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Role of nitric oxide on the increased vascular permeability and neutrophil accumulation induced by staphylococcal enterotoxin B into the mouse paw

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Abstract

The role of nitric oxide (NO) on the increase in vascular permeability and neutrophil migration induced by staphylococcal enterotoxin B (SEB; 25 μg/paw) in the mouse was investigated in this study. The NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) [but not its inactive enantiomer N^{ω}-nitro-D-arginine methyl ester (D-NAME)], given intravenously (25–100 μmol/kg) or subplantarly (0.25–1.0 μmol/paw), reduced SEB-induced paw oedema significantly. A similar response was observed with aminoguanidine, given either intravenously (200–600 μmol/kg) or subplantarly (2 μmol/paw). In contrast to paw oedema, the plasma exudation in response to SEB was not affected by the subplantar injection of L-NAME or aminoguanidine. The inhibition of oedema and plasma exudation by systemic treatment with L-NAME or aminoguanidine was reversed by co-injection of the vasodilator iloprost (0.3 nmol/paw). Subplantar injection of SEB (25 μg/paw) increased by 69% the myeloperoxidase (MPO) activity of SEB-treated paws, indicating the presence of neutrophils. Intravenous (12.5–50 μmol/kg) or subplantar (0.125–0.5 μmol/paw) administration of L-NAME (but not of its inactive enantiomer, D-NAME) largely reduced the MPO activity in SEB-treated paws. Similarly, intravenous (200–600 μmol/kg) or subplantar (2 μmol/paw) administration of aminoguanidine significantly reduced the MPO values of the SEB-injected paws. The vasodilator iloprost (0.3 nmol/paw) completely reversed the inhibition by L-NAME or aminoguanidine of the MPO activity in SEB-injected paws. Our results show that the increased vascular permeability and neutrophil accumulation in response to subplantar injection of SEB in the mouse are inhibited by L-NAME and aminoguanidine by mechanisms probably involving reduction of local microvascular blood flow.

Keywords: Plasma exudation; Paw oedema; Neutrophils; Enterotoxins; Aminoguanidine

1. Introduction

Staphylococcal enterotoxins are the most common cause of acute food poisoning in humans [1]. They represent a family of structurally related exoproteins (25–30 kDa) produced by several strains of *Staphylococcus aureus* and are classified into eight distinct immunological types (A-E and G-I) [2]. The physiopathological responses to staphylococcal enterotoxins involve activation of a number of cell types such as mast cells, macrophages, and lymphocytes, causing the release of different mediators including histamine, serotonin, leukotrienes, and various cytokines [3–5]. Among the staphylococcal toxins, SEB can be easily obtained in relatively large amounts and purity, and therefore has been used extensively in experimental investigations. Previous studies demonstrated that SEB induces hind-paw oedema and peritoneal neutrophil migration in the mouse, both of which are believed to be mediated by platelet-activating factor, histamine, leukotrienes, and neuropeptides [6,7]. In addition, SEB causes acute inflammatory lung injury characterized by an increase in vascular permeability, granulocyte infiltration, and induction of a number of cell adhesion

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Abbreviations: SEB, staphylococcal enterotoxin B; NO, nitric oxide; NOS, nitric oxide synthase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; D-NAME, N^{ω}-nitro-D-arginine methyl ester; MPO, myeloperoxidase; IL, interleukin; TNF, tumor necrosis factor; and IFN, interferon.

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molecules [8]. In the air pouch model in mice, SEB was able to recruit leukocytes (mainly neutrophils) in the pouch exudate [9]. Neutrophils have been considered the major cell type involved in the host's defense against *S. aureus* [10], and accumulation of neutrophils is a prominent feature of staphylococcal enterotoxin-induced gastroenteritis [11].

NO is produced in physiopathological conditions by three distinct isoforms of NOS, namely eNOS (isoform III), bNOS (isoform I), and iNOS (isoform II). The first two isoforms are expressed constitutively, whereas iNOS is expressed following exposure to diverse stimuli, such as cytokines (IL-1, TNF, IFN-γ) and lipopolysaccharide. SEB is able to release NO both *in vivo* [12] and *in vitro* [13], and this seems to play a major role in SEB-induced shock. Since NO has been shown to modulate acute and chronic inflammatory processes [14], in this study we examined the role of NO on the increase in vascular permeability (plasma exudation and paw oedema) and neutrophil migration induced by SEB in the mouse paw.

