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Inhibition by nitric oxide-releasing compounds of E-selectin expression in and neutrophil adhesion to human endothelial cells

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Abstract

The effects of two chemically unrelated nitric oxide (NO)-releasing compounds were studied on adhesion molecule expression in and neutrophil adhesion to human umbilical vein endothelial cells. Incubation of confluent monolayers of endothelial cells with increasing concentrations of lipopolysaccharide stimulated the adhesion of polymorphonuclear leukocytes to endothelial cells. Flow cytometric analysis showed that lipopolysaccharide treatment upregulated the expression of adhesion molecules E-selectin and intercellular adhesion molecule-1 (ICAM-1) in human umbilical vein endothelial cells. A novel NO-releasing compound GEA 3175 (1,2,3,4-oxatriazolium,-3-(3-chloro-2-methylphenyl)-5-[[(4-methylphenyl)sulfonyl]amino]-, hydroxide inner salt) inhibited lipopolysaccharide-induced adhesion being more potent than the earlier known NO donor *S*-nitroso-*N*-acetylpenicillamine. The increased E-selectin expression induced by lipopolysaccharide was significantly attenuated by the two NO donors tested whereas ICAM-1 expression remained unaltered. The present data show that NO donors inhibit E-selectin expression in and neutrophil adhesion to lipopolysaccharide-stimulated vascular endothelial cells. Thus, by inhibiting leukocyte adhesion NO donors may reduce leukocyte infiltration and leukocyte-mediated tissue injury in inflammation and ischemia–reperfusion injury.

Keywords: Nitric oxide (NO); Nitric oxide-releasing compound; Adhesion; E-Selectin; ICAM-1; Lipopolysaccharide

1. Introduction

Adhesion molecules play a critical role in the regulation of leukocyte migration from the bloodstream into inflamed tissues (Carlos and Harlan, 1994; Henricks and Nijkamp, 1998). Soluble mediators or bacterial products activate endothelial cells to express adhesion molecules such as E-selectin and intercellular adhesion molecule-1 (ICAM-1) (Gonzalez-Amaro et al., 1998). Inhibition of leukocyte extravasation by blocking expression or activation of adhesion molecules serves as a novel approach in anti-inflammatory drug design.

Nitric oxide (NO) is a biologically active factor constitutively produced by the endothelium which, in addition to maintaining vascular perfusion, acts as an anti-adhesive molecule for leukocytes (Hickey and Kubes, 1997). Inhibition of NO production promotes leukocyte rolling, adhesion in postcapillary venules and transmigration into tissues (Kubes et al., 1991; Davenpeck et al., 1994). Delivery of exogenous NO has been demonstrated to reverse these phenomena and to reduce leukocyte recruitment in acute inflammation and ischemia–reperfusion injury (Gauthier et al., 1994; Gaboury et al., 1996). Inducible nitric oxide synthase (iNOS) deficient mice have enhanced leukocyte–endothelium interactions in endotoxemia (Hickey et al., 1997) indicating that induction of iNOS may also act as a homeostatic regulator of leukocyte adhesion.

This study sought to determine whether NO donors can limit endothelial activation by inhibiting adhesion molecule expression and neutrophil adhesion in lipopolysaccharide-stimulated human endothelial cells. We selectively exposed cultured endothelial cells to NO donors in order to separate the actions of NO on hemodynamics and leuko-

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cytes from its direct effects on endothelial cell functions. Two chemically unrelated NO donors, the mesoionic oxatriazole derivative GEA 3175 (1,2,3,4-oxatriazolium,-3-(3-chloro-2-methylphenyl)-5-[[(4-methylphenyl)sulfonyl]-amino]-, hydroxide inner salt; Corell et al., 1994; Karup et al., 1994; Kankaanranta et al., 1996) and *S*-nitroso-*N*-acetylpenicillamine (Feelisch, 1993) were used as NO donors.

