell adhesion to TNF-α-stimulated cells

As shown in Fig. 6, stimulation with TNF-α for 4 h significantly enhanced the HL-60 cell adhesion to human PMVEC. Pretreatment of endothelial cells with PDTC (Fig. 6A) or Sper-NO (Fig. 6C) for 1 h caused significant inhibition of the TNF-α-induced adhesion, while NAC (Fig. 6B) or SNAP (Fig. 6D) had no such effect. Pretreatment with PDTC, NAC, Sper-NO or SNAP alone had no effect. The enhanced HL-60 cell adhesion to TNF-α-stimulated cells was abolished by pretreatment with anti-E-selectin antibody before the adhesion assay (Fig. 7A), while pretreatment with anti-ICAM-1 (Fig. 7B) or anti-VCAM-1 antibodies had no such effect (Fig. 7C). Isotype-matched control antibody did not affect the HL-60 cell adhesion.

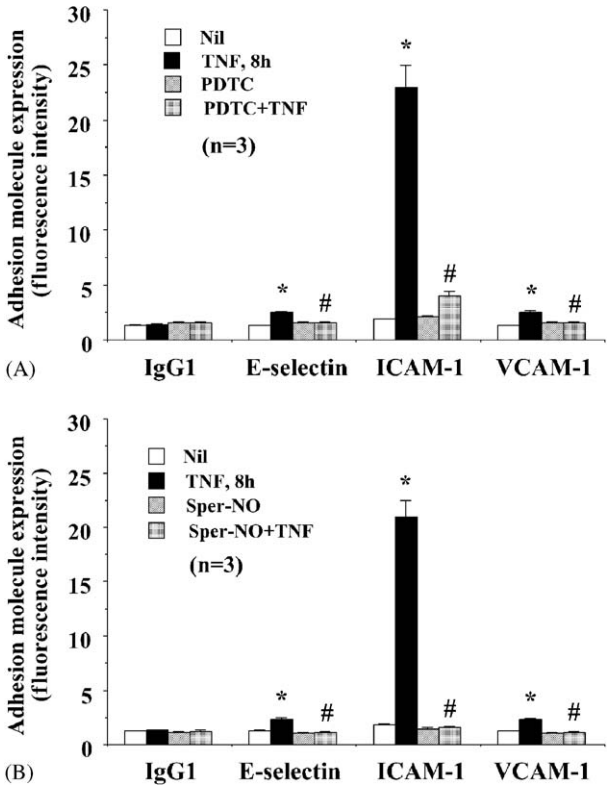

Figure 2 Effects of PDTC and Sper-NO on the cell surface expression of E-selectin, ICAM-1 and VCAM-1 in human PMVEC stimulated by TNF-α for 8 h. Cells were without pretreatment or pretreated with 0.1 mM PDTC or 1 mM Sper-NO for 1 h and then not stimulated or stimulated with TNF-α (10 ng/ml) for 8 h. The Nil group consisted of cells incubated with medium alone. These cells were immunostained with specific antibodies against adhesion molecules and analyzed by flow cytometry. Immunostaining with isotype-matched IgG_1 served as internal control. Results are expressed as the mean ± SEM of three independent experiments. $^{*}P<0.001$ vs. Nil; $^{\#}P<0.001$ vs. TNF-α alone.

Discussion

Positioned at the interface between blood and tissue, the endothelium is equipped to respond quickly to local changes in biological needs caused by inflammation. Endothelial cells express cell adhesion molecules that orchestrate the trafficking of circulating blood cells.[1–3] These adhesion molecules accelerate the migration of leukocytes toward sites of inflammation in response to a variety of stimuli. The TNF-α-induced upregulation of endothelial–leukocyte adhesion molecules and consequent leukocyte adhesion have been well documented to occur in vivo as well as in vitro.[1,2,6,7,9,11–14,16,22–25] However, little information is yet available as to redox mechanisms in the adhesion molecule expression in human PMVEC.[7]