2. Materials and methods

2.1. Drugs

SEB (purchased from the Sigma Chemical Co.) was dissolved in sterile saline (0.9%, w/v) and stored in a refrigerator at a concentration of 1 mg/mL. L-NAME, D-NAME, aminoguanidine, hexadecyltrimethylammonium (HTAB), *o*-dianisidine dihydrochloride, and hydrogen peroxide were also obtained from Sigma. Iloprost was obtained from Schering. L-[2,3,4,5-^{3}H]Arginine (specific activity 60.0 Ci/mmol) was supplied by Amersham. All drugs were diluted in saline.

2.2. Animals

All experiments were carried out in accordance with the guidelines of the State University of Campinas (UNICAMP). Experimental procedures were performed in adult male Swiss mice (25–30 g) obtained from the Central Animal House (CEMIB).

2.3. Measurement of paw oedema and vascular permeability

Groups of five mice were injected intravenously with Evans blue (25 mg/kg) immediately before subplantar injection of 0.05 mL (25 μg/paw) of SEB in the left hind-paw under light ether anesthesia. The right hind-paw of each mouse served as the control and received an injection of 0.05 mL of vehicle alone (saline). The oedema and the dye exudate were measured 4 hr after the stimulus. Briefly, after being killed, the paws of the animals were amputated at the tarsocrural joint and weighed on an analytical balance. The oedema, expressed in milligrams, was the weight variation between the left (treated) and right (untreated) paw. Each paw was then chopped into small pieces and placed in a test tube with 3.0 mL of formamide. The tubes were then incubated in a water bath at 57° for 24 hr. The absorbance of the supernatants was measured at 619 nm. The concentration of Evans blue present in the extracts was determined from a standard curve of the dye prepared in formamide.

2.4. MPO assay

MPO, a hemoprotein located in azurophil granules of neutrophils, has been used as a biochemical marker for neutrophil infiltration into tissues. Briefly, each paw was weighed, chopped into small pieces, and placed in a test tube in the presence of 0.5% HTAB in 50 mM potassium phosphate buffer, pH 6.0. Each tissue sample was homogenized for 15 sec, then vortexed, and 1-mL aliquots of the homogenate were decanted into Eppendorf tubes. These tubes were then centrifuged (Eppendorf centrifuge) for 2 min at maximum speed, and the supernatants were collected. An MPO assay was performed using a microliter plate scanner (Spectra Max 34, Molecular Devices). This consisted of mixing 10 μL of sample with 200 μL of *o*-dianisidine solution (0.167 mg/mL of *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide) prior to reading the plate. The changes in absorbance were measured at 460 nm for 30 sec over a period of 5 min. One unit of MPO activity was defined as that degrading 1 μmol of peroxide/min at 25° [15].

2.5. NOS assay

NOS activity was determined according to the method described by Förstermann *et al.* [16], which is based upon the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. Briefly, hind-paws were thawed, weighed, and homogenized with 50 mM Tris–HCl, pH 7.4, containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM L-citrulline in a polytron tissue homogenizer. The homogenates were centrifuged (10,000 *g*, 10 min), and 50 μL of each supernatant was incubated for 30 min in the presence of 1 mM NADPH, 2 mM $CaCl_2$, 10 μg/mL of calmodulin, 10 μM FAD, 100 μM BH_4, and 10 μM L-arginine containing 200,000 dpm of [2,3,4,5-^{3}H]L-arginine monohydrochloride at 37°. Pharmacological controls of NOS activity were carried out in parallel and consisted of either the omission of $CaCl_2$ and the addition of 1 mM EGTA, or the addition of 1 mM L-NAME to the incubation medium. The protein content of samples was determined according to the method of Peterson [17], and NOS activity was expressed as picomoles of L-citrulline per minute per milligram of protein.

2.6. Experimental protocols

For the plasma exudation and paw oedema measurements, the animals received L-NAME (25–100 μmol/kg),

D-NAME (100 μmol/kg), or aminoguanidine (200–600 μmol/kg) intravenously immediately before subplantar injection of SEB (25 μg/paw; N = 5 in each group). In a separate set of experiments, L-NAME (0.25–1.0 μmol/paw), D-NAME (1.0 μmol/paw), and aminoguanidine (2 μmol/paw) were co-injected with SEB into the paws (N = 5 in each group). In the experiments designed to examine the effects of the vasodilator iloprost on SEB-induced responses, iloprost (0.3 nmol/paw) was injected 1 hr before the measurement of paw oedema. The increases in paw weight and plasma exudation were always measured 4 hr after the subplantar injection of SEB.

