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Nitric oxide-releasing compounds inhibit neutrophil adhesion to endothelial cells

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Abstract

In the present work, we demonstrated that chemically different nitric oxide (NO)-releasing compounds inhibit tumor necrosis factor α (TNF-α)-induced polymorphonuclear leukocyte adhesion to endothelial cells in vitro. Two mesoionic oxatriazole derivatives GEA 3162 (1,2,3,4-oxatriazolium,5-amino-3(3,4-dichlorophenyl)-chloride) and GEA 3175 (1,2,3,4-oxatriazolium,-3-(3-chloro-2-methylphenyl)-5-[[(4-methylphenyl)sulfonyl]amino]-, hydroxide inner salt) were compared to the earlier-known NO donor SIN-1 (3-morpholino-sydnonimine). GEA 3162 (3–10 μM) and GEA 3175 (10–30 μM) inhibited human polymorphonuclear leukocyte adhesion to B_4 endothelial cells in a dose-dependent manner being more potent than SIN-1. In the present model, leukocytes rather than endothelial cells seemed to be the target of the effect of NO. Flow cytometric analysis showed that NO-releasing compounds did not alter TNF-α induced CD11/CD18 surface expression in polymorphonuclear leukocytes. The inhibitory action of NO-releasing compounds on adhesion paralleled with the increased synthesis of cGMP in polymorphonuclear leukocytes. Analogues of cGMP inhibited polymorphonuclear leukocyte adhesion indicating a role for cGMP in the action of NO donors. The results suggest that exogenous NO in the form of NO-releasing compounds inhibits polymorphonuclear leukocyte adhesion to endothelial cells, which may be implicated in the regulation of leukocyte migration and leukocyte-mediated tissue injury.

Keywords: Nitric oxide (NO); Nitric oxide (NO)-releasing compound; Adhesion; CD11/CD18

1. Introduction

Nitric oxide (NO) is a signalling molecule involved in the regulation of human immune response. Depending on the type and phase of inflammation and the individual vascular or cellular response studied NO seems to have both proinflammatory and anti-inflammatory properties (Moilanen and Vapaatalo, 1995; Moilanen et al., 1999; Grisham et al., 1999). NO attenuates neutrophil accumulation and neutrophil-mediated tissue damage in ischemia–reperfusion injury (Kurose et al., 1994; Fukuda et al., 1995; Grisham et al., 1998). Although the mechanism by which NO regulates neutrophil-mediated inflammatory response remains unclear, some mechanisms have been proposed. Evidence is accumulating that NO modulates leukocyte–endothelial cell interactions, and may down-regulate specific cell adhesion molecules (Lefer and Lefer, 1996; Armstead et al., 1997; Spiecker et al., 1998). Inhibition of NO synthesis stimulates also mast cells in contact with endothelium to release proadhesive agents including platelet activating factor (PAF; Niu et al.,1996; Hickey and Kubes, 1997).

GEA 3162 (1,2,3,4-oxatriazolium,5-amino-3(3,4-dichlorophenyl)-chloride) and GEA 3175 (1,2,3,4-oxatriazolium,-3-(3-chloro-2-methylphenyl)-5-[[(4-methylphenyl)-sulfonyl]amino]-, hydroxide inner salt) are two mesoionic oxatriazole derivatives known to release NO in aqueous solutions. We have earlier found that these compounds have NO-dependent effects in inflammatory cells (Moilanen et al., 1993; Corell et al., 1994; Kosonen et al., 1997, 1998a). NO-releasing properties of GEA 3162 and GEA 3175 have been characterized by documenting 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 complex (Karup et al., 1994;

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Kankaanranta et al., 1996). The aim of the present study was to clarify the effects of these NO-releasing compounds as well as an earlier known NO donor 3-morpholino-sydnonimine (SIN-1) on polymorphonuclear leukocyte adhesion to endothelial cells.

