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Nitric oxide regulates interactions of PMN with human brain microvessel endothelial cells

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Abstract

The hypothesis that the NO/cGMP pathway modulates PMN adhesion to human brain microvessel endothelial cells (HBMEC) was examined. Human PMN were incubated with resting or TNF-α-treated endothelial monolayers, and adhesion was quantified by light microscopy. TNF-α upregulated PMN adhesion in a time-dependent manner. Treatment of HBMEC with the NO donors SNP and DETA NONOate for 4 or 24 h decreased PMN adhesion. This was completely reversed by the guanylyl cyclase inhibitor ODQ, while addition of a cGMP agonist (8-Br-cGMP) decreased PMN adhesion. NO donors did not affect the levels of E-selectin or ICAM-1 in HBMEC. However, pre-treatment of PMN with NO donors or 8-Br-cGMP decreased their adhesion to recombinant E-selectin and ICAM-1, suggesting an effect of NO on PMN. These findings indicate that NO modulates PMN–HBMEC interactions through cGMP and decreases the binding of PMN to the adhesion molecules E-selectin and ICAM-1.

Keywords: Polymorphonuclear leukocytes; cGMP-dependent protein kinase; Adhesion molecules; Tumor necrosis factor-α; E-selectin; ICAM-1

Nitric oxide functions as a physiological messenger in addition to being a cytotoxic substance. Cells produce NO from arginine using enzymes termed nitric oxide synthases (NOS). There are two types of this enzyme: constitutive (cNOS) and inducible NOS (iNOS). cNOS include an endothelial cell form and a neuronal form. These calcium-dependent enzymes are constitutively expressed and generate small amounts of NO phasically. In contrast, iNOS is produced after the cells are stimulated. A large amount of NO is continuously generated by this calcium-independent enzyme. Many cell types can express iNOS activity, including endothelial cells, astrocytes and inflammatory neutrophils, and monocytes/macrophages. In vivo, high levels of NO and NOS are found in lesions and peripheral areas of lesions following ischemia/reperfusion (stroke) and during central inflammatory reactions.

There has been some controversy concerning the effect of NOS inhibitors on ischemia. It is now clear that NO has differential effects on neurons, EC, and other cell types. NO overproduction is neurotoxic. Thus, neuronal NOS inhibitors can reduce the damage due to ischemia [1–3]. Inhibition of NOS increases leukocyte adhesion to and migration across feline mesenteric venules [4]. In cases of experimental ischemia/reperfusion, diminished basal NO release precedes increased leukocyte adhesion to the endothelium [5]. The leukocyte adhesion molecule CD11/CD18 is upregulated by nitric oxide inhibitors [4]. Whether this also occurs with endothelial adhesion molecules is not known. In rats, NO generators reduce tissue damage by reducing leukocyte infiltration [6–11]. It is not known how this applies to human cerebral endothelium.

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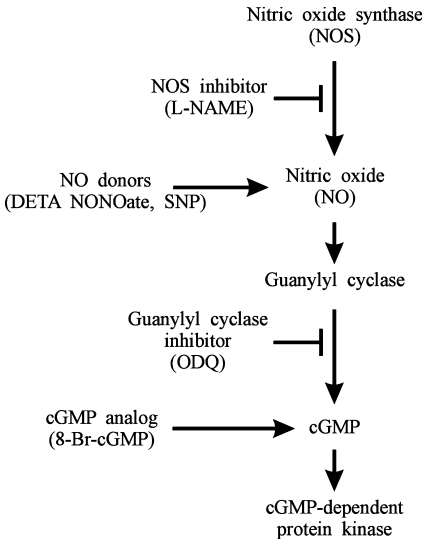

Fig. 1. The NO signal transduction pathway. The cytoplasmic enzyme nitric oxide synthase (NOS) produces NO. This is a gas which can diffuse through the cell membrane. It activates the enzyme guanylyl cyclase in the cytoplasm to produce cGMP, which in turn, regulates the cGMP-dependent protein kinases. A number of compounds were used to investigate the action of NO: the NOS inhibitor, L-NAME; the NO donors DETA NONOate and SNP which release exogenous NO; ODQ which inhibits the action of guanylyl cyclase; and 8-Br-cGMP, a membrane permeable analog of cGMP.

