

Regulation by Nitric Oxide of Endotoxin-Induced Tissue Factor and Plasminogen Activator Inhibitor-1 in Endothelial Cells

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Keywords

HUVEC, Sepsis, Nitric oxide, Tissue factor, Plasminogen activator inhibitor-1

Summary

The increase in nitric oxide (NO) production in lipopolysaccharide (LPS)-induced sepsis is thought to contribute to the development of shock. However, NO could also play an antithrombotic role. Little is known about the modulating effect of NO on the endothelial overexpression and production of tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) occurring in endotoxemia. We analyzed the effect of N(G)-nitro-L-arginine-methyl-ester (L-NAME), an inhibitor of NO synthases, and S-nitroso-N-acetyl-D,L-penicillamine (SNAP), a NO donor, on the expression and synthesis of TF and PAI-1 by LPS-challenged human umbilical vein endothelial cells (HUVEC): L-NAME enhanced the increase in TF mRNA and antigen levels ($P < 0.05$) observed in LPS-treated HUVEC; SNAP down-regulated the LPS-induced TF increment ($p < 0.05$). However, no effects of NO on regulation of the LPS-dependent increase in PAI-1 could be seen. Thus, NO could play an antithrombotic role in sepsis by down-regulating the endothelial overexpression and production of TF.

Introduction

The endothelium is a key organ involved in the pathogenesis of sepsis (1). The hemostatic funtions of the vascular beds are strongly influenced by the properties of the vascular endothelium: under physiologic conditions, it plays a significant role in maintaining blood fluidity through both anticoagulant protein C and antithrombin systems and fibrinolysis (2-4). However, sepsis stimuli such as lipopolysaccharide (LPS) modify the endothelial properties making the endothelium act as a prothrombotic surface. Among the changes induced by LPS in the endothelial cells, the increase in the expression and synthesis of tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) could contribute to the generation and deposition of fibrin thrombi in the microvasculature (5-7).

Nitric oxide (NO) displays several important effects on the vasculature, such as maintaining basal tone, inhibiting leukocyte adhesion to the endothelium and inhibiting vascular smooth muscle cell migration and proliferation (8). Endothelial cells are able to synthesize NO by NO synthases (NOS). Under physiologic conditions, low levels of NO are synthesized by endothelial-NOS (e-NOS). Pathogenic stimuli such as LPS induce the endothelial expression of the inducible-NOS (i-NOS) which generates high amounts of NO, which, in turn, could be partly responsible for hypotension and the subsequent septic shock (9). However, experimental data suggest that NO could play an antithrombotic role in sepsis: in previous experiments we found that rats challenged with LPS exhibited a higher plasma level of PAI-1 when simultaneously treated with N(G)-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS; such an increase in PAI-1 was associated with increased fibrin deposition in kidneys (10). Other studies in endotoxemic animals have shown both that NO inhibition induces thrombosis in different vascular territories (11-14) and that endogenous NO and the administration of NO donors decrease glomerular thrombosis (15, 16). Moreover, there are several evidences of the down-regulating effect of NO on the TF synthesis by monocytes, which is an important source of TF (17-19); however, little is known about the endothelial TF modulation by NO: recently, Yang and Loscalzo described how L-arginine was able to reduce the LPS-induced synthesis of TF by endothelial cells in culture (20), although they were not able to demonstrate that such an effect was due to an increase in the production of NO (21). In the present study the effects of a NOS inhibitor, L-NAME, and a NO donor, S-nitroso-N-acetyl-D,L-penicillamine (SNAP), on the expression and the synthesis of TF and PAI-1 by human vein endothelial cells (HUVEC) have been assessed. Our data suggest that NO may protect against LPS-induced thrombus formation in the microvasculature by down-regulating the expression and synthesis of endothelial TF.

Materials and Methods

Materials

Medium 199 with Earl's salts (MEM 199), Hank's Balanced Salts Solution (HBSS), phosphate buffered saline (PBS), trypsin-EDTA, penicillin-streptomycin mixture, endothelial cell serum free medium (SFM), and fetal calf serum (FCS) were purchased from Gibco-BRL (UK); collagenase A from *Clostridium histolyticum*, LPS from Escherichia coli 0127:B8, bovine gelatine, L-NAME and SNAP were obtained from Sigma (USA).