2. Materials and methods

2.1. Co-cultures of endothelial cells and polymorphonuclear leukocytes

Rabbit B_4 endothelial cells (Buonassisi and Venter, 1976) were cultured on 24-multiwell plates to confluence. Human polymorphonuclear leukocytes were isolated from citrated blood of healthy donors by density gradient centrifugation on Ficoll–Paque as described earlier (Moilanen et al., 1988). Polymorphonuclear leukocytes (1×10^6 cells in 1 ml RPMI medium supplemented with 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B) were added to the endothelial cell cultures supplemented with or without the NO donors or other compounds tested. After 30 min incubation at 37°C in a humidified atmosphere of 5% CO_2, tumor necrosis factor α (TNF-α;10 U/ml) was added to induce adhesion. The co-cultures were washed twice with phosphate-buffered saline (PBS) after 30 min to remove non-adherent leukocytes. Hexadecyltrimethylammonium bromide (HTAB; 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).

To evaluate a direct cytotoxicity of the NO donors, Trypan blue staining and the measurements of released lactate dehydrogenase (The Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology, 1974) were included in the protocol. None of the NO donors tested decreased cell viability as measured by these tests in the incubation conditions described above. The NO donors did neither alter the morphology of endothelial cell monolayers as examined under a phase-contrast microscope.

2.2. Determination of cyclic guanosine 3′:5′-monophosphate (cGMP) production

Polymorphonuclear leukocytes (5×10^6 cells in 500 μl of Dulbecco's phosphate-buffered saline) were incubated with the NO donor for 30 min at 37°C. The incubations were terminated by addition of ice cold trichloroacetic acid (final concentration 6%), and the samples were centrifuged ($10{,}000 \times g$ for 10 min). The supernatants were washed four times with water saturated ether, diluted with an equal volume of 100 mM sodium acetate buffer (pH 6.2) and stored at −20°C until assayed for cGMP. The cGMP samples were acetylated and measured by radioimmunoassay as described earlier (Axelsson et al.,1988).

2.3. Direct immunofluorescence and flow cytometry

Polymorphonuclear leukocytes (2×10^6 cells in 1000 μl of Dulbecco's phosphate-buffered saline + 0.25 % bovine serum albumin) were incubated with the NO donor at 37°C. After 10 min incubation, TNF-α (10 U/ml) was added for 30 min. The incubations were stopped by addition of cold PBS (2 ml) and then the cells were washed with cold PBS. Cells were then stained with fluorescein isothiocyanate (FITC) labelled CD18 monoclonal antibody (MHM23) or with negative mouse IgG1 control mAb and analysed by flow cytometry (FACScan; Becton Dickinson).

2.4. Drugs and chemicals

GEA 3162 and GEA 3175 as well as SIN-1 and an analogue of cGMP, 8-*p*-chlorophenylthio-cGMP (Miller et al. 1973, Butt et al. 1992) were kindly provided by GEA (Copenhagen, Denmark). Culture media and media supplements (Gibco, Paisley, UK), Ficoll–Paque (Pharmacia, Uppsala, Sweden), recombinant human TNF-α (Genzyme, Cambridge, MA, USA), 8-bromo-cGMP (Sigma, St. Louis, MO, USA), ^{125}I-labelled cGMP (DuPont, Boston, MA, USA), FITC-conjugated anti-human CD18 mAb and negative control mAb (Dako, Glostrup, Denmark) were obtained as indicated.

2.5. Statistics

Results are expressed as mean ± S.E. Statistical significance was calculated by analysis of variance for repeated

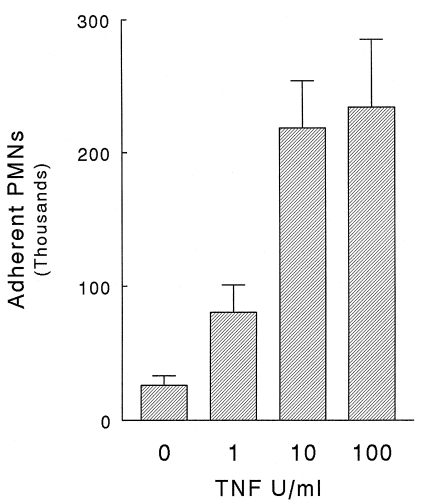

Fig. 1. The dose–response curve of the stimulatory effect of TNF-α on polymorphonuclear leukocyte adhesion to endothelial cells. The co-cultures were incubated for 30 min at 37°C with TNF-α and thereafter non-adherent leukocytes were removed by washing. The number of adherent leukocytes was quantitated by myeloperoxidase assay. The results are expressed as mean ± S.E. of three quadruplicate experiments.

measures supported by Dunnett's multiple comparisons test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Effects of NO donors on polymorphonuclear leukocyte adhesion to endothelial cells