We have previously demonstrated that primary cultures of HBMEC can be induced by cytokines such as TNF-α to upregulate the expression of the adhesion molecules E-selectin, VCAM-1, and ICAM-1 [12,13]. PMN adhere to TNF-α treated HBMEC through E-selectin and ICAM-1, and T cells through VCAM-1, ICAM-1, and PECAM-1 [14]. NO has been reported to modulate PMN inflammatory responses and to play a protective role in priming and activation processes of inflammatory PMN [15]. Although other groups have found no effect of NO donors or NOS inhibitors on chemotaxis by human PMN [16]. In the present study, we examined how the interactions of PMN with HBMEC can be modulated by NO and the mechanisms involved. The signal transduction pathway we have examined involves the production of NO by the cytoplasmic enzyme NOS (Fig. 1). NO is a dissolved gas which can diffuse through the cell membrane. It activates the enzyme guanylyl cyclase in the cytoplasm to produce cGMP, which in turn, regulates cGMP-dependent protein kinases.

Materials and methods

Human brain microvessel endothelial cells. Primary cultures of human brain microvessel endothelial cells (HBMEC) were established from brains at autopsy as previously described [17]. The endothelial nature of these cells was confirmed by the positive staining for Factor VIII related antigen and binding of *Ulex europeaus* agglutinin. Cells were grown on fibronectin coated 96-well plates and cultured in Medium 199 with 10% horse serum. Nine- to ten-day-old confluent cultures were used. Several primary cultures from different autopsy brains were utilized.

Polymorphonuclear leukocyte isolation. Polymorphonuclear leukocyte (PMN) were isolated from anti-coagulated peripheral blood of several healthy donors by centrifugation in a gradient of lympholytepoly (Cedarlane, Hornby, ON). By this method, we routinely obtained cell fractions containing 99% PMN as determined on Giemsa-stained smears. Viability was 99% by trypan blue exclusion test.

Reagents. Tumor necrosis factor-α (TNF-α) was from Sigma. ^{N}G-nitro-L-arginine methyl ester (L-NAME), 8-Br-cGMP, and 1H[1,2,4]oxadiazolo[4,3-a]quinoxaliin-1-one (ODQ) were from Biomol (Plymouth meeting, PA). DETA NONOate was from Cayman Chemicals (Ann Arbor, MI). Sodium nitroprusside (SNP) was from BDH/VWR (Mississauga, ON). cGMP ELISA kit was from Biomol. Rabbit anti-cGMP-dependent protein kinase I was from Dr. S. Pelech (Vancouver, BC). Recombinant human E-selectin was from Dr. D. Lyons (Boulder, CO). Recombinant human ICAM-1 was from Dr. M. Dustin (St. Louis, MO). Anti-ICAM-1 (CA7) was from Dr. Rothlein (Ridgefield, CI).

Controls. Monolayers grown in the absence of TNF-α or in the presence of TNF-α only, as well as monolayers incubated with NO donor solutions prepared 24 h earlier served as controls.

Adhesion assay to HBMEC. Each adhesion molecule was maximally induced on HBMEC by treatment with 100 U TNF-α/ml for 4–24 h (4 h for E-selectin, 24 h for ICAM-1) along with the various donors and inhibitors. PMN (2×10^6 cells/ml) were then added to the wells and incubated with HBMEC in the presence of the donors and inhibitors for 30 min at 37 °C. At the end of the incubation period, the supernatants with the nonadherent PMN were removed and the monolayers with the adherent PMN were fixed in 1:1 acetone:ethanol, and stained with the immunoperoxidase technique for leukocyte common antigen. The number of leukocytes bound to the monolayers was determined by counting the number of adherent PMN per mm^2 of the culture dish in 5 fields by light microscopy.

Adhesion assay to recombinant adhesion molecules. Ninety-six-well ELISA plates (Dynex, Chantilly, VA) were coated with rhE-selectin at 3 μg/ml in PBS or anti-ICAM-1 (CA7) antibody at 10 μg/ml followed by rhICAM-1 at 10 μg/ml in bicarbonate buffer at 4 °C overnight. Unbound recombinant adhesion molecules were removed by two washes with PBS. PMN were incubated with TNF-α and the donors or inhibitors for 30 min or 24 h at 37 °C. At the end of the incubation period, PMN (2×10^6 cells/ml) were added to the ELISA plates coated with individual adhesion molecules and allowed to adhere for 30 min. After washing to remove nonadherent PMN and fixation with acetone:ethanol, adherent PMN were stained with hematoxylin and the number of cells was counted as above.