2. Materials and methods

2.1. Endothelial cell culture

Human umbilical vein endothelial cells were isolated by treatment of human umbilical veins with 0.01% collagenase (Zimmerman et al., 1990). The cells were suspended in RPMI 1640 Glutamax-1 supplemented with 20% heat-inactivated fetal bovine serum, endothelial cell growth supplement (10 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml). Cells were grown to confluence in plastic dishes, then removed by treatment with trypsin–EDTA (ethylenediaminetetraacetic acid), and seeded to gelatin-coated 24-well plates for adhesion experiments.

2.2. Adhesion assay

The endothelial cells were incubated with lipopolysaccharide for 6 h at 37°C in a humified atmosphere of 5% CO_2 either in the presence or absence of NO donors or a nitric oxide synthase (NOS) inhibitor. None of the compounds tested altered the morphology of endothelial cell monolayers as examined under a phase-contrast microscope. Thereafter, the cells were washed and human polymorphonuclear leukocytes (5×10^5 cells/500 μl) were added into the culture. Human polymorphonuclear leukocytes were isolated by Ficoll-Paque gradient centrifugation (Moilanen et al., 1988) from venous blood obtained from healthy volunteers who had abstained from any drugs for at least 1 week before sampling. After 20-min incubation, the co-cultures were washed twice with phosphate-buffered saline to remove non-adherent leukocytes. Hexadecyltrimethylammonium bromide (0.5% w/v; 1 ml/well) was added to lyse cells. The number of adherent leukocytes was quantitated by myeloperoxidase assay (Bailey and Fletcher, 1988). In every experiment, myeloperoxidase activity of known numbers of leukocytes was analysed and the number of leukocytes in a sample was calculated from standard curve (number of leukocytes vs. myeloperoxidase activity).

2.3. E-selectin and ICAM-1 expression

Endothelial cells were incubated with the culture medium (unstimulated cells) or lipopolysaccharide (10 ng/ml) for 6 h either in the presence or absence of NO donors. The cells were detached from flasks using 0.02% EDTA in phosphate buffered saline, washed and suspended in phosphate buffered saline. Then the cells were incubated (4°C, 30 min) with fluorescein isothiocyanate labelled anti-CD62E (E-selectin) antibody (1.2B6), *R*-phycoerythrin labelled anti-CD54 (ICAM-1) antibody (HA58) or with similarly labelled negative mouse IgG_1 control antibody. After two washes with phosphate-buffered saline containing 2% bovine serum albumin, cells were fixed in 1% paraformaldehyde in 0.15 M NaCl. Expression of surface antigens was analysed by flow cytometry (FACScan; Becton Dickinson) using the channel number (log scale) representing the mean fluoresence intensity of 10 000 cells. The specific mean fluorescence intensity for cells stained by each antibody was calculated after subtracting the mean fluorescence intensity of the cells exposed to the negative control antibody.

2.4. Nitrite assay

Nitrite, a stable product of NO in aqueous solutions was measured in the incubation medium with Griess reagent (Green et al., 1982). The detection limit of the assay was 1 μM (1 nmol/10^6 cells).

2.5. Drugs and chemicals

GEA 3175 as well as *S*-nitroso-*N*-acetylpenicillamine were kindly provided by GEA, Copenhagen, Denmark. Culture media, fetal bovine serum, antibiotics and trypsin–EDTA (Gibco, Paisley, Scotland, UK), collage-