Polymorphonuclear leukocyte adhesion to endothelial cells was induced by addition of TNF-α (10 U/ml) into the co-cultures. Fig. 1 shows the dose–response curve of the stimulatory effect of TNF-α (1–100 U/ml) on polymorphonuclear leukocyte adhesion to endothelial cells in these incubation conditions. The three NO donors inhibited the adhesion process in a concentration-dependent manner (Fig. 2a) On a molar basis, the two new mesoionic oxatriazole derivatives (GEA 3162 and GEA 3175) were more potent than the earlier known NO-releasing compound 3-morpholino-sydnonimine (SIN-1).

In order to study which cell type, leukocyte, endothelial cell or both might act as the target of the action of NO donors, a set of experiments using two NO donors (GEA 3175 and SIN-1) were run. The results are summarised in Table 1. In the control experiments, the co-cultures of polymorphonuclear leukocytes and endothelial cells were exposed to the NO donors for 30 min before TNF-α was added to induce adhesion. When endothelial cells alone were exposed to the NO donor for 30 min, then washed to remove the NO donor before addition of leukocytes and TNF-α, the inhibitory action of NO donors was totally abolished. When leukocytes alone were incubated with the NO donor for 30 min, then washed and placed in the co-culture the inhibitory action of the NO donors was still present although at somewhat lower degree. On the basis of these experiments, we suggest that leukocytes were the primary target of the action of NO in these co-culture conditions.

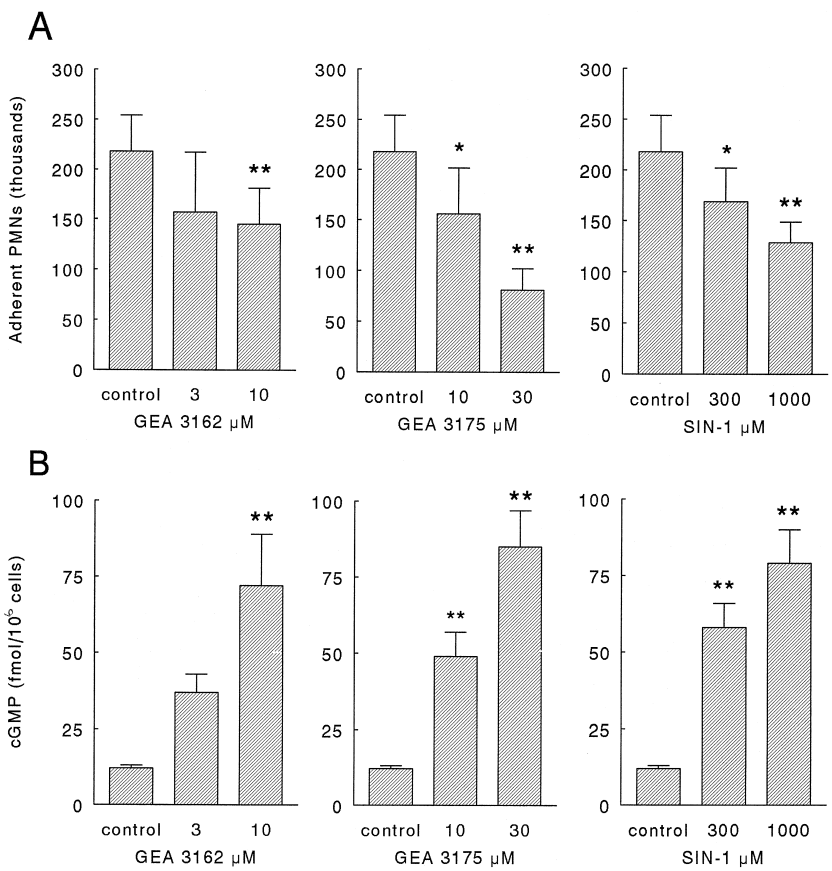

Fig. 2. In (A), the inhibitory action of three NO donors on TNF-α-induced polymorphonuclear leukocyte adhesion to endothelial cells is shown. The co-cultures were incubated for 30 min at 37°C with the NO donor tested and thereafter the adhesion process was induced by adding TNF-α (10 U/ml). After another 30 min incubation, non-adherent leukocytes were removed by washing. The number of adherent leukocytes was quantitated by myeloperoxidase assay. The results are expressed as mean ± S.E. of three quadruplicate experiments. In (B), the stimulatory effects of NO donors on cGMP production in human polymorphonuclear leukocytes are shown. The cells were incubated with the NO donor for 30 min at 37°C. Thereafter, the incubations were terminated by addition of trichloroacetic acid. Cyclic GMP was assayed by radioimmunoassay. The results are expressed as mean ± S.E. of six duplicate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control without NO donor.