Western blot. PMN were treated with TNF-α as above. 1×10^7 cells were used per lane. Cells were lysed in 100 mM PMSF with aprotinin, pepstatin, and leupeptin, and then denatured by boiling in SDS for 5 min. Both the pellet and the supernatant were run together on SDS–PAGE minigels with 5% stacking gel and 10% running gel, and then transferred to nitrocellulose overnight (Amersham Hybond-C extra). The membrane was blocked for 2 h and incubated with primary antibody overnight and then secondary antibody for 2 h. The protein was detected using the Amersham ECL Western Blotting System (Amersham). Antibodies used were rabbit anti-cGMP-dependent protein kinase I at 1:4000 dilution and donkey anti-rabbit-HRP at 1:5000 dilution (Amersham).

cGMP quantification. The amount of cGMP was determined using the cGMP assay kit from Biomol (Plymouth Meeting, PA). Briefly, PMN were treated with the TNF, NO donors or inhibitors as above. Cells were then lysed with 0.1 M HCl and centrifuged. The supernatant containing cGMP was incubated in the ELISA plate along with the anti-cGMP antibody for 2 h and then by shaking with the *p*-nitrophenyl phosphate substrate. The optical density was determined at 405 nm and the amount of cGMP in the samples was calculated from the cGMP standard curve.

Statistics. Data from the assays were examined by ANOVA. Where significant differences were found, Student's *t* test was applied.

Results

HBMEC were first treated with TNF-α, with or without the various donors and inhibitors for 4 or 24 h; 4 h for maximal upregulation of E-selectin and 24 h for ICAM-1. PMN were then placed on top of the monolayers and allowed to adhere for 20 min. After fixation and staining, the amount of adhesion was quantified by counting the number of PMN bound in 4 peripheral and 1 central field under the microscope. TNF-α treatment increased adhesion by threefold after 4 h and fivefold after 24 h. Adhesion was also assessed after treatment with the NOS inhibitor L-NAME, guanylyl cyclase inhibitor ODQ, NO donors DETA NONOate, and SNP, 8-Br-cGMP. Changes are shown relative to cells receiving only TNF-α for 4 h (Fig. 2). The NOS inhibitor, L-NAME, significantly increased PMN adhesion. Both NO donors significantly decreased adhesion. This effect was prevented by the guanylyl cyclase inhibitor ODQ, suggesting it involves cGMP production. Addition of exogenous 8-Br-cGMP mimicked the NO effect. After 24 h of HBMEC treatment, L-NAME again increased adhesion (Fig. 3). The NO donors significantly inhibited adhesion. ODQ prevented this action. 8-Br-cGMP acted similar to NO.

We have previously shown that none of the NO treatments affected the expression of E-selectin or ICAM-1. It is possible that the NO and cGMP are affecting some other function of the HBMEC or they

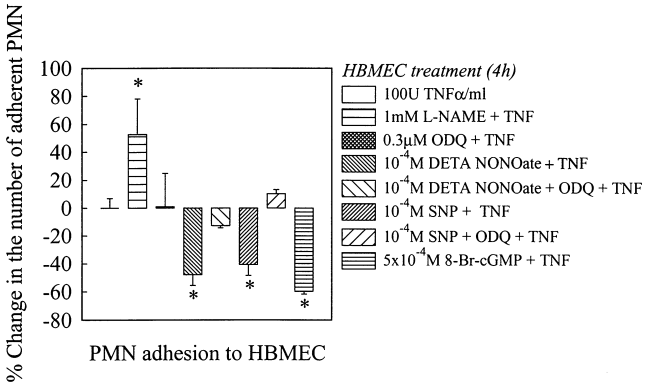

Fig. 2. Quantification of PMN adhesion to HBMEC treated with TNF-α and various combinations of the donors and inhibitors. Changes are shown relative to cells receiving only TNF-α for 4 h. Values represent means ± SEM of two experiments, each performed in duplicate wells. *Significant ($p < 0.05$) change in adhesion compared to HBMEC treated only with TNF-α. Both NO donors significantly decreased PMN adhesion. This action was reversed by ODQ, suggesting cGMP is involved. Exogenous 8-Br-cGMP has an even greater effect than NO.