For the MPO assay, groups of five mice were injected intravenously with L-NAME (12.5–50 μmol/kg), D-NAME (50 μmol/kg), or aminoguanidine (200–600 μmol/kg) immediately before the subplantar injection of SEB (25 μg/paw). In a separate set of experiments, L-NAME (0.125–0.5 μmol/paw), D-NAME (0.5 μmol/paw), and aminoguanidine (2 μmol/paw) were co-injected with SEB into the paws (N = 5 in each group). In the experiments designed to examine the effects of the vasodilator iloprost on SEB-induced responses, iloprost (0.3 nmol/paw) was injected 1 hr before the measurement of MPO activity. The MPO activity in the paw homogenates was determined 4 hr after the subplantar injection of SEB.

2.7. Statistical analysis

Data are reported as the means ± SEM of 4–5 animals. Results were compared using ANOVA followed by the Bonferroni test. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Plasma exudation and paw oedema

Subplantar injection of SEB in the hind-paws of mice caused a dose-dependent paw oedema (2 ± 2, 10 ± 0.1, 30 ± 3.1, and 32 ± 2 mg for 6.25, 12.5, 25, and 50 μg/paw at 4 hr, respectively) and plasma exudation (18 ± 2, 32 ± 2, and 14 ± 2.5 mg for 25 μg/paw at 2, 4, and 24 hr, respectively). The time-course for oedema formation (18 ± 2, 32 ± 2, and 14 ± 2.5 mg for 25 μg/paw at 2, 4, and 24 hr, respectively) and plasma exudation (1.4 ± 0.4, 4.3 ± 0.6, and 1.8 ± 0.2 μg/g of Evans blue for 25 μg/paw at 2, 4, and 24 hr, respectively; N = 5) revealed that maximal responses were observed at 4 hr post-SEB injection. For further studies, SEB was administered routinely at a dose of 25 μg/paw, and both plasma protein exudation and paw oedema were evaluated 4 hr later.

Figure 1A shows that intravenous administration of L-NAME (25–100 μmol/kg) caused a dose-dependent inhibition of the SEB-induced paw oedema. A significant reduction ($P < 0.05$) in dye exudation was observed only at the highest dose of L-NAME (Fig. 1B). The intravenous administration of the inactive enantiomer D-NAME (100 μmol/kg) affected neither paw oedema (Fig. 1A) nor plasma exudation (Fig. 1B). At the doses used, intravenous administration of L-NAME had no significant effect on basal plasma exudation in saline-injected paws (not shown).

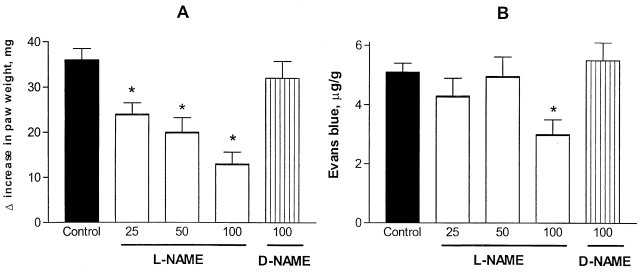

Fig. 1. Effects of L-NAME (25–100 μmol/kg) and D-NAME (100 μmol/kg) on SEB-induced paw oedema and plasma exudation. The changes in paw weight (A) and Evans blue exudation (B) were measured 4 hr after the injection of SEB (25 μg/paw). Each column represents the mean ± SEM of five mice. Key: (*) $P < 0.05$ compared with the control group (untreated animals).

The subplantar administration of L-NAME (0.25–1.0 μmol/paw), but not D-NAME (1.0 μmol/paw), also reduced SEB-induced paw oedema significantly without affecting plasma exudation (Table 1). At the doses used, subplantar administration of L-NAME had no significant effect on the basal plasma exudation of the saline-injected paws (not shown).

The intravenous administration of the iNOS inhibitor aminoguanidine (200–600 μmol/kg) significantly reduced ($P < 0.05$) both paw oedema (Fig. 2A) and plasma exudation (Fig. 2B) induced by SEB. When given locally into the paws, aminoguanidine (2 μmol/paw) reduced paw oedema by 44% ($P < 0.05$), but failed to affect plasma exudation (4.6 ± 0.8 and 5.6 ± 0.5 μg/g of Evans blue for control and treated-paws, respectively). At the doses used, subplantarly or intravenously, aminoguanidine had no significant effect on basal plasma exudation in saline-injected paws (not shown).