Table 1
The role of endothelial cells (EC) and polymorphonuclear leukocytes (PMN) as a target of the inhibitory action of NO donors GEA 3175 and SIN-1 on TNF-α induced polymorphonuclear leukocytes adhesion to endothelial cells. The number of adherent cells is expressed as percentage of control, i.e., experiment run without NO donors. The values are the mean ± S.E. of three quadruplicate experiments

	Adherent PMNs (percentage of control)				
	Without NO donors	GEA 3175 10 μM	GEA 3175 30 μM	SIN-1 300 μM	SIN-1 1000 μM
EC + PMN + NO[a]	100	72 ± 17	44 ± 8	97 ± 6	63 ± 5
EC + NO[b]	100	95 ± 10	94 ± 4	101 ± 11	104 ± 8
PMN + NO[c]	100	92 ± 8	57 ± 4	108 ± 14	85 ± 5

[a] EC + PMN + NO donor for 30 min; + TNF-α (10 U/ml) for 30 min.
[b] EC + NO donor for 30 min; wash; + PMN + TNF-α (10 U/ml) for 30 min.
[c] PMN + NO donor for 30 min; wash; EC + TNF-α (10 U/ml) for 30 min.

3.2. Effects of NO donors on cGMP production in polymorphonuclear leukocytes

cGMP production in human polymorphonuclear leukocytes during 30 min incubation with the three NO donors was measured. GEA 3162, GEA 3175 and SIN-1 induced a dose-dependent increase in cGMP synthesis the two new compounds causing their effects at lower concentrations than SIN-1 (Fig. 2b). The NO donors increased cGMP production in a parallel concentration-dependent manner as they inhibited polymorphonuclear leukocyte adhesion to endothelial cells.

3.3. Effects of analogues of cGMP on polymorphonuclear leukocyte adhesion to endothelial cells

To find out if cGMP could mediate the NO donor-induced inhibition of polymorphonuclear leukocyte adhesion to endothelial cells, the effects of two analogues of cGMP, 8-bromo-cGMP and 8-*p*-chlorophenylthio-cGMP, were studied in the co-culture. Both of these analogues inhibited the polymorphonuclear leukocyte adhesion to endothelial cells (Table 2). 8-*p*-chlorophenylthio-cGMP was more potent than 8-bromo-cGMP.

Table 2
The effects of two analogues of cGMP, 8-bromo-cGMP (8-br-cGMP) and 8-*p*-chlorophenylthio-cGMP (8-pCPT-cGMP) on TNF-α-induced polymorphonuclear leukocytes (PMNs) adhesion to endothelial cells. The co-cultures were incubated with the cGMP analogue for 30 min at 37°C before the adhesion process was induced by adding TNF-α (10 U/ml). The results are expressed as percentage of control (i.e., the cells incubated without an analogue of cGMP). The values are the means ± S.E. of four (8-br-cGMP) or three (8-pCPT-cGMP) quadruplicate experiments

	Adherent PMNs (percentage of control)
Control	100
8-Br-cGMP (1 mM)	74 ± 14
8-Br-cGMP (3 mM)	59 ± 10[a]
8-pCPT-cGMP (10 μM)	82 ± 17
8-pCPT-cGMP (100 μM)	58 ± 13[b]

[a] $P < 0.01$ vs. control without cGMP analogue.
[b] $P < 0.05$ vs. control without cGMP analogue.

3.4. Effects of NO donors on CD11/CD18 expression on TNF-α-stimulated polymorphonuclear leukocytes

Since polymorphonuclear leukocytes seemed to be the target of NO in these culture conditions the possibility that NO decreases the surface expression of the main adhesion molecules (CD11/CD18-integrins) in neutrophils was investigated by flow cytometry. Stimulation of polymorphonuclear leukocytes with TNF-α (10 U/ml) resulted in a 25% increase in the binding of mAb MHM23 (directed to CD18) as compared to unstimulated cells. This increase was not altered by treatment of the cells with NO donors (Fig. 3).