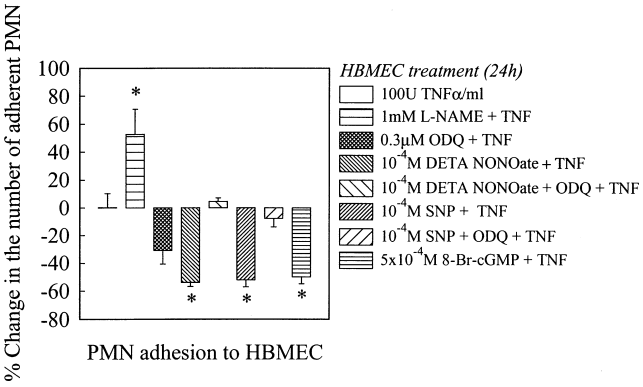

Fig. 3. Quantification of PMN adhesion to HBMEC treated with TNF-α and various combinations of the donors and inhibitors. Changes are shown relative to cells receiving only TNF-α for 24 h. Values represent means ± SEM of two experiments, each performed in duplicate wells. *Significant ($p < 0.05$) change in adhesion compared to HBMEC treated only with TNF-α. Both NO donors significantly decreased PMN adhesion. This action was reversed by ODQ, suggesting cGMP is involved. Exogenous 8-Br-cGMP has an even greater effect than NO.

are affecting the PMN. We therefore examined the PMN. First, we determined if PMN contain all the machinery in this pathway, specifically, the generation of cGMP and the cGMP-dependent protein kinases. For the former, the level of cGMP was measured by ELISA. TNF treatment for 20 min did not significantly affect cGMP production (Table 1). The guanylyl cyclase inhibitor ODQ significantly decreased the level of cGMP by threefold. Treatment with the NO donors raised the cGMP level by twofold. ODQ reduced the effect of the NO donors. Additionally, Type I cGMP-dependent protein kinase was present in unstimulated and TNF treated PMN as demonstrated by Western blot (Fig. 4). Having shown that this signal transduction pathway is present in PMN, we subsequently examined the effect of NO and 8-Br-cGMP on the adhesion of PMN to recombinant E-selectin and ICAM-1 in ELISA plates.

Table 1
Production of cGMP by PMN after 20 min of treatment

Treatment	[cGMP] (pmol/ml)
Untreated	0.437 ± 0.017
100 U TNFα/ml	0.447 ± 0.050
TNF + 0.3 μM ODQ	0.151 ± 0.023*
TNF + 10^{-4} M DETA NONOate	0.792 ± 0.077*
TNF + DETA NONOate + ODQ	0.735 ± 0.057*
TNF + 10^{-4} M SNP	1.007 ± 0.020*
TNF + SNP + ODQ	0.218 ± 0.024*

Quantification of the level of cGMP in PMN by ELISA after 20 min of treatment. TNF-α did not significantly change the level of cGMP in PMN. Both NO donors significantly increased the level of cGMP. This is inhibited by the guanylyl cyclase inhibitor ODQ.

* Significant ($p < 0.05$) change in the level of cGMP compared to untreated cells.

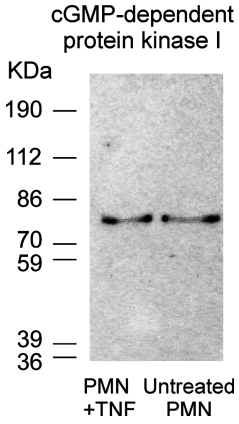

Fig. 4. Detection of cGMP-dependent protein kinase by Western blot. Type I cGMP-dependent protein kinase is expressed in both unstimulated and TNF-treated PMN.

First, PMN were treated with TNF-α for 20 min and then allowed to adhere to E-selectin. TNF-α treatment increased adhesion to E-selectin by twofold. Both NO donors reduced this level of adhesion (Fig. 5A). ODQ partially, but not completely, reversed this change. 8-Br-cGMP is even more effective than NO. Twenty minutes of TNF-α treatment increased adhesion to ICAM-1. Both NO donors significantly reduced this adhesion (Fig. 5B). The guanylyl cyclase inhibitor ODQ reversed this effect so that the level of adhesion is no longer significantly different from cells not treated with NO. 8-Br-cGMP also reduced PMN adhesion.

In addition, we examined whether these effects are transient. PMN were treated for 4 h with TNF-α and with or without the donors before the adhesion assays. Four hours of TNF treatment increased PMN adhesion to E-selectin. Both NO and 8-Br-cGMP significantly reduced PMN adhesion to E-selectin (Fig. 6A), 8-Br-cGMP and SNP being slightly more effective. Four hours of TNF-α treatment also increased PMN adhesion to ICAM-1. NO donors and 8-Br-cGMP were all effective in reducing this adhesion (Fig. 6B).