Endothelial Cell Cultures

Endothelial cells (HUVEC) were isolated from human umbilical vein cords obtained less than 8 h after delivery, essentially as described by Jaffe et al. (22). The umbilical cord was cannulated, perfused with PBS, and incubated at 37° C with collagenase A (0.5 mg/mL) for 20 min. Cells thus obtained were centrifuged at 250 g for 5 min, resuspended in MEM 199 containing 20% pooled human

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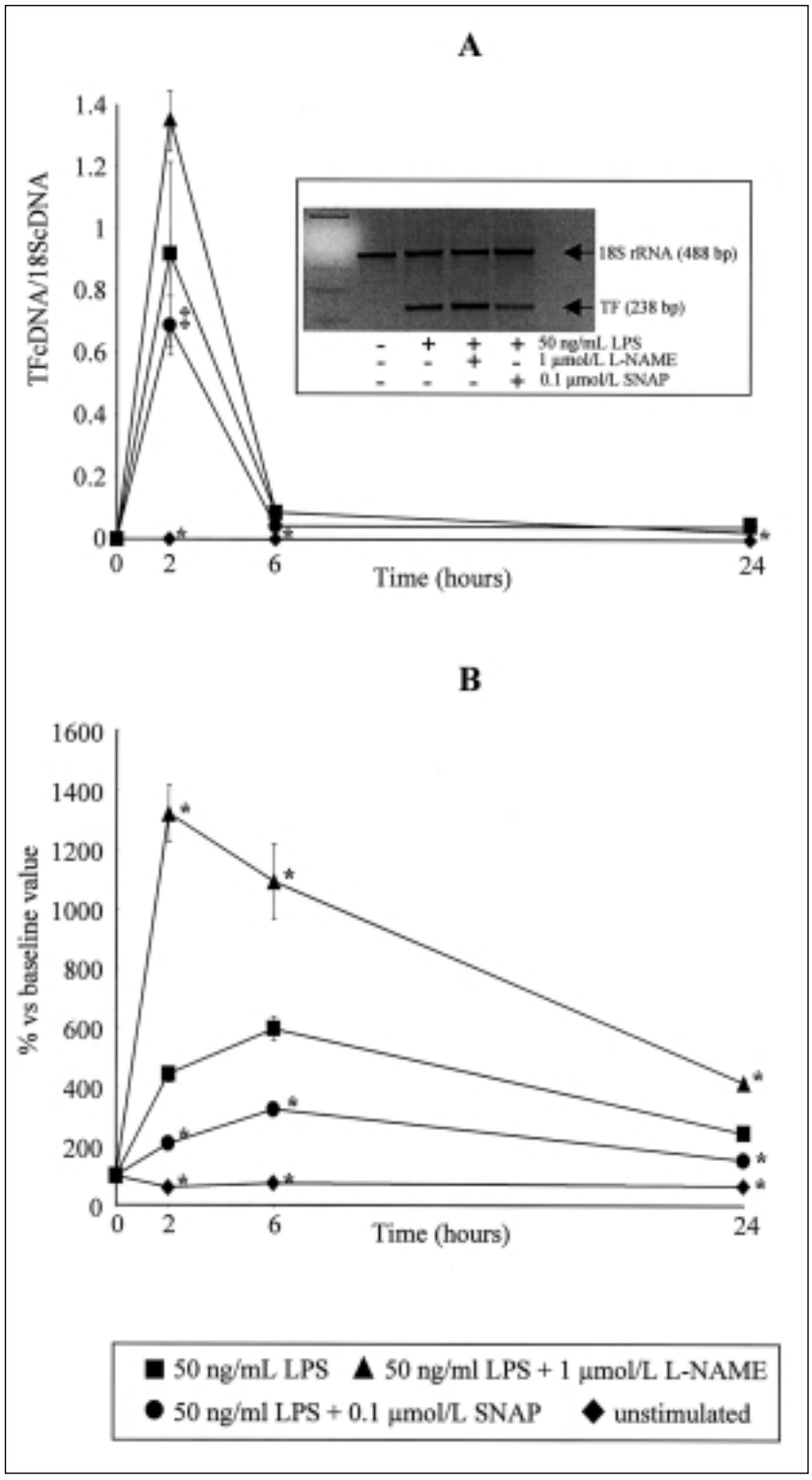

Fig. 1 TF mRNA expression (panel A) and TF protein levels (panel B) in HUVEC challenged with LPS, LPS + L-NAME and LPS + SNAP. Results are mean ± SEM. * $P < 0.05$ with respect to the LPS group (inset: TF cDNA electrophoresis at 2 h)