Present experiments using cultured human PMVEC demonstrated that cell surface expression of E-selectin, ICAM-1 and VCAM-1 were induced significantly after stimulation with TNF-α for 8 h, but that the expression of VCAM-1 did not increase significantly after 4-h treatment with TNF-α. Treatment of endothelial cells with TNF-α for 2 h also induced a significant increase in the mRNA level of E-selectin, ICAM-1 and VCAM-1. Preliminary experiments indicated that cell surface expression of ICAM-1 and VCAM-1 was markedly induced after stimulation with TNF-α (10 ng/ml) for 24 h. The mean (± SEM) fluorescence intensity was 1.4 ± 0.2 and 2.2 ± 0.3 for E-selectin, 2.6 ± 0.4 and 76.0 ± 3.1 for ICAM-1, and 1.4 ± 0.2 and 17.1 ± 3.6 for VCAM-1 in non-stimulated and TNF-α-stimulated cells, respectively ($n = 4$ in each) (MZ Jiang and H Tsukahara, unpublished observations). In support of this, Blease et al.[22] have revealed that TNF-α induces maximal E-selectin expression at 6 h that is reduced at 16 h, and also significant ICAM-1 expression at 6 h and maximal expression at 24 h, while TNF-α-induced VCAM-1 expression peaks at 24 h that are sustained at 72 h. It appears, therefore, that the time-dependent kinetics of adhesion molecule expression are diversified in TNF-α-stimulated human PMVEC.

Regulation of adhesion molecule expression is under the control of the endothelial NF-κB system.[2,3,5,6] Analysis of promoter elements of adhesion molecule genes has revealed that E-selectin, ICAM-1 and VCAM-1 contain from one to three NF-κB binding sites. Activation of this transcription factor involves phosphorylation of the main inhibitory protein IκBα followed by dissociation of NF-κB from IκBα and degradation of IκBα. NF-κB then moves to the nucleus and activates the expression of a plethora of genes. The activation pathway is widespread and possibly ubiquitous in vascular endothelial cells.[9,11–14,31] This is consistent with our finding that stimulation by TNF-α for 2 h caused the translocation of NF-κB p65 to the nucleus in human PMVEC. The functional significance of the adhesion molecules induced by TNF-α was evaluated with a static adhesion assay using the promyelocytic leukemic cell line HL-60. As expected, treatment with TNF-α for 4 h stimulated endothelial cells to increase the HL-60 binding. Monoclonal antibody against E-selectin, but not ICAM-1 or VCAM-1, abolished the HL-60 adhesion to TNF-α-stimulated cells, which indicated the major contribution of E-selectin to the adhesion process of HL-60 to TNF-α-stimulated endothelial cells under these experimental conditions.

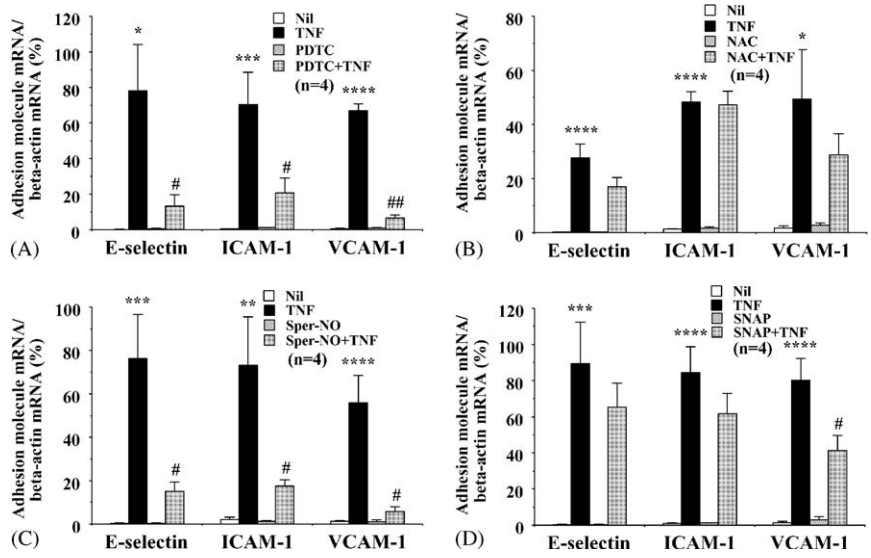

Figure 3 Effects of antioxidants and NO donors on the mRNA expression of E-selectin, ICAM-1 and VCAM-1 in TNF-α-stimulated human PMVEC. Cells were without pretreatment or pretreated with antioxidants (0.1 mM PDTC, 10 mM NAC) or NO donors (1 mM Sper-NO, 1 mM SNAP) for 1 h and then not stimulated or stimulated with TNF-α (10 ng/ml) for 2 h. The Nil group consisted of cells incubated with medium alone. The mRNA levels of adhesion molecules 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; $^{\#}P<0.05$, $^{\#\#}P<0.001$ vs. TNF-α alone.