Discussion

The blood–brain barrier (BBB), formed by the endothelial cells of the cerebral microvessels, regulates what

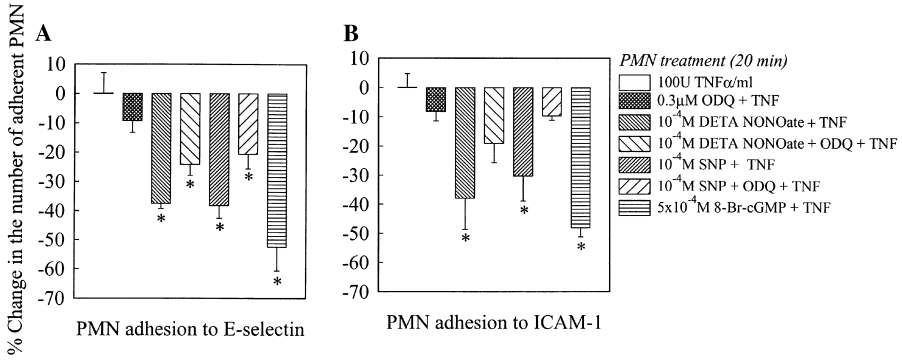

Fig. 5. Quantification of PMN adhesion to recombinant adhesion molecules after PMN treatment with TNF-α and various donors and inhibitors for 20 min. Values represent means ± SEM of three experiments, each performed in duplicate wells. *Significant ($p < 0.05$) change in adhesion. Both NO donors decreased adhesion to E-selectin and ICAM-1. ODQ partly reversed this effect, while 8-Br-cGMP had a similar effect compared to NO.

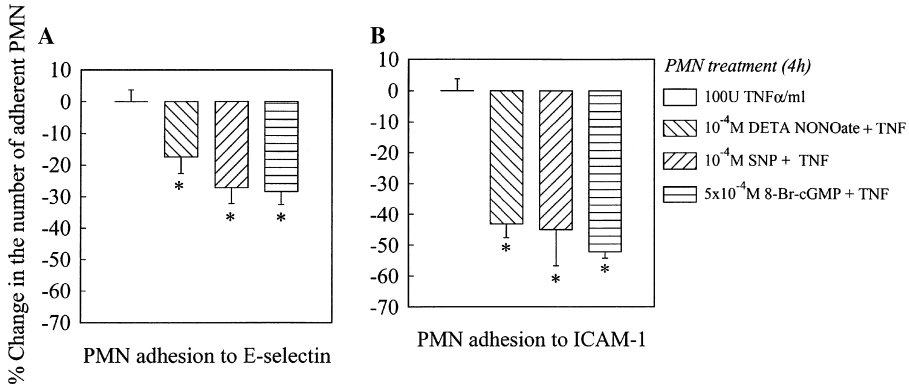

Fig. 6. Quantification of PMN adhesion to recombinant adhesion molecules after PMN treatment with TNF-α and various donors and inhibitors for 4 h. Values represent means ± SEM of three experiments, each performed in duplicate wells. *Significant ($p < 0.05$) change in adhesion. Treatment with DETA NONOate, SNP, and 8-Br-cGMP consistently decreased PMN adhesion to E-selectin and ICAM-1.

enters the brain. Adhesion and activation of circulating leukocytes is an important aspect of the inflammatory response. The BBB normally prevents the movement of leukocytes into the brain. In acute cerebral inflammatory reactions, PMN infiltrate the brain. We have previously shown that human brain microvessel endothelial cells (HBMEC) express the adhesion molecules E-selectin and ICAM-1 after treatment with LPS and cytokines [12,13], maximally at 4 and 24 h, respectively, and human peripheral blood PMN adhere to HBMEC via E-selectin and ICAM-1. Nitric oxide is a dissolved gas that has been shown to affect the PMN infiltration process in various species and organs. The literature has been rather complicated because of differences between species and organ systems, compounded by opposite effects depending on concentration [18,19]. The present study examined this phenomenon in HBMEC for the first time.

TNF-α treatment of HBMEC upregulated PMN adhesion, while NO treatment for 4 or 24 h reduced PMN adhesion by a pathway involving cGMP production and the activation of cGMP-dependent protein kinase. Various other NO donors have been shown to have similar effects. For example, CAS1609, SIN-1, GEA3175, SNAP, and S-NO-alb all reduced PMN adhesion to LPS or TNF-α stimulated HUVEC [20–22]. GEA3162, GEA3175, and SIN-1 also inhibited human PMN adhesion to rabbit endothelial cells treated with TNF-α [23]. This was associated with an increase in cGMP levels in the PMN. Furthermore, the membrane permeable analogs of cGMP, 8-Br-cGMP, and 8-pCPT-cGMP similarly decrease adhesion, consistent with our observations.