Table 1 TF and PAI-1 antigen levels before the start of the experiment and at 2, 6 and 24 h. Results are expressed as the mean ± SEM

	TF (pg/µg protein 10^6 cells)				PAI-1 (ng/mL · 10^6 cells)			
	LPS	LPS + L-NAME	LPS + SNAP	Unstimulated	LPS	LPS + L-NAME	LPS + SNAP	Unstimulated
Basal value	50.2 ± 2.6	28.7 ± 0.3	41.9 ± 2.3	63.3 ± 3.2	10,378 ± 545	10,563 ± 743	11,836 ± 476	11,225 ± 480
2 hours	220. 7 ± 16.5	378.8 ± 27.9	86.7 ± 5.3	38.4 ± 4.6	15,287 ± 1,252	13,633 ± 867	14,210 ± 647	11,378 ± 672
6 hours	296.8 ± 23.7	314.1 ± 37.2	136.0 ± 10.0	47.0 ± 5.1	14,623 ± 778	14,513 ± 701	15,214 ± 689	14,325 ± 865
24 hours	121.8 ± 4.3	119.1 ± 5.0	64.6 ± 3.5	41.5 ± 3.4	37,872 ± 2,359	42,743 ± 3,797	37,014 ± 7,904	18,354 ± 1,863

serum and 1% penicillin/streptomycin, and seeded in a 25 cm^2 culture flask precoated with gelatine at 0.1% (v/v) in PBS. Cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2. The medium was changed 24 h after seeding and then every 48 h. When cultures reached confluence, 1 mL trypsin-EDTA was added. After 2 min at 37° C, cells were collected and seeded into a 75 cm^2 flask. When they became confluent, cells were subsequently spread into four 150 cm^2 flasks.

Experimental Design

All studies were performed with confluent cultures in the third passage, using the four 150 cm^2 flasks coming from the same umbilical cord for each single experiment. Cultures were washed with HBSS after which fresh SFM supplemented with 1% penicillin/streptomycin was added. Culture supernates and cells were harvested from one flask before the start of the experiment. The three remaining cultures were simultaneously challenged with the same stimulus, and supernates and cells were harvested, from one flask at each time, at 2, 6 and 24 h after the start of the experiment. The stimuli tested were: LPS (50 ng/mL); LPS (50 ng/mL) + L-NAME (1 μmol/L); LPS (50 ng/mL) + SNAP (0.1 μmol/L). Doses were chosen according to previous *in vivo* and *in vitro* experiments (10, 23). Cell viability was not altered by these compounds when used at these concentrations (data not shown). Unstimulated cultures were used as controls. Three (FT analysis) and six (PAI-1 analysis) independent experiments were performed for each experimental condition.

Isolation of Total RNA

2-3 × 10^6 cells were scraped from the flask into PBS and then lysed by repetitive pipetting in 1 mL Tripure™ Isolation Reagent (Boehringer Mannheim, Germany) using RNAse-free polypropylene tubes. After 5 min incubation, the sample was mixed vigorously with 0.2 mL chloroform and incubated for 15 min, then centrifuged at 12,000 g for 15 min. The aqueous phase was mixed with 0.5 mL isopropanol and stored for 10 min, and then centrifuged to precipitate RNA. The RNA pellet was subsequently washed with 1 mL 75% ethanol, centrifuged at 7,500 g for 5 min, air-dried for 10 min, and dissolved in 25 μL diethyl pyrocarbonate (DEPC)-treated water by pipetting and incubating at 55-60° C for 10 min. Thus, total RNA (DNA-free and protein-free) was obtained.