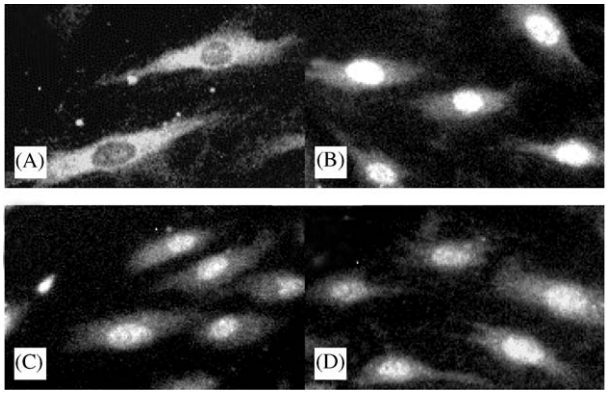

Figure 4 Effects of PDTC and Sper-NO on the nuclear translocation of NF-κB p65 in TNF-α-stimulated human PMVEC. Cells were without pretreatment or pretreated with 0.1 mM PDTC or 1 mM Sper-NO for 1 h and then not stimulated or stimulated with TNF-α (10 ng/ml) for 2 h. The NF-κB subunit p65 was immunostained with the specific antibody and visualized as detailed in Methods. Representative immunofluorescence studies are shown. (A) unstimulated cells; (B) TNF-α-stimulated cells; (C) and (D) cells pretreated with PDTC (C) or Sper-NO (D) and then stimulated by TNF-α.

TNF-α stimulates production of ROI, including superoxide anions, hydrogen peroxide and hydroxyl radicals, in a variety of cell types.[9,12,13] ROI generated in excess amounts damage cells by

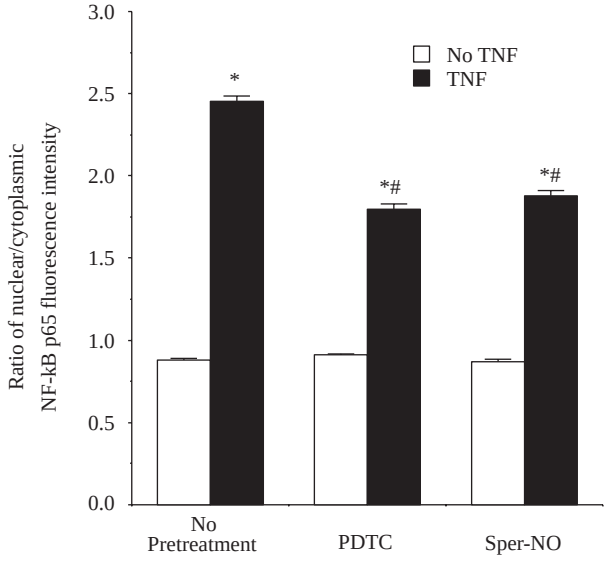

Figure 5 Effects of PDTC and Sper-NO on the TNF-α-induced high ratio of nuclear/cytoplasmic NF-κB p65 fluorescence in human PMVEC. Cells were without pretreatment or pretreated with 0.1 mM PDTC or 1 mM Sper-NO for 1 h and then not stimulated or stimulated with TNF-α (10 ng/ml) for 2 h. The NF-κB subunit p65 was immunostained with the specific antibody and the fluorescence intensity was quantified as detailed in Methods. Results are expressed as the mean ± SEM of three independent experiments (20 cells for each experiment). $^{*}P<0.001$ vs. non-treatment (medium alone); $^{\#}P<0.001$ vs. treatment with TNF-α alone.