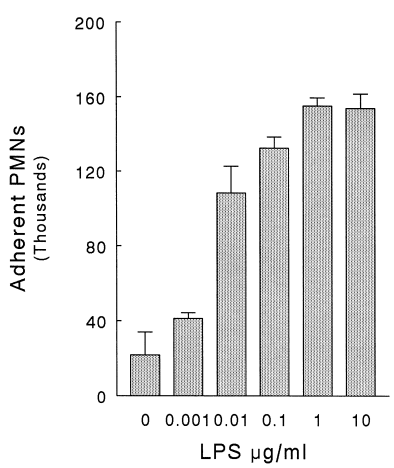

Fig. 1. The dose–response curve of the stimulatory effects of lipopolysaccharide on polymorphonuclear leukocyte adhesion to endothelial cells. The human umbilical vein endothelial cells were incubated with lipopolysaccharide for 6 h. Thereafter, the cells were washed and leukocytes were added into the culture. After 20-min incubation, non-adherent leukocytes were removed by washing. The number of adherent leukocytes was quantitated by myeloperoxidase assay. The values are the mean ± S.E. of three quadruplicate experiments ($n = 3$).

nase A (Boehringer Mannheim, Germany), endothelial cell growth supplement, hexadecyltrimethylammonium bromide and lipopolysaccharide (Sigma, St. Louis, MO, USA), *N*-monomethyl-L-arginine (L-NMMA; Clinalfa, Läufelfingen, Switzerland), Ficoll-Paque (Pharmacia, Uppsala, Sweden), anti-human CD54 antibody (Pharmingen, San Diego, CA, USA) and anti-human CD62E antibody (Calbiochem, La Jolla, CA, USA) were obtained as indicated.

2.6. Statistics

Results are expressed as mean ± S.E. Statistical significance was calculated by analysis of variance for repeated measures supported by Dunnett's multiple comparisons test. Differences were considered significant when $P < 0.05$. The *n* refers to number of separate experiments with endothelial cells from different donors.

3. Results

3.1. Stimulation of leukocyte adhesion to endothelial cells by lipopolysaccharide

Lipopolysaccharide (0.001–10 μg/ml) stimulated human polymorphonuclear leukocyte adhesion to umbilical vein endothelial cells in a concentration-dependent manner (Fig. 1). In the absence of lipopolysaccharide 30 850 ± 5670 ($n = 8$) neutrophils/well adhered to confluent layers of endothelial cells. Lipolysaccharide (10 ng/ml) increased the number of adherent leukocytes up to 112 600 ± 14 020 ($n = 13$). The submaximally activating concentration of 10 ng/ml of lipopolysaccharide was used in the further studies. Flow cytometric analysis showed that lipopolysaccharide treatment of endothelial cells upregulated the expression of adhesion molecules E-selectin and