Table 1
Effects of subplantar administration of L-NAME or D-NAME on paw oedema and plasma exudation induced by SEB (25 μg/paw)

	Δ Increase in paw weight (mg)	Evans blue (μg/g)
Control	34 ± 2.4	5.0 ± 0.3
L-NAME (μmol/paw)		
0.25	18 ± 2.0*	4.6 ± 1.1
0.5	20 ± 3.2*	4.7 ± 0.9
1.0	20 ± 4.5*	4.8 ± 0.6
D-NAME (μmol/paw)		
1.0	30 ± 3.2	4.9 ± 0.9

The increase in paw weight and Evans blue exudation was measured 4 hr after SEB injection. Data represent the means ± SEM of five mice.
*$P < 0.05$ compared with the control group.

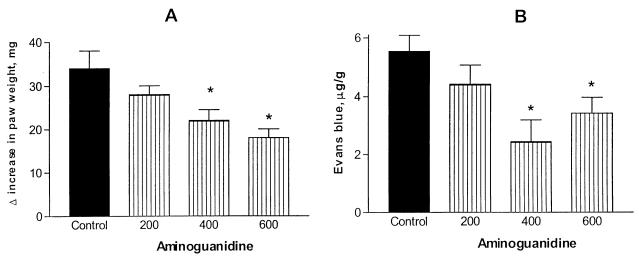

Fig. 2. Effect of aminoguanidine (200–600 μmol/kg, i.v.) on SEB-induced paw oedema and plasma exudation. The changes in paw weight (A) and Evans blue exudation (B) were measured 4 hr after injection of SEB (25 μg/paw). Each column represents the mean ± SEM of five mice. Key: (*) $P < 0.05$ compared with the control group (untreated animals).

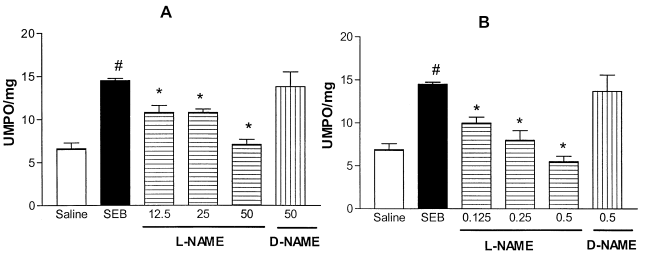

Fig. 4. Effects of intravenous (A) and subplantar (B) administration of L-NAME and D-NAME on the MPO activity of SEB-treated paws. The measurement of MPO activity (UMPO/mg) was carried out at 4 hr after injection of SEB (25 μg/paw). UMPO = MOP units. Each column represents the mean ± SEM of five mice. Key: (#) $P < 0.05$ compared with the saline group; and (*) $P < 0.05$ compared with the SEB group.

The subplantar injection of the prostacyclin analogue iloprost (0.3 nmol/paw) potentiated the SEB-induced paw oedema and plasma exudation by 65.7 and 42.7% ($P < 0.05$), respectively, as expected. In addition, iloprost significantly reversed the inhibition by L-NAME or aminoguanidine of the SEB-induced paw oedema (Fig. 3A) and plasma exudation (Fig. 3B). Iloprost did not evoke paw oedema or plasma exudation when injected alone (not shown).

3.2. Measurement of MPO activity

Subplantar injection of SEB (25 μg/paw) in the hindpaws of the mice caused a significant increase ($P < 0.05$) in MPO activity, as determined at 4 and 12 hr post-SEB injection (12.5 ± 0.8 and 15.7 ± 1.1 MPO units/mg) in comparison with the MPO values in the contralateral paws injected with sterile saline (7.44 ± 0.5 MPO units/mg). For further studies, MPO activity was determined routinely at 4 hr post-SEB injection.