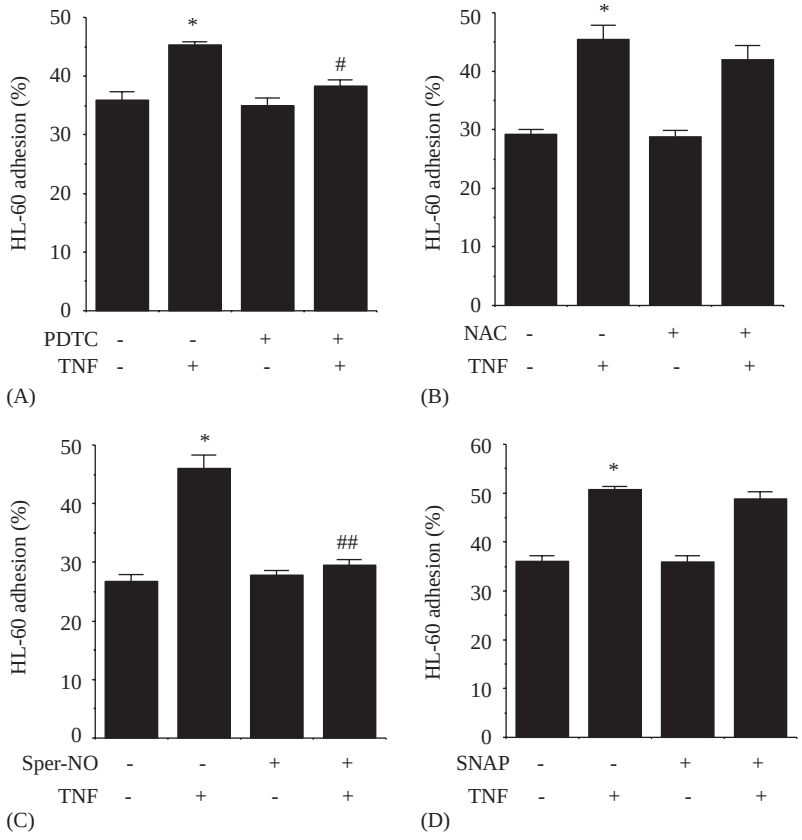

Figure 6 Effects of antioxidants and NO donors on HL-60 cell adhesion to TNF-α-stimulated human PMVEC. Cells were without pretreatment or pretreated with antioxidants (0.1 mM PDTC, 10 mM NAC) or NO donors (1 mM Sper-NO, 1 mM SNAP) for 1 h and then not stimulated or stimulated with TNF-α (10 ng/ml) for 4 h. Fluorescence-labeled HL 60 cells were added to endothelial cells as suspension, co-incubated for 15 min and the percentage of HL-60 adhesion was calculated. Results are expressed as the mean ± SEM of two independent experiments, each consisting of six replicates. $^{*}P<0.001$ vs. non-treatment (medium alone); $^{\#}P<0.005$, $^{\#\#}P<0.001$ vs. TNF-α alone.

peroxidizing lipids and disrupting proteins and nucleic acids. ROI in lower concentrations may function as second messengers in mediating TNF-α- and interleukin-1β-activated signal transduction pathways that regulate the NF-κB system.[3,6] Because the nuclear translocation of NF-κB is essential for the regulation of adhesion molecules in cytokine-activated endothelial cells,[9,11–14,31] it is plausible that there would be a common redox-sensitive mechanism controlling the expression of E-selectin, ICAM-1 and VCAM-1 in these cells.