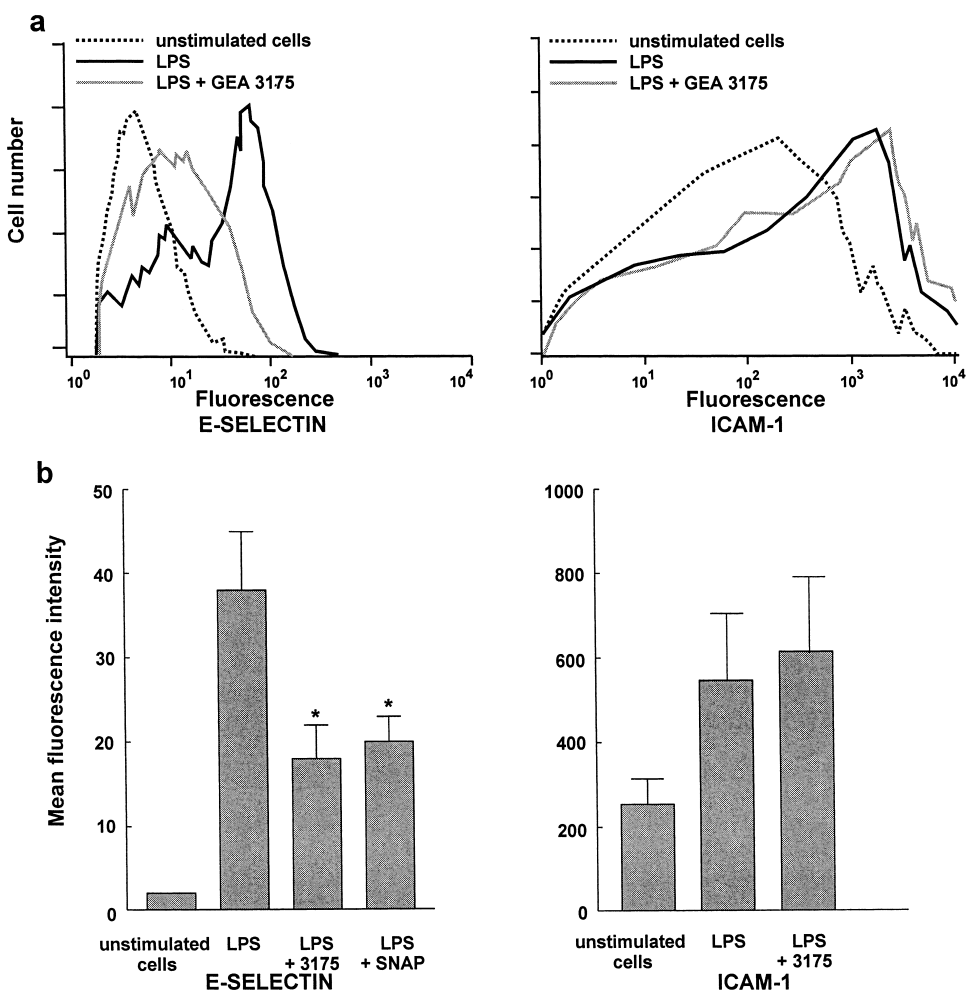

Fig. 2. The effect of NO donors on E-selectin and ICAM-1 expression on lipopolysaccharide-stimulated human umbilical vein endothelial cells. The endothelial cells were incubated with the culture medium (unstimulated cells) or lipopolysaccharide (10 ng/ml) for 6 h either in the presence or in the absence of NO donor GEA 3175 (30 μM) or *S*-nitroso-*N*-acetylpenicillamine (1000 μM). E-selectin and ICAM-1 expression was measured by direct immunofluorescence and flow cytometry. In (a) representative histograms are shown. Results were plotted as intensity of fluorescence (arbitrary units, on logarithmic scale) versus cell number (total cell count 10 000). In (b) the specific mean fluorescence intensity for cells stained by each antibody was calculated after subtracting the mean fluorescence intensity of the cells exposed to the negative control antibody. The values are the mean ± S.E. of five separate experiments. * $P < 0.05$ versus lipopolysaccharide-stimulated value without NO donor.

ICAM-1 (Fig. 2). Unstimulated endothelial cells expressed little E-selectin and lipopolysaccharide (10 ng/ml) caused 15.1 ± 3.2-fold increase ($n = 8$) in the fluorescence intensity. ICAM-1 was expressed also in unstimulated endothelial cells and lipopolysaccharide treatment further increased its expression up to 2.2 ± 0.5-fold ($n = 5$).

3.2. Effects of NO donors on leukocyte adhesion to endothelial cells

The two NO donors tested inhibited lipopolysaccharide (10 ng/ml)-induced adhesion of polymorphonuclear leukocytes to endothelial cells in a concentration-dependent manner (Fig. 3). On molar basis the new mesoionic oxatriazole derivative GEA 3175 was more potent than the earlier known NO-releasing compound *S*-nitroso-*N*-acetylpenicillamine. The increased E-selectin expression induced by lipopolysaccharide was significantly attenuated by both NO donors. GEA 3175 (30 μM) and *S*-nitroso-*N*-acetylpenicillamine (1000 μM) inhibited lipopolysaccharide-induced E-selectin expression by 54 ± 6% ($n = 5$) and 49 ± 3% ($n = 5$), respectively (Fig. 2). Exposure of endothelial cells to NO donor GEA 3175 (30 μM) did not attenuate the lipopolysaccharide-induced rise in ICAM-1 expression (Fig. 2).