Intravenous administration of L-NAME (12.5–50 μmol/kg) largely reduced the MPO activity in SEB-treated paws. At the highest dose (50 μmol/kg), the increased MPO activity was nearly abolished by L-NAME (Fig. 4A). Similarly, subplantar administration of L-NAME (0.125–0.5 μmol/paw) dose-dependently reduced the increased MPO activity in SEB-treated paws. A total reversal of the increased MPO activity was observed with 0.5 μmol/paw of L-NAME (Fig. 4B). The inactive enantiomer D-NAME, given either intravenously (50 μmol/kg) or subplantarly (0.5 μmol/paw), had no effect on the increased MPO activity in SEB-treated paws (Fig. 4). In addition, L-NAME, given either intravenously (50 μmol/kg) or subplantarly (0.5 μmol/paw), failed to affect the MPO activity of saline-injected paws significantly (not shown).

The intravenous (200–600 μmol/kg) or subplantar (2 μmol/paw) administration of the iNOS inhibitor aminoguanidine significantly reduced ($P < 0.05$) MPO activity in the SEB-treated paws. However, a complete reduction of MPO activity was not observed with the use of aminoguanidine (Table 2).

Subplantar injection of iloprost (0.3 nmol/paw) potentiated by 40% ($P < 0.05$) the MPO activity of SEB-treated paws. In addition, iloprost completely reversed the inhibition by L-NAME (50 μmol/kg, i.v.) or aminoguanidine (600

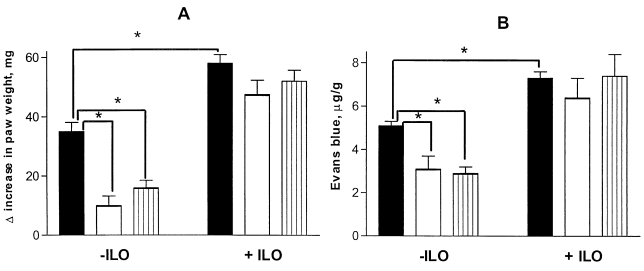

Fig. 3. Effects of iloprost on the inhibition of SEB-induced paw oedema (A) and plasma exudation (B) by L-NAME (open columns) or aminoguanidine (striped columns). The mice were injected intravenously with either L-NAME (100 μmol/kg) or aminoguanidine (600 μmol/kg) immediately before SEB (25 μg/paw). Control (untreated) mice were injected intravenously with saline (solid columns). The mice received either iloprost (0.3 nmol/paw; + ILO) or saline instead of iloprost (-ILO) 1 hr before paw oedema analysis. The changes in paw weight and dye exudation were measured 4 hr after the injection of SEB. Each column represents the mean ± SEM of five mice. Key: (*) $P < 0.05$ compared with the control group.

Table 2
Effects of intravenous (200–600 μmol-kg) or subplantar (2 μmol/paw) administration of aminoguanidine on MPO activity of SEB-treated paws (25 μg/paw)

	MPO activity (MPO units/mg)
Saline	6.9 ± 0.7
SEB	22.1 ± 1.5**
Aminoguanidine (μmol/kg)	
200	17.2 ± 0.6**
400	14.9 ± 1.0**
600	14.8 ± 1.1**
Aminoguanidine (μmol/paw)	
2	12.7 ± 1.2**

Changes in MPO activity were measured 4 hr after SEB injection. Data represent the means ± SEM of five mice.

* $P < 0.05$ compared with the saline group.

** $P < 0.05$ compared with the SEB-treated group.

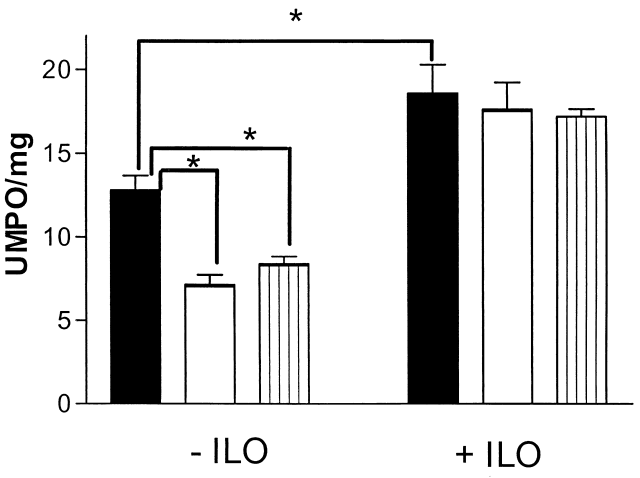

Fig. 5. Effects of iloprost on the reduction of MPO activity by L-NAME (open columns) or aminoguanidine (striped columns). The mice were injected intravenously with either L-NAME (50 μmol/kg) or aminoguanidine (600 μmol/kg) immediately before SEB (25 μg/paw). Control (untreated) mice were injected intravenously with saline (solid columns). The mice received either iloprost (0.3 nmol/paw; + ILO) or sterile saline instead of iloprost (-ILO). The changes in MPO activity (UMPO/mg) were measured 4 hr after the injection of SEB. UMPO = MPO units. Each column represents the mean ± SEM of five mice. Key: (*) $P < 0.05$ compared with the control group.