Isolation of TF and PAI-1 cDNA by Reverse Transcription-PCR

The reverse transcription (RT) reaction was performed to determine whether the stimuli applied to HUVEC were able to modify the transcription level of TF and PAI-1 genes. RT was performed using 1 μg RNA in a final volume of 20 μL including 200 U Moloney murine leukemia virus RT (Gibco BRL), 2 μL RT buffer, 100 ng/μL random hexamers (Boehringer Mannheim), 1 mmol/L dNTPs (Amersham Pharmacia Biotech, Sweden), 20 U RNAse inhibitor (Amersham Pharmacia Biotech), 5 mmol/L DTT (Gibco BRL). The reaction was performed at 37° C for 1 h. PCR primers pairs 5´-tcctgctcggctgggtctcttc-3´ and 5´-acatccttcacaatctcgtcg-3´, were used to amplify a 238 bp fragment for human TF cDNA located between exons 1 and 3. PCR primers pairs 5´-acaggaggagaaacccagcag-3´ and 5´-ccgtctgatttgtggaagagg-3´, were used to amplify a 434 bp fragment of human PAI-1 cDNA located between exons 2 and 4. Since the amount of ribosomal RNA (rRNA) in the RNA sample is high enough to maintain its expression constant in spite of variations among samples during the RT-PCR process, 18S rRNA (Ambion, USA) was used as an internal control of PCR reactions, giving a PCR product of 488 bp. TF/18S rRNA or PAI-1/18S rRNA PCR reactions were performed in the same tube in a final volume of 50 μL containing 240 pmol/L and 200 pmol/L of TF and PAI-1 primer pairs respectively. PCR master mix consisted of 1 U Taq polymerase (Roche, Germany), 1.5 mmol/L $MgCl_2$, 40 mmol/L KCl, 16 mmol/L Tris-ClH, pH 8.3. PCR reactions were performed using the GeneAmp 2400 PCR system (Perkin Elmer, USA). Touch-down cycles were used during the amplification to increase specificity (24). The thermal cycling conditions included initial denaturation at 94° C for 40 seconds (s), followed by ten touch-down cycles of 20 s denaturation at 94° C, 15 s annealing (70-62° C) and 20 s elongation at 72° C, followed by 24 or 15 cycles (20 s at 94° C, 15 s at 60° C, 20 s at 72° C) for TF/18S or PAI-1/18S respectively, and a final extension at 72° C for 2 min. PCR products were electrophoresed in 2.5% agarose gel and visualised by ethidium bromide. Intensity of the PCR product was determined by densitometric analysis with the Gel Doc 1000 UV fluorescent system and Molecular Analyst software for image quantification (BioRad, USA). Values corresponding to TF and PAI-1 amplification were normalized with those corresponding to 18S cDNA.

TF Levels Measurement

TF antigen content in cell lysates was quantified using a commercial sandwich enzyme immunoassay (Immubind®-TF, American Diagnostica, USA) following the manufacturer's specifications. Cells were counted by means of a Neubauer chamber, washed and lysed with a solution containing 0.1% Triton-X-100 in 0.5 mol/L Tris-HCl, pH 8.1, 10 mmol/L EDTA-Na_2, 1 mmol/L and 100 U/mL Trasylol® (Bayer, Germany). TF antigen content in each sample was expressed as pg/10^6 cells and then normalized to cell protein concentration, which was determined as described by Bradford (25).

PAI-1 Antigen Measurement

PAI-1 antigen level was measured in the media harvested at 0, 2, 6 and 24 h by an ELISA based on a specific monoclonal antibody able to detect PAI-1 in its active and latent forms, as well as t-PA/PAI-1 complexes (Asserachrom PAI-1, Diagnostica Stago, France).

Statistical Analysis

Results are expressed as the mean ± standard error of the mean (SEM) of three (FT) or six (PAI-1) independent experiments. For statistical purposes, the results of PAI-1 mRNA, TF levels in lysates and PAI-1 antigen in media at 2, 6 and 24 h were expressed as percentages of their baseline value, which is considered 100%. TF mRNA data were not expressed as percentages but as counts/mm^2 because baseline values were undetectable. The Kruskal-Wallis followed by Mann-Whitney U test was applied for purposes of group comparison.

Results

Effect of NOS Inhibition and NO Supplementation on TF Expression

As Fig. 1A shows, whereas TF gene expression at baseline condition was undetectable, challenging cultures with LPS resulted in an increase in mRNA levels, especially 2 h after stimulation (0.92 ± 0.3; $p < 0.05$ with respect to unstimulated cultures). Such an increase was enhanced when HUVEC were challenged simultaneously with LPS and the NOS inhibitor L-NAME (1.35 ± 0.1; $p = 0.08$ when compared to the LPS group). However, the NO donor SNAP attenuated the effect of LPS on TF gene expression (0.69 ± 0.1) so that a statistically significant difference was observed between mRNA levels of cultures challenged with LPS + L-NAME and LPS + SNAP ($P < 0.05$).