3.3. Effects of an inhibitor of NO synthase on leukocyte adhesion to endothelial cells

L-NMMA, an inhibitor of NO synthase, did not significantly augment lipopolysaccharide (10 ng/ml)-stimulated human polymorphonuclear leukocyte adhesion to umbilical vein endothelial cells (Fig. 4). L-NMMA did not either alter the expression of E-selectin or ICAM-1 in lipopolysaccharide-stimulated endothelial cells (Fig. 5). In addition, lipopolysaccharide (10 ng/ml) failed to induce detectable nitrite production in endothelial cells (the detection limit was 1 μM).

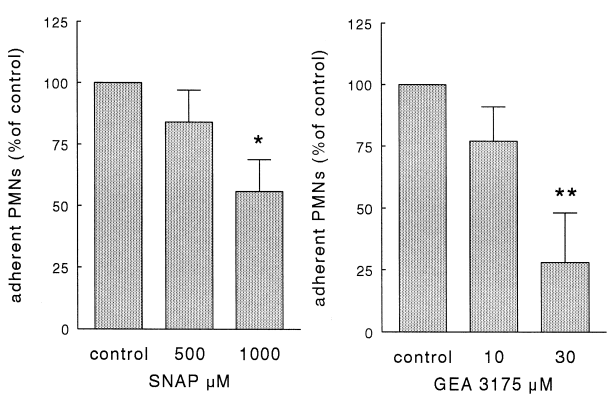

Fig. 3. The effect of NO donors on polymorphonuclear leukocyte adhesion to endothelial cells. The human umbilical vein endothelial cells were stimulated with lipopolysaccharide (10 ng/ml) either in the presence or absence of NO donor. After 6-h incubation, the cells were washed and leukocytes were added into the culture. After 20-min incubation, non-adherent leukocytes were removed by washing. The number of adherent leukocytes was quantitated by myeloperoxidase assay. The results are expressd as percent of control (i.e. the cells cultured without NO donor). The values are the mean ± S.E. of three quadruplicate experiments ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control without NO donor.

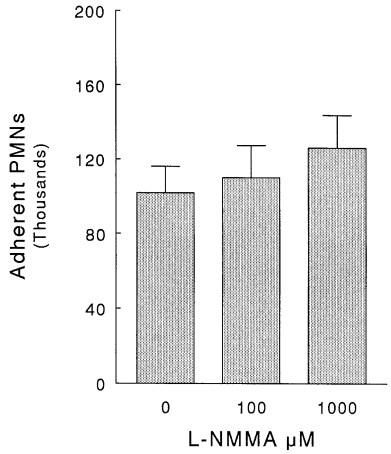

Fig. 4. The effect of NO-synthase inhibitor L-NMMA on polymorphonuclear leukocyte adhesion to endothelial cells. The human umbilical vein endothelial cells were stimulated with lipopolysaccharide 10 ng/ml for 6 h in the presence or absence of L-NMMA. Then the cells were washed and leukocytes were added into the culture. After 20-min incubation, non-adherent leukocytes were removed by washing. The number of adherent leukocytes was quantitated by myeloperoxidase assay. The results are the mean ± S.E. of 6–8 quadruplicate experiments ($n = 6$–8).

4. Discussion

The importance of NO as an endogenous inhibitor of neutrophil adhesion to vascular endothelium has been described (Kubes et al., 1991; Hickey and Kubes, 1997). In addition, NO donors have been successively used to inhibit leukocyte adhesion to endothelial cells (Lefer et al., 1993; Kubes et al., 1994; Kurose et al., 1994). The present study extends the data by showing that a novel NO-releasing compound GEA 3175 as well as a reference compound *S*-nitroso-*N*-acetylpenicillamine inhibit E-selectin expression in and neutrophil adhesion to lipopolysaccharide-stimulated human endothelial cells, whereas ICAM-1 expression remained unaltered.