μmol/kg, i.v.) of the MPO activity in SEB-injected paws (Fig. 5). When injected alone, iloprost did not affect MPO activity compared with the saline control (7.5 ± 1.1 and 7.6 ± 0.4 MPO units/mg, respectively).

3.3. Measurement of NOS activity

cNOS activity in the SEB-injected mouse paws was enhanced markedly (1.7 ± 0.4 pmol citrulline/min/mg) compared with that of the saline-injected paws (0.4 ± 0.09 pmol citrulline/min/mg, N = 3; $P < 0.05$). The omission of Ca^{2+} and the addition of EGTA to the paw homogenates abolished the increased NOS activity of SEB-injected paws (0.3 ± 0.1 pmol citrulline/min/mg), thus indicating that the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was essentially due to constitutive NOS.

4. Discussion

Our results clearly show that increased vascular permeability and neutrophil accumulation in response to SEB in the mouse paw were inhibited significantly by local or systemic treatment of the animals with the NOS inhibitors L-NAME and aminoguanidine, strongly indicating that NO modulates both of these phenomena. The failure of the inactive enantiomer D-NAME to affect the SEB-induced inflammatory responses confirms the hypothesis that inhibition by L-NAME was, in fact, due to the inhibition of NO synthesis.

The role of NO in inflammation is still controversial. Depending on the type and the phase of inflammation and the individual vascular or cellular response studied, NO seems to have both pro-inflammatory and anti-inflammatory properties, suggesting that NO, like other inflammatory mediators, has a dualistic function in inflammation [14]. NO modulates vascular permeability and oedema formation by mechanisms dependent on, and independent of, local blood flow [18]. Our results showing that L-NAME, given intravenously or subplantarly, significantly reduced SEB-induced paw oedema are in agreement with previous studies that demonstrated that this compound reduces oedema formation in a number of animal species, including the mouse [19,20]. The reduction of inflammatory oedema by NOS inhibitors has been attributed to their ability to prevent vasodilatation and decrease regional microvascular blood flow as a consequence of the inhibition of NO synthesis in vascular endothelium [21–24]. We speculated, therefore, that inhibition of SEB-induced paw oedema by L-NAME reflects a decrease in blood flow of the paw microcirculation (vasoconstriction) without directly affecting the permeability. The finding that iloprost, an analogue of the vasodilator prostacyclin [25], completely reversed the inhibition by L-NAME of SEB-induced paw oedema is consistent with this proposal, as previously suggested for carrageenan-induced rat paw oedema [26]. Aminoguanidine is a compound known to preferentially inhibit the iNOS isoform with little or no inhibitory effect on the constitutive (endothelial and neuronal) NOS isoforms [27–29]. Interestingly, systemic or subplantar treatment of the mice with aminoguanidine also reduced SEB-induced paw oedema significantly, and such reduction was completely reversed by iloprost. This suggests that inhibition of SEB-induced oedema by aminoguanidine cannot be explained solely by virtue of its ability to selectively inhibit the iNOS isoform, but rather involves inhibition of constitutive NOS activity. Indeed, previous studies reported that, depending on the dose employed, aminoguanidine can inhibit constitutive NOS activity [30]. Our findings that subplantar injection of SEB markedly increased constitutive NOS activity in the paw homogenates further support the involvement of this isoform in oedema development. The participation of an inducible, calcium-independent NOS isoform was excluded since omission of calcium and addition of EGTA to the paw homogenates completely prevented the increased NOS activity. Although the evidence that SEB increases the activity of a constitutive (but not an inducible) NOS isoform is surprising, previous studies showed that rat paw oedema induced by carrageenan is accompanied by a marked increase in constitutive (but not inducible) NOS in paw homogenates at early (0–4 hr) phases of oedema [31], an effect recently attributed to NO synthesized by a neuronal NOS isoform, most probably within sensory nerves [32]. Accordingly, a recent study showed that SEB-induced mouse paw oedema is a consequence of a complex neurogenic response involving local activation of sensory nerves [33].