The changes in TF mRNA were followed by changes in protein level, as can be seen in Fig. 1B and Table 1. Fig. 1B shows that the TF antigen results from lysates followed the same trend as the mRNA data: very low levels could be detected in unstimulated cultures; TF antigen level increased shortly after the start of the LPS challenge. However, such an increase was about three-fold higher in LPS + L-NAME cultures with respect to LPS group at 2 h (1320 ± 95% vs. 440 ± 25% respectively, $p < 0.05$), while levels in LPS + SNAP cultures were about two-fold lower than LPS group (207 ± 3% vs. 440 ± 25% respectively, $p < 0.05$). TF antigen levels at 6 and 24 h in LPS + L-NAME and LPS + SNAP cultures were also significantly higher or

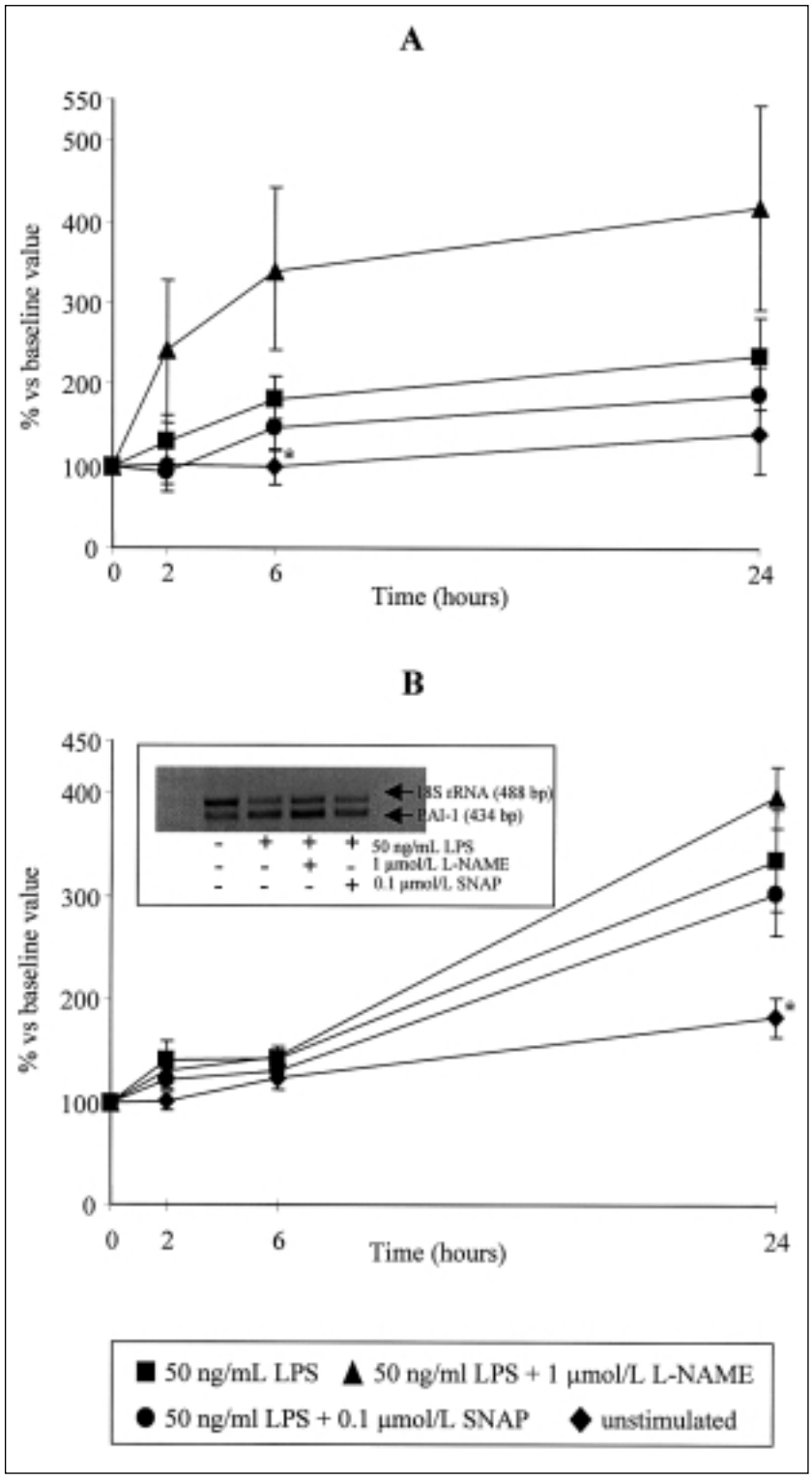

Fig. 2 PAI-1 mRNA expression (panel A) and PAI-1 protein levels (panel B) in HUVEC challenged with LPS, LPS + L-NAME and LPS + SNAP. Results are mean ± SEM (inset: PAI-1 cDNA electrophoresis at 6 h). * $P < 0.05$ with respect to the LPS group

lower respectively than those observed in LPS group ($p < 0.05$). Thus, these data support the view that NO modulation could play an anti-thrombotic role in endotoxemia by lowering the TF level.