GEA 3175 belongs to a family of recently characterized NO-releasing mesoionic oxatriazole derivatives. The NO-releasing properties of these compounds have been characterized by their ability to inhibit platelet aggregation, induce cGMP synthesis in platelets, convert oxyhemoglobin to methemoglobin, generate nitrite and nitrate in aqueous solutions and to form nitrosyl-hemoglobin complexes (Karup et al., 1994; Kankaanranta et al., 1996). The mesoionic oxatriazole derivatives have been shown to have

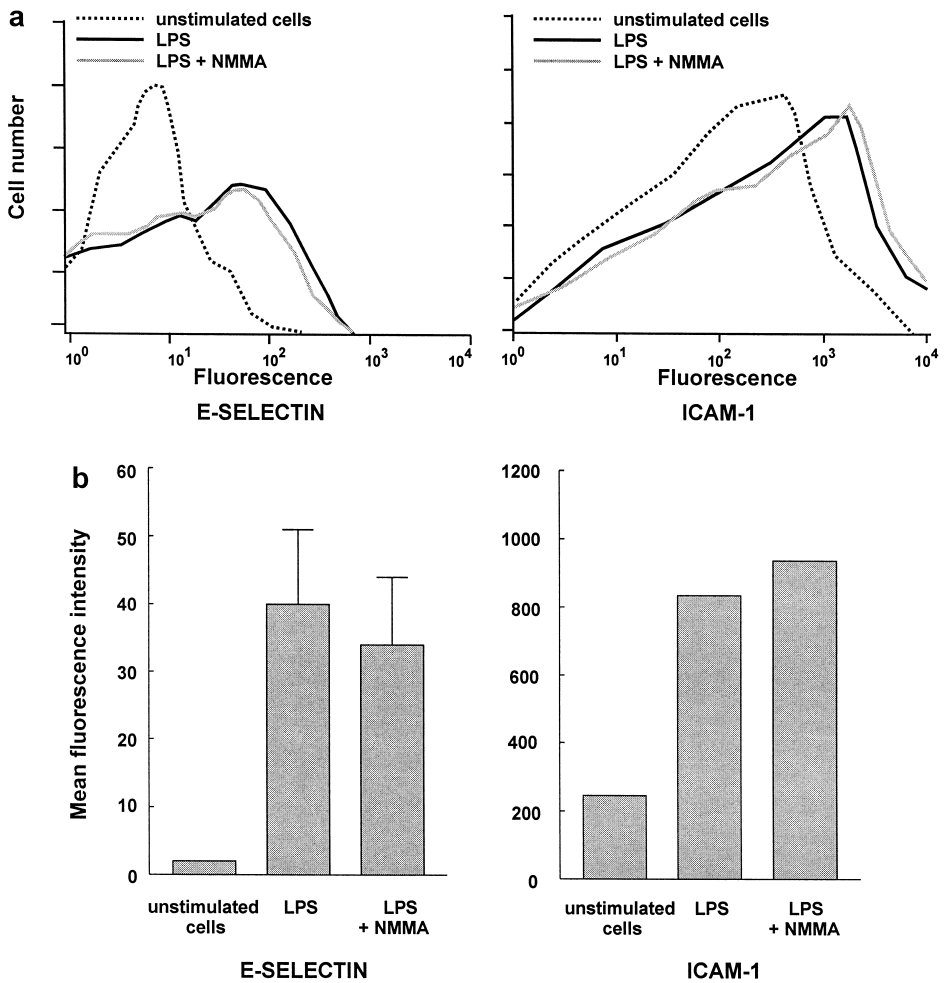

Fig. 5. The effect of NO-synthase inhibitor L-NMMA on E-selectin and ICAM-1 expression on lipopolysaccharide-stimulated endothelial cells. The human umbilical vein endothelial cells were incubated with the culture medium (unstimulated cells) or lipopolysaccharide (10 ng/ml) for 6 h either in the presence or in the absence of L-NMMA (1000 μM). E-selectin and ICAM-1 expression was measured by direct immunofluorescence and flow cytometry. In (a) representative histograms are shown. Results were plotted as intensity of fluorescence (arbitrary units, on logarithmic scale) versus cell number (total cell count 10 000). In (b) the specific mean fluorescence intensity for cells stained by each antibody was calculated after subtracting the mean fluorescence intensity of the cells exposed to the negative control antibody. The values are the mean ± S.E. of three (E-selectin) or mean of two (ICAM-1) separate experiments.