What is the mechanism by which NO regulates PMN adhesion to PBMEC? We have found that the expression of cell adhesion molecules by HBMEC is unaffected by NO. Similarly, Cartwright et al. [24] found that NO donors, GSNO, SNP, and spermine-NO, did not change the expression of E-selectin or ICAM-1 in a HUVEC cell line, SGHEC-7. However, Kosonen et al. [21] found that SNAP and GEA3175 (NO donors) reduced LPS induced E-selectin expression on HUVEC but did not affect ICAM-1 expression. De Caterina et al. [25] came to similar findings with GSNO, SIN-1, and SNP treatment of IL-1β stimulated human saphenous vein EC. On the other hand, Khan et al. [26] found that DETA-NO reduced ICAM-1 but not E-selectin expression. The NO donors SIN-1, GSNO, SNAP, DETA-NO, SNAP, and CAS1609 inhibited the expression of both ICAM-1 and E-selectin by cytokines and LPS treated HUVEC and saphenous vein EC [20,27–29]. The mechanisms by which NO regulates adhesion molecule expression have not been fully elucidated, but the interactions of NO with the transcription factor NF-κB, which is involved in inflammation and regulates synthesis of cytokines, receptors, and adhesion molecules, may be involved (De Caterina et al. [25]. Variations between EC from different vascular beds may partly account for the reported discrepancies in NO action.

Although the actions of NO on PMN adhesion might be thought to arise from changes in the expression of some endothelial cell adhesion molecules, there is also evidence that NO can affect the adhesive properties of PMN directly. We have found that treatment of PMN with NO for 20 min or 4 h decreased the number of PMN adherent to recombinant E-selectin and ICAM-1 through pathways involving cGMP. This may be due to a change in the level of E-selectin ligand or integrin expression by the PMN. Indeed, some studies have found that exogenous NO causes a reduction in CD11b/CD18 expression in PMN [30], although others have reported no change [21,31]. Another possible mechanism for NO action on PMN adhesion might involve interactions with the cytoskeleton. Clancy et al. [32] found that NO donors inhibit PMN adhesion by a mechanism associated with ADP ribosylation of actin. We found that PMN can synthesize cGMP and express cGMP-dependent protein kinase I. Both are components of the NO signal transduction pathway. The literature supports our finding that PMN express the signal transduction pathway needed to respond to NO. PMN have been shown to produce cGMP [22,23]. Vimentin and cGMP-dependent protein kinase are colocalized in a calcium-dependent manner in neutrophils and vimentin is transiently phosphorylated by cGMP-dependent protein kinase in activated neutrophils [33,34]. Furthermore, NO also has additional effects on PMN. It reduced TNF-α primed reactive oxygen release [22] and reduced PMN migration at the range we used [35]. NO donors SIN-1, GEA3162, and GEA5024 inhibited fMLP induced chemotaxis, correlating to increases in cGMP levels [36]. Those donors also inhibit the degranulation of PMN, calcium ionophore A23187 induced LTB_4 and β-glucuronidase release, and opsonized zymosan-triggered chemiluminescence [37].

Treatment of animal models with NO has similarly found positive effects. Treatment of the coronary artery of dogs with the NO donor SPM-5185 after ischemia–reperfusion reduced PMN adhesion, infiltration, and necrosis [38]. In ischemic isolated perfused rat kidney, SNP reduced PMN retention and glomerular dysfunction [39]. Inhaled NO also showed good effects. It reduced PMN sequestration in the lung of rabbits infused with complement fragments [40]. Changes were also seen in the peripheral vasculature. Fox-Robichaud et al. [41,42] found a reduction in the activation of the endothelium of the mesenteric circulation leading to a decrease in leukocyte rolling, adhesion and migration. Intravenous infusion of sodium nitroprusside reduced leukocyte adhesion and emigration in the rat mesentary after hemorrhagic shock without exerting a significant vasodilatory effect [43]. In addition, NO gas reduced

adhesion of PMN to the endothelium of the extracorporeal circulation [30]. It is clear that NO and members of its signal transduction pathway may reduce the interaction between circulating PMN and the BBB. They may thus be therapeutic for ischemia–reperfusion and other acute CNS inflammatory reactions.

Acknowledgments

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