4. Discussion

Reduced endothelial NO is involved in the mechanisms leading to inflammatory response and tissue injury occurring in ischemia and reperfusion (Lefer and Lefer, 1996, 1999). Several studies have shown that inhibition of NO synthesis with analogues of L-arginine enhances cell adhesion in the microcirculation (Kubes et al., 1991; Arndt et al., 1993; Kurose et al., 1993) and mimics the effects of ischemia–reperfusion whereas NO-releasing compounds have therapeutic potential in preventing ischemia–reperfusion injury (Fukuda et al., 1995; Liu et al., 1998).

The present results demonstrated that chemically different NO-releasing compounds inhibited TNF-α-induced polymorphonuclear leukocyte adhesion to endothelial cells in vitro. On molar basis, the two NO-releasing mesoionic oxatriazole derivatives GEA 3162 and GEA 3175 were more potent than an earlier-known NO donor SIN-1. GEA 3162 and GEA 3175 have previously been reported to have 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) and lymphocyte proliferation (Kosonen et al., 1997, 1998a), suppress tumor cell growth (Vilpo et al., 1994), regulate glycosaminoglycan synthesis in articular cartilage (Järvinen et al., 1995), inhibit oxidation of low density

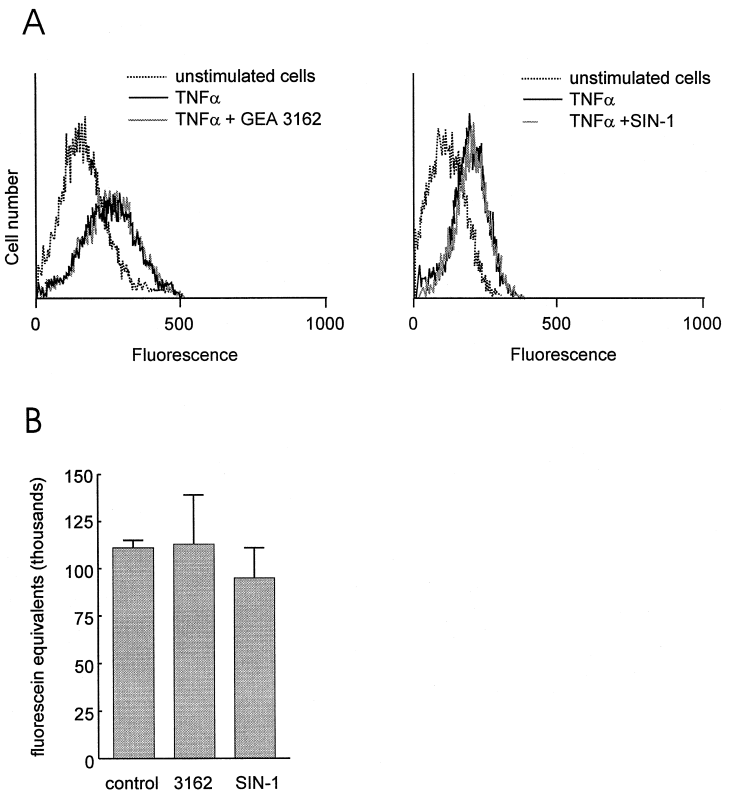

Fig. 3. The effects of NO donors on TNF-α stimulated CD11/CD18 expression on polymorphonuclear leukocytes. Leukocytes were incubated with GEA 3162 (10 μM) or SIN-1 (1000 μM) for 10 min at 37°C before the addition of TNF-α (10 U/ml) for 30 min. CD11/CD18 expression was measured by direct immunofluorescence and flow cytometry. In (A), two representative histograms are shown. In (B), the results are expressed as mean ± S.E. of three experiments. The control cells were stimulated with TNF-α without the NO donor.

lipoprotein (Malo-Ranta et al., 1994) and regulate COX-2 activity in human endothelial cells (Kosonen et al., 1998b).