It is well documented that development of inflammatory oedema is due primarily to an increase in protein efflux, which decreases the lymph-to-plasma total-protein ratio (L/P ratio), thus virtually eliminating the transmural colloid osmotic pressure gradient, whereas the transmural hydrostatic pressure gradient is increased markedly. The rise in microvascular pressure (P_{mv}) is not accepted as an important determinant of oedema formation [34]. Our results showed that while L-NAME (or aminoguanidine) reduced SEB-induced paw oedema, the plasma exudation was reduced only by the higher doses of these compounds (in the case of systemic treatment) or was not affected (in the case of subplantar treatment). This implies that in situations where paw oedema is reduced, the amount of extravasated proteins is not modified significantly. Although these results are unclear, they suggest that L-NAME and aminoguanidine reduce SEB-induced paw oedema mainly by reducing the transmural hydrostatic pressure gradient; the findings that the vasodilator iloprost reversed the inhibition of paw oedema by L-NAME and aminoguanidine reinforce this proposal. Furthermore, L-NAME inhibits carrageenan-induced paw oedema without affecting the permeability kinetics [26]. In this respect, it is interesting to note that L-NAME has been shown to increase the microvascular protein efflux by mechanisms dependent on, and independent of, leukocyte adhesion [35,36]. However, it is unlikely that this mechanism contributes to the overall phenomenon in the mouse paw microcirculation since L-NAME and aminoguanidine failed to affect basal plasma exudation, as observed in the saline-treated paws.

Neutrophil migration from circulating blood to sites of injury is a crucial event during inflammatory processes, a phenomenon mediated by various substances including C5a, leukotriene B_4, IL-1, IL-8, TNF-α, and IFN-γ. Some of these cytokines such as IL-1, TNF-α, and IFN-γ can induce iNOS activity in a variety of cells (including neutrophils), leading to enhanced NO formation [30]. The role of NO on leukocyte recruitment has been investigated extensively, but results are still conflicting. Inhibitors of NOS such as N^G-monomethyl-L-arginine attenuated *in vitro* human neutrophil chemotaxis [37,38]. In the *in vivo* pleurisy model, L-NAME can either inhibit [39,40] or have no effect on [41] pleural neutrophil accumulation. Additionally, in both cat mesenteric [42] and rat hindlimb muscle [43] preparations *in vivo*, L-NAME increases leukocyte adhesion to vascular endothelium. Our results showed that subplantar injection of SEB markedly increased MPO activity in mouse paw homogenates at 4 hr, indicating that the number of neutrophils in the paws was enhanced by this enterotoxin. The increased MPO activity values of SEB-treated paws were reduced significantly by L-NAME (but not D-NAME) and aminoguanidine, indicating that NO modulates this phenomenon. Since L-NAME (and aminoguanidine) reduced SEB-induced paw oedema by a mechanism probably related to decrease of local blood flow, we next examined whether attenuation of infiltrating neutrophils by the NO inhibitors also reflects a decrease of regional microvascular blood flow, a condition that may restrict the number of infiltrating cells into inflammatory sites. Our findings that the vasodilator iloprost potentiated the MPO activity in SEB-treated paws and completely reversed the inhibitory effects of L-NAME (and aminoguanidine) on the SEB-induced increase in MPO activity are consistent with the suggestion that reduction of basal blood flow is the mechanism by which L-NAME and aminoguanidine inhibit neutrophil accumulation in SEB-treated paws. In a similar way, the vasodilators sodium nitroprusside and prostaglandin E_1 also reversed the inhibitory effects of L-NAME on the neutrophil accumulation induced by zymosan-activated serum in guinea-pig skin [23], thus reinforcing our proposal. Furthermore, the failure of L-NAME to affect the MPO activity in the contralateral paws injected with saline may rule out that increased MPO activity in SEB-treated paws reflects an enhancement of neutrophil adhesion to vascular endothelium.

In conclusion, our results demonstrate that the increased vascular permeability and neutrophil accumulation observed in response to subplantar injection of SEB in mice were inhibited significantly by NOS inhibitors such as L-NAME and aminoguanidine. Reduction of local blood flow seems to be the main mechanism by which the NO inhibitors exert their anti-inflammatory effects.

Acknowledgments

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