Effect of NOS Inhibitors and NO Donors on PAI-1 Expression

LPS challenge resulted in a continuous increase in PAI-1 mRNA expression throughout the experiment (Fig. 2A). When L-NAME was applied together with LPS the mRNA expression was about two-fold higher than that observed in LPS group, although the differences were not statistically significant (130 ± 32% vs. 242 ± 87% at 2 h, 182 ± 28% vs. 338 ± 105% at 6 h, and 235 ± 46% vs. 418 ± 125% at 24 h in LPS group and in LPS + L-NAME group respectively). When given together with LPS, SNAP slightly attenuated the mRNA increase at 2 (92 ± 17%), 6 (147 ± 10%; P = 0.068 when compared to LPS + L-NAME group) and 24 h (187 ± 36%). PAI-1 antigen results are shown in Table 1 and Fig. 2B. As can be seen in Fig. 2B, PAI-1 antigen levels increased after the LPS challenge, specially at 24 h (335 ± 49%); such

an increase was slightly enhanced by L-NAME (397 ± 29%), while SNAP exerted no effect (303 ± 41%). Thus, an effect of NO on the PAI-1 expression subsequent to the LPS challenge is not supported by these results.

Discussion

LPS transforms the thromboresistant phenotype of vascular endothelium into a surface with prothrombotic properties, among other mechanisms, through the increased expression and synthesis of TF and PAI-1 (5, 6). Endothelium-derived NO levels are also modified by LPS: NO is produced at a low level by the eNOS of quiescent endothelial cells, but LPS importantly enhances its production by increasing the expression of iNOS (9). NO plays an antithrombotic role by preventing platelet aggregation (26). A down-regulating effect of NO on TF expression at the monocyte surface (17-19) and on PAI-1 expression in several tissues has also been described (27-31). The effect of modulating NO level on the expression and production of TF and PAI-1 at the endothelial level in the presence of LPS is less known. In the present study we have shown that the endothelial LPS-induced upregulation of TF might be regulated by NO.

First, our results suggest that NO is able to attenuate the endothelial LPS-induced TF increase: TF gene expression and protein were enhanced by L-NAME, an inhibitor of the NOS, specially at 2 h, a time at which the expression of TF has been described to be maximum (32-34), thus suggesting that NOS are involved in the TF modulation; moreover, a NO donor, SNAP, was able to revert the enhanced endothelial TF expression and production, thus supporting the notion that NO does play a role in down-modulating TF expression in the vascular endothelium. Thus, our findings support the concept that NO, in addition to its down-regulating effect on TF expression at the monocyte surface (17-19), also exerts a similar effect on TF expression at the endothelial level. This ability of NO would help to explain the remarkable increase in renal fibrin deposits that we had observed in LPS-challenged rats after treatment with NOS inhibitors (10).

Fibrinolysis is largely offset in sepsis due to the sustained LPS-induced increase in PAI-1 levels (7, 35). In spite of previous findings suggesting a modulating role of NO in attenuating PAI-1 overexpression in sepsis (10), our results are not conclusive enough to confirm such a role: although a trend towards an increase in the PAI-1 mRNA expression was observed when incubating HUVEC with LPS and L-NAME, such an expression was not significantly higher than the obtained with LPS only; furthermore, the PAI-1 mRNA expression with SNAP and LPS was similar to the observed with LPS only. Thus, it can be concluded that our results do not support a role for NO in the regulation of the endothelial PAI-1 expression in endotoxemia. Although other studies have described a down-regulating effect of NO on PAI-1 expression in other cell types, it must be noted that the experiments were performed in absence of LPS (27-31).

In summary, our data suggest that NOS inhibition would enhance the endothelial expression of TF in endotoxemia, while NO supplementation would attenuate the endothelial expression of TF. Therefore, although inhibition of NO has been suggested as a useful therapeutic tool in preventing the LPS-induced hypotension and subsequent shock, we provide evidence that NO inhibition could worsen the hemostatic abnormalities induced by sepsis.

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