vasodilator, antiplatelet, fibrinolytic (Corell et al., 1994) and antibacterial (Virta et al., 1994) activities as well as to inhibit neutrophil functions (Moilanen et al., 1993), suppress lymphocyte proliferation (Kosonen et al., 1997, Kosonen et al., 1998a), decrease tumour cell growth (Vilpo et al., 1997), regulate glycosaminoglycan synthesis in articular cartilage (Järvinen et al., 1995), inhibit oxidation of low density lipoprotein (Malo-Ranta et al., 1994) and regulate cyclooxygenase-2 activity in human endothelial cells (Kosonen et al., 1998b). In the present study, GEA 3175 was found to inhibit neutrophil adhesion to endothelial cells, and on molar basis it was more potent than *S*-nitroso-*N*-acetylpenicillamine. This agrees with our previous data on the order of potency of these NO donors in their ability to increase cGMP synthesis (Corell et al., 1994) and augment other NO-like actions (Kosonen et al., 1997; Kosonen et al., 1998b).

The detailed mechanisms involved in the inhibitory action of NO on the leukocyte adhesion are not known. The significance of the inactivation of superoxide anion by NO and suppression of mast cell functions has been suggested (Kubes et al., 1993; Gaboury et al., 1993). More recently, inhibition of certain adhesion molecules in endothelial cells has been described. NO has been shown to inhibit cytokine stimulated vascular cell adhesion molecule-1 (VCAM-1) expression and mRNA accumulation in endothelial cells (De Caterina et al., 1995; Khan et al., 1996). VCAM-1 mediates the adhesion of mononuclear leukocytes to endothelial cells. NO is believed to repress VCAM-1 gene transcription, in part, by inhibiting nuclear factor κB (NF-κB) (De Caterina et al., 1995; Khan et al., 1996). Increased levels of P-selectin protein and mRNA were found in human endothelial cells cultured in the presence of NO synthase inhibitor L-*N*-nitroarginine methyl

ester (L-NAME) suggesting a regulatory role of NO also in the early leukocyte–endothelial interactions (Armstead et al., 1997). In the present study, by selectively exposing cultured endothelial cells to NO donors, we have identified adhesion molecule expression in endothelial cells as a potential target of NO.

In human umbilical vein endothelial cells lipopolysaccharide induces the expression of adhesion molecules E-selectin and ICAM-1 which take part in neutrophil adhesion to endothelial cells. E-selectin is induced on the transcriptional level, maximal levels of E-selectin protein are expressed within 4 h after stimulation at the cell surface (Bevilacqua et al., 1987; Vestweber and Blanks, 1999). ICAM-1 is constitutively expressed in human endothelial cells and the expression is increased by lipopolysaccharide (Dustin and Springer, 1988; Lee et al., 1995). In our study stimulation of human umbilical vein endothelial cells for 6 h with lipopolysaccharide increased surface expression of both E-selectin and ICAM-1. We found that GEA 3175 and S-nitroso-*N*-acetylpenicillamine suppressed lipopolysaccharide-induced expression of E-selectin in endothelial cells. De Caterina et al. (1995) and Spiecker et al. (1998) have reported that NO donors inhibit also interleukin 1α- and tumor necrosis factor-α (TNF-α)-induced E-selectin expression in human saphenous vein endothelial cells. These results suggest that NO reduces E-selectin-mediated adhesion process in stimulus-independent manner. In the present study, flowcytometric analysis of E-selectin suggested that there are two different cell populations: high and low expressing cells. Treatment with NO-donors seemed to reduce the number of the high expressing cells and only the low expressing cells remained.