Based on the above considerations, we tested the impact of two antioxidants, PDTC and NAC, and two NO donors, Sper-NO and SNAP, on TNF-α-induced expression of adhesion molecules in human PMVEC. The antioxidants, PDTC and NAC, are structurally different but both prevent NF-κB activation through their antioxidant property.[7–13] Marui et al.[11] and Weber et al.[13] reported that PDTC or NAC inhibited TNF-α-induced VCAM-1 surface protein and mRNA expression, in part, by blocking NF-κB activation in human umbilical vein endothelial cells. Ferran et al.[9] reported that stimulation of porcine aortic endothelial cells with TNF-α resulted in ROI production and that pretreatment with PDTC inhibited TNF-α-induced generation of ROI, activation of NF-κB and expression of E-selectin, ICAM-1 and VCAM-1. Rahman et al.[12] also demonstrated that stimulation of human pulmonary artery endothelial cells with TNF-α resulted in ROI production and that pretreatment with PDTC or NAC inhibited TNF-α-induced generation of ROI, and activation of NF-κB and E-selectin expression. These results support the contention that generation of ROI in endothelial cells induced by pro-inflammatory cytokines such as TNF-α is a critical signal mediating cell adhesion molecule expression. Activation of NF-κB DNA binding activity was also inhibited by treatment with NO donors in studies using vascular endothelial cells. Liao and associates[18,19] showed that NO donors, sodium nitroprusside and S-nitrosoglutathione, inhibited TNF-α-induced NF-κB activation by stabilization

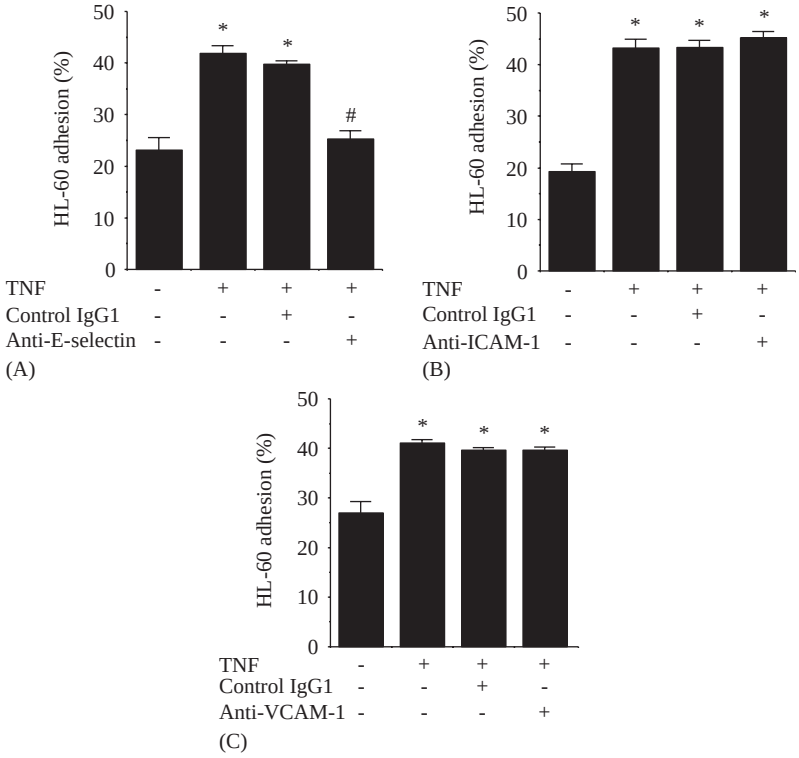

Figure 7 Effects of specific monoclonal antibodies against E-selectin, ICAM-1 and VCAM-1 on HL-60 cell adhesion to TNF-α-treated human PMVEC. After stimulation with TNF-α (10 ng/ml) for 4 h, endothelial cells were incubated with blocking monoclonal antibodies against E-selectin, ICAM-1, VCAM-1 or control isotype-matched IgG_1. Thereafter, fluorescence-labeled HL-60 cells were plated on endothelial cells, incubated for 15 min and the percentage of HL-60 adhesion was calculated. The Nil group consisted of cells incubated with medium alone. Results are expressed as the mean ± SEM of two independent experiments, each consisting of six replicates. $^{*}P<0.001$ vs. non-treatment (medium alone); $^{\#}P<0.001$ vs. TNF-α alone.

and transcriptional induction of IκBα in human saphenous vein endothelial cells, and that macrophage-derived inducible NO also attenuated VCAM-1 gene expression in the same cells, in part, by the inhibitory effect of NO on κB cis-acting elements. They also reported that the same NO donors inhibited cytokine (TNF-α, interleukin-1β)-induced adhesion molecule (E-selectin, ICAM-1, VCAM-1) expression, in part, by blocking NF-κB activation in human saphenous vein endothelial cells, and that this inhibition was unaffected by cGMP analogues and paralleled by reduced monocyte adhesion to endothelial monolayers.[14] Khan et al.[31] showed that in human umbilical vein endothelial cells and dermal microvascular endothelial cells, an NO donor (diethyl-amine-NO) reduced NF-κB activation and VCAM-1 expression at the cell surface and mRNA levels induced by TNF-α. Therefore, it was expected that antioxidants or NO donors would limit the TNF-α-induced activation of NF-κB and resultant expression of cell adhesion molecules in human PMVEC.