Unlike GEA 3162 and *S*-nitroso-*N*-acetylpenicillamine (SNAP) (Holm et al., 1998) SIN-1 is known to produce both NO and superoxide anion, and the reaction between these two molecules results in the formation of peroxynitrite (Feelisch, 1991; Hogg et al., 1992). The reaction between NO and superoxide anion may be regarded as an inactivation route of these two reactive molecules (Gryglewski et al., 1986) and could explain the lower potency of SIN-1 found in the present experiments. On the other hand, the product formed in this reaction, i.e., peroxynitrite, is an active oxidant and nitrating agent, which may be responsible for some of the effects of NO in conditions where superoxide anion is also formed (Crow and Beckman, 1995). Activated neutrophils produce superoxide anion, and we have recently shown that when activated but not resting neutrophils are exposed to a NO donor, detectable amounts of peroxynitrite are formed (Holm et al., 1999). TNF-α has been shown to activate neutrophils to produce superoxide anion (Tsujimoto et al., 1986; Menegazzi et al., 1994). Therefore, peroxynitrite may be formed in these culture conditions after addition of either SIN-1 or GEA compounds and peroxynitrite may be implicated in the actions of NO donors.

In the present in vitro model of neutrophil/endothelial cell interactions, neutrophils rather than endothelial cells seemed to be the target of the effect of NO. Leukocyte adhesion induced by proinflammatory mediators such as cytokines and chemoattractants is primarily mediated via leukocyte ß2-integrins (Carlos and Harlan, 1994). Neutrophils express three ß2-integrins and their surface expression is increased by a variety of agonists: phorphol esters, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), leukotriene B_4 and TNF-α (Carlos and Harlan, 1994). Kubes et al. (1991) and Niu et al. (1994) reported that antibodies directed against ß2-integrin CD18 prevented the leukocyte adhesion enhanced by nitric oxide synthase (NOS)-inhibitors. This suggests that NO mediates its anti-adhesive effect through the leukocyte adhesion molecule CD11/CD18. In our study stimulation of neutrophils with TNF-α resulted in an increase in surface expression of CD11/CD18 confirming the earlier findings (Gamble et al., 1985; Lo et al., 1989). NO donors did not suppress TNF-α-induced surface expression of CD11/CD18 which is consistent with previous studies (Kubes et al., 1994; Ohashi et al., 1997). Both quantitative and qualitative changes occur in ß2-integrins after cell activation (Arnaout, 1990). However, it has been demonstrated that upregulation of CD11/CD18 surface expression is neither neces-

sary nor sufficient for the stimulated neutrophils to adhere to cultured endothelial cells (Vedder and Harlan, 1988; Philips et al., 1988; Schleiffenbaum et al., 1989). Qualitative changes in adhesion molecule avidity play a more critical role in regulation of ß2-integrin function (Carlos and Harlan, 1994). NO has been shown to inhibit ß2-integrin associated signalling pathways (Banick et al., 1997) suggesting that NO may regulate ß2-integrin activation. The present results show that NO donors do not alter CD11/CD18 surface expression in TNF-α-stimulated polymorphonuclear leukocytes but they may cause changes in the ß2-integrin avidity. In addition, NO-donors may have indirect effects, e.g., through altered mediator release. As potential mechanisms, the inactivation of superoxide anion by NO and suppression of PAF release have been suggested (Gaboury et al., 1993; Kubes et al., 1993).

The data presented in our study also indicate a role for cGMP in the modulation of neutrophil adhesion by NO donors. The concentration–response curves of the cGMP-enhancing effect by various NO donors correlated with their inhibitory action of neutrophil adhesion. Two analogues of cGMP, 8-*p*-chlorophenylthio-cGMP and 8-bromo-cGMP, inhibited neutrophil adhesion. 8-*p*-chlorophenylthio-cGMP caused the effect at lower concentrations than 8-bromo-cGMP probably because it penetrates well into the cells and is a poor substrate for cGMP-degrading phosphodiesterases (Butt et al., 1992). These results suggest that the inhibitory action of NO donors on neutrophil adhesion might be a cGMP-mediated process. That assumption is consistent with the studies of Kurose et al. (1993) and Davenpeck et al. (1994) who found that analogues of cGMP prevented leukocyte influx elicited by NOS-inhibitor L-N^G-nitroarginine methyl ester (L-NAME).

In conclusion, the present data suggest that exogenous NO in the form of NO donors inhibits neutrophil adhesion to vascular endothelium, which may be implicated in the regulation of neutrophil function in ischemia–reperfusion syndrome and inflammation.

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