The published effects of NO donors on adhesion molecule expression are in many parts conflicting. In the present study, NO-releasing compounds did not alter lipopolysaccharide-induced ICAM-1 expression, confirming the studies of Biffl et al. (1996) and Tsao et al. (1996) who studied lipopolysaccharide-stimulated human umbilical vein endothelial cells and human aortic endothelial cells. However, NO donors have been reported to inhibit ICAM-1 expression in human endothelial cells after stimulation with cytokines interleukin-1ß and TNF-α (Takahashi et al., 1996; Spiecker et al., 1998). These variable effects may be partly explained by differencies in the endothelial cells studied. In addition, the present data show that lipopolysaccharide-induced ICAM-1 expression in human vascular endothelial cells is resistant to NO leaving open the possibility that NO regulates ICAM-1 expression in a stimulus dependent manner. This may well be the case as lipopolysaccharide-induced ICAM-1 expression was not inhibited by interleukin-1 or TNF-α antibodies, indicating that it was not an autocrine effect mediated by the lipopolysaccharide-induced interleukin-1 or TNF-α (Lee et al., 1995). Accordingly, dexamethasone has been shown to regulate ICAM-1 expression in a stimulus-dependent manner (Burke-Gaffney and Hellewell, 1996).

Lipopolysaccharide induces expression of iNOS and production of high amounts of NO in several rodent cell types whereas in human cells additional stimuli are usually needed (Mossalayi et al., 1994; Vouldoukis et al., 1995; Moilanen et al., 1999). Expression of iNOS in freshly isolated human umbilical vein endothelial cells treated with a mixture of cytokines TNFα, interleukin-1β and lipopolysaccharide was recently demonstrated (Orpana et al., 1997; Ranta et al., 1998). However, cultured human umbilical vein endothelial cells do not express iNOS when exposed to proinflammatory stimuli (Rosenkranz-Weiss et al., 1994). This may imply that endothelial cells that are conventionally cultured for several passages have lost their ability to express iNOS, whereas freshly isolated cells maintain this capacity. In the present study, no measurable nitrite production (detection limit 1 nmol/10^6 cells/6 h) as a marker of NO synthesis was found. L-NMMA, an inhibitor of NOS, failed to alter neutrophil adhesion to endothelial cells. These results suggest that in human umbilical vein endothelial cells cultured for two passages lipopolysaccharide induces low, if any, NO production and that endogenous NO does not regulate neutrophil adhesion. This does not exclude the possibility that endogenous NO might regulate neutrophil adhesion in human endothelium in vivo.

NO donors have therapeutic potential in stable and unstable angina, coronary vasospasm and myocardial infarction (Moncada and Higgs, 1995). Restitution of blood flow to ischemic tissues is associated with neutrophil-mediated ischemia–reperfusion injury (Granger and Korthuis, 1995). It is now well recognized that the accumulation of neutrophils to post-ischemic tissues is mediated by various coordinately regulated adhesion molecules on the surface of leukocytes and endothelial cells (Lefer and Lefer, 1996). Expression of E-selectin on coronary endothelium after myocardial ischemia–reperfusion has been described (Shen and Verrier, 1994). Therefore, the present findings on the inhibitory action of NO donors on E-selectin expression in endothelial cells may be implicated also in the mechanisms of the protective action of NO donors in ischemia–reperfusion injury.

In conclusion, the present data show that NO donors inhibit E-selectin expression in and neutrophil adhesion to lipopolysaccharide-stimulated vascular endothelial cells and NO donors may thus reduce the damaging effects of ischemia–reperfusion and inappropriate inflammatory response by inhibiting leukocyte infiltration.

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