In the present experiments, pretreatment with PDTC or Sper-NO significantly decreased the level of NF-κB p65 presented in the nucleus of TNF-α-stimulated human PMVEC 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 human PMVEC. We also assessed the inhibitory effect of PDTC or Sper-NO on TNF-α-induced adhesion molecule expression in these cells. PDTC and Sper-NO significantly decreased the mRNA level of E-selectin, ICAM-1 and VCAM-1 induced by treatment with TNF-α for 2 h. They also significantly suppressed the surface expression of the adhesion molecules induced by treatment with TNF-α for periods of 4 or 8 h. NAC and SNAP also tended to reduce the level of mRNA and surface expression of some adhesion molecules, but their inhibitory effect did not reach statistical significance except for the effect of SNAP on the VCAM-1 mRNA level. NAC is an antioxidant that can increase the intracellular concentration of glutathione and augment cellular antioxidant capacity and can also

directly scavenge ROI. PDTC has a radical-scavenging, antioxidant property and also a metal-chelating property that decreases oxidative stress by preventing formation of hydroxyl radicals from hydrogen peroxide via the Fenton reaction.[32,33] We assume that the more potent effect of PDTC than NAC may be related to its metal-chelating property, which may be independent of the effect on the cellular redox state. The more potent 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.[34–36] It is interesting to note that PDTC and Sper-NO suppress the TNF-α-induced cell surface and mRNA expression of adhesion molecules in a most profound way, whereas the inhibition of NF-κB nuclear translocation is much less profound. It is possible that the other redox-sensitive transcription factors, such as activator protein-1, are involved in the upregulation of adhesion molecules.[4] The effects of antioxidants and NO on the other transcription factors in TNF-α-stimulated human PMVEC will be investigated in the future.

It is well known that NO activates soluble guanylate cyclase to generate cGMP, which alters contractile responses of cells.[15] However, the addition of a cell membrane-permeable analogue of cGMP, 8-Br-cGMP, did not affect the adhesion molecule expression in our cell culture, suggesting that the NO effect is independent of cGMP formation. NO may offer similar mechanisms to those of antioxidants in terms of suppressing ROI production and inhibiting NF-κB because NO can bind the superoxide anion with extremely high affinity and thereby decrease its dismutated product, hydrogen peroxide.[3,14,15,18,31] Alternatively, NO may directly affect protein kinases and/or phosphatases that regulate IκBα phosphorylation[18] or S-nitrosylate a critical thiol in the DNA-interacting NF-κB p50 subunit.[17] One attractive speculation is that inducible-type NO synthase expression by macrophages and vascular smooth and endothelial cells would produce relatively large amounts of NO, implicating an auto-protective regulatory mechanism because the induction of NO synthase in these cells requires activation of NF-κB.[15,19,20] An additional notable role of NO is that this molecule may regulate the cell–matrix and/or cell–cell adhesion and thus modify microvascular permeability in human PMVEC.[30]

We also observed that pretreatment of endothelial cells with PDTC or Sper-NO significantly inhibited HL-60 adhesion to TNF-α-stimulated cells, which corresponded to the functional significance of the inhibition of E-selectin induction by these chemicals.

After the completion of this work, we found that both cell surface and mRNA expression of adhesion molecules (E-selectin, ICAM-1, VCAM-1) in human dermal microvascular endothelial cells induced by TNF-α ere inhibited significantly by pretreatment with PDTC or Sper-NO, possibly via blocking redox-regulated NF-κB activation.[37] These results are consistent with the findings of the present work, and provide some evidence that the redox-sensitive NF-κB activation is essential for TNF-α-induced cell adhesion molecule upregulation in human microvascular endothelial cells.

In summary, triggering of the pulmonary vascular inflammatory response by TNF-α via upregulation of cell adhesion molecules appears to require ROI and to be attenuated by PDTC or Sper-NO. Although our in vitro results cannot be directly extrapolated to the in vivo situation, they suggest a potentially effective approach to intervene with and down-regulate the biological effects of endothelial–leukocyte adhesion in the pulmonary inflammatory reaction in vivo.

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References

1. Cines DB, Pollak ES, Buck CA, et al. Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* 1998;**91**(10):3527–61.
2. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers. *FASEB J* 1995;**9**(10):899–909.
3. Grisham MB, Granger DN, Lefer DJ. Modulation of leukocyte-endothelial interactions by reactive metabolites of oxygen and nitrogen: relevance to ischemic heart disease. *Free Radic Biol Med* 1998;**25**(4–5):404–33.
4. Comhair SA, Erzurum SC. Antioxidant responses to oxidant-mediated lung diseases. *Am J Physiol Lung Cell Mol Physiol* 2002;**283**(2):L246–55.
5. Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. *Cytokine Growth Factor Rev* 2002;**13**(4–5):413–21.
6. Janssen-Heininger YMW, Poynter ME, Baeuerle PA. Recent advances towards understanding redox mechanisms in the activation of nuclear factor κB. *Free Radic Biol Med* 2000;**28**(9):1317–27.
7. Blackwell TS, Blackwell TR, Holden EP, Christman BW, Christman JW. In vivo antioxidant treatment suppresses nuclear factor-kappa B activation and neutrophilic lung inflammation. *J Immunol* 1996;**157**(4):1630–7.

8. Cuzzocrea S, Chatterjee PK, Mazzon E, et al. Pyrrolidine dithiocarbamate attenuates the development of acute and chronic inflammation. *Br J Pharmacol* 2002;**135**(2):496–510.
9. Ferran C, Millan MT, Csizmadia V, et al. Inhibition of NF-κB by pyrrolidine dithiocarbamate blocks endothelial cell activation. *Biochem Biophys Res Commun* 1995;**214**(1): 212–23.
10. Kawai M, Nishikomori R, Jung EY, et al. Pyrrolidine dithiocarbamate inhibits intercellular adhesion molecule-1 biosynthesis induced by cytokines in human fibroblasts. *J Immunol* 1995;**154**(5):2333–41.
11. Marui N, Offermann MK, Swerlick R, et al. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J Clin Invest* 1993;**92**(4):1866–74.
12. Rahman A, Kefer J, Bando M, Niles WD, Malik AB. E-selectin expression in human endothelial cells by TNF-α-induced oxidant generation and NF-κB activation. *Am J Physiol* 1998; **275** (3 Part 1) (*Lung Cell Mol Physiol* 19): L533–44.
13. Weber C, Erl W, Pietsch A, Strobel M, Ziegler-Heitbrock HW, Weber PC. Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-κB mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. *Arterioscler Thromb* 1994;**14**(10):1665–73.
14. De Caterina R, Libby P, Peng HB, et al. Nitric oxide decreases cytokine-induced endothelial activation: nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* 1995;**96**(1):60–8.
15. Gaston B, Drazen JM, Loscalzo J, Stamler JS. The biology of nitrogen oxides in the airways. *Am J Respir Crit Care Med* 1994;**149**(2 Part 1):538–51.
16. Katsuyama K, Shichiri M, Marumo F, Hirata Y. NO inhibits cytokine-induced iNOS expression and NF-κB activation by interfering with phosphorylation and degradation of IκB-α. *Arterioscler Thromb Vasc Biol* 1998;**18**(11):1796–802.
17. Marshall HE, Stamler JS. Inhibition of NF-κB by S-nitrosylation. *Biochemistry* 2001;**40**(6):1688–93.
18. Peng HB, Libby P, Liao JK. Induction and stabilization of IκBα by nitric oxide mediates inhibition of NF-κB. *J Biol Chem* 1995;**270**(23):14214–9.
19. Peng HB, Spiecker M, Liao JK. Inducible nitric oxide: an autoregulatory feedback inhibitor of vascular inflammation. *J Immunol* 1998;**161**(4):1970–6.
20. Raychaudhuri B, Dweik R, Connors MJ, et al. Nitric oxide blocks nuclear factor-κB activation in alveolar macrophages. *Am J Respir Cell Mol Biol* 1999;**21**(3):311–6.
21. Lum H, Malik AB. Regulation of vascular endothelial barrier function. *Am J Physiol* 1994; **267** (3 Part 1) (*Lung Cell Mol Physiol* 11): L223–41.
22. Blease K, Seybold J, Adcock IM, Hellewell PG, Burke-Gaffney A. Interleukin-4 and lipopolysaccharide synergize to induce vascular cell adhesion molecule-1 expression in human lung microvascular endothelial cells. *Am J Respir Cell Mol Biol* 1998;**18**(5):620–30.
23. Shen J, Ham RG, Karmiol S. Expression of adhesion molecules in cultured human pulmonary microvascular endothelial cells. *Microvasc Res* 1995;**50**(3):360–72.
24. Blease K, Burke-Gaffney A, Hellewell PG. Modulation of cell adhesion molecule expression and function on human lung microvascular endothelial cells by inhibition of phosphodiesterases 3 and 4. *Br J Pharmacol* 1998;**124**(1):229–37.
25. Burke-Gaffney A, Hellewell PG. Tumour necrosis factor-α-induced ICAM-1 expression in human vascular endothelial and lung epithelial cells: modulation by tyrosine kinase inhibitors. *Br J Pharmacol* 1996;**119**(6):1149–58.
26. Lentsch AB, Shanley TP, Sarma V, Ward PA. In vivo suppression of NF-κB and preservation of IκBα by interleukin-10 and interleukin-13. *J Clin Invest* 1997;**100**(10): 2443–8.
27. Kinnula VL, Crapo JD, Raivio KO. Generation and disposal of reactive oxygen metabolites in the lung. *Lab Invest* 1995;**73**(1):3–19.
28. Barnes PJ, Adcock IM. NF-κB: a pivotal role in asthma and a new target for therapy. *Trends Pharmacol Sci* 1997;**18**(2):46–50.
29. Hart LA, Krishnan VL, Adcock IM, Barnes PJ, Chung KF. Activation and localization of transcription factor, nuclear factor-κB, in asthma. *Am J Respir Crit Care Med* 1998;**158**(5 Part 1):1585–92.
30. Tsukahara H, Noiri E, Jiang MZ, Hiraoka M, Mayumi M. Role of nitric oxide in human pulmonary microvascular endothelial cell adhesion. *Life Sci* 2000;**67**(1):1–11.
31. Khan BV, Harrison DG, Olbrych MT, Alexander RW, Medford RM. Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. *Proc Natl Acad Sci USA* 1996;**93**(17):9114–9.
32. Bowie A, O'Neill LA. Oxidative stress and nuclear factor-κB activation: a reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol* 2000;**59**(1): 13–23.
33. Kim CH, Kim JH, Lee J, Hsu CY, Ahn YS. Thiol antioxidant reversal of pyrrolidine dithiocarbamate-induced reciprocal regulation of AP-1 and NF-κB. *Biol Chem* 2003;**384**(1): 143–50.
34. Fitzhugh AL, Keefer LK. Diazeniumdiolates: pro- and antioxidant applications of the ''NONOates''. *Free Radic Biol Med* 2000;**28**(10):1463–9.
35. Ignarro LJ, Lippton H, Edwards JC, et al. Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of *S*-nitrosothiols as active intermediates. *J Pharmacol Exp Ther* 1981;**218**(3):739–49.
36. Mathews WR, Kerr SW. Biological activity of *S*-nitrosothiols: the role of nitric oxide. *J Pharmacol Exp Ther* 1993;**267**(3): 1529–37.
37. Jiang MZ, Tsukahara H, Ohshima Y, et al. Effects of antioxidants and nitric oxide on TNF-α-induced cell adhesion molecule expression and NF-κB activation in human dermal microvascular endothelial cells. *Life Sci* 2004;**75**(10): 1159–70.