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N^{ω}-NITRO-L-ARGININE METHYL ESTER EFFECTS ON NEUTROPHIL FUNCTION AND BACTERIAL CLEARANCE

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ABSTRACT—Nitric oxide synthase (NOS) inhibitors are considered promising as a therapeutic option in severe septic shock. The aim of this study was to investigate the effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME) application on neutrophil (PMN) respiratory burst, phagocytosis, and elimination of *Escherichia coli* from blood and tissue in rabbits. Twenty-eight female chinchilla rabbits were randomized to a treatment and control group. To quantify the bacterial clearance process, 10^8 colony forming units (CFU) of *E. coli* were injected intravenously into anesthetized rabbits. Animals in the L-NAME group had a significantly higher mortality compared with controls. NOS inhibition resulted in a significant delay of bacterial clearance ($P < 0.001$). These findings correlated with a significant augmentation of all organ *E. coli* findings ($P = 0.002$-0.035). PMN phagocytosis activity was notably reduced by L-NAME treatment during the experimental observation. Neutrophil burst, on the other hand, was amplified by NOS inhibition ($P = 0.008$). Our findings point to an interference with the PMN-dependent immune mechanisms after L-NAME treatment. The augmented PMN burst reaction could be a compensatory mechanism, potentially leading to tissue damage. Therefore, in this model, we find sufficient evidence pointing to a possible cause for the deleterious effect of early nonselective NOS inhibition in critically ill patients.

KEYWORDS—L-NAME, respiratory burst, phagocytosis, neutrophils, bacterial clearance, bacterial killing, *E. coli*

INTRODUCTION

Ever since nitric oxide (NO) has been considered to play an important role as a vasodilator in conductance and resistance vessels during sepsis, therapeutic synthesis inhibition or inactivation of NO has been attempted in various experimental and clinical studies (1–3). In septic conditions, overshooting enzyme activation causes systemic vasodilation through large amounts of NO. Consequently, blood pressure decreases due to volume maldistribution and decreased responsiveness to vasopressors, worsening the septic situation and, possibly, inducing multiorgan failure. Endotoxin, in particular, but also other proinflammatory stimuli induce excess NO production (3), contributing to a poor outcome in sepsis (4).

NO-induced hemodynamic effects (5), particularly a drop in systemic vascular resistance, can be inhibited by NO synthase (NOS) inhibitor (NOSI) therapy (6–8). Influences of NOSI on sepsis hemodynamics have been studied with mixed success since the early 1990s. Although NOSI therapy in septic shock had positive effects on systemic blood pressure (2, 3), negative inotropy (9), reduction of cardiac output (CO), and a consecutive drop of oxygen delivery (DO_2) (7, 8) were also noted (6).

Data concerning the *in vivo* effects of NOSI on host defense mechanisms are not available. Although NO as a signal molecule most probably has immune-regulatory properties, NO produced by macrophages, directly or through intermediate products, has a cytotoxic (10) and bacteriostatic function. NO and superoxide anions, produced during oxidative burst, cooperate to generate peroxynitrite and other highly toxic bactericidal products (11).

Therefore, NO, on one hand, takes on the function of a closely adjusted intercellular signal and regulator (1), and on the other hand, that of a potent effector molecule (11). Thus, this double function of NO makes it difficult to restore the disturbed regional vasoregulation during sepsis and the imbalance of bactericidal systems by systemic administration of NOSI at the same time (12).

In previous investigations, our group found a significant negative effect of vasoconstrictive agents such as endothelin-1 (13) and norepinephrine (14) on host defense. Therefore, in the present study, we investigated bacterial clearance mechanisms in an animal model of endotoxemia when NO synthesis is inhibited by N^{ω}-nitro-L-arginine methyl ester (L-NAME).

We hypothesized that L-NAME application on the one hand would result in positive hemodynamic effects. On the other hand, though, we hypothesized that due to interference with host defense, mortality would be higher in the L-NAME-treated group. Respiratory burst activity and granulocyte phagocytosis, as well as systemic *Escherichia coli* and endotoxin (LPS) elimination kinetics, and bacterial tissue distribution in the liver, spleen, kidney, and lung were studied.

MATERIALS AND METHODS

Animal model

The experiments were performed in agreement with the local government's commission for animal protection. Care and handling of the animals were in accordance with the National Institutes of Health guidelines.

Twenty-eight female chinchilla rabbits weighing between 2 and 3 kg were anesthetized with 50 mg/kg ketamine (Parke Davis, Freiburg, Germany) and 4 mg/kg xylazine (Bayer, Leverkusen, Germany) and were anticoagulated with heparin-sodium (1000 I.E./kg) injected into an ear vein catheter as described previously (15).

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Briefly, the animals were placed in a supine position on a temperature-controlled (35°C) operating table. After tracheotomy and intubation, the rabbits were mechanically ventilated with room air (tidal volume 30 mL, frequency 30/min) via respirator (Servo ventilator 900D; Siemens Elema, Solna, Sweden) during the entire observation period. A PVC catheter (ID, 1.4 mm) was inserted into the right carotid artery for measurements of the arterial blood pressure and for collection of blood samples. Anesthesia was maintained by a continuous infusion of ketamine (5-10 mg/kg/h) and xylazine (0.5-1.5 mg/kg/h). In addition to the basal fluid requirement of 3-4 mL/kg/h, blood loss from sampling was replaced by isovolemic injection of normal saline (resulting in replacement of 10 mL/kg/h).

Monitoring

After instrumentation of the rabbits, arterial and airway pressures were continuously monitored via Statham strain gauge transducers connected to a Sirecust 404A recorder (Siemens, Munich, Germany). CO was not monitored. To ensure adequate mechanical ventilation, blood samples were drawn intermittently for measurements of O_2 saturation, pH, partial oxygen pressure (PaO_2), and partial carbon dioxide pressure ($PaCO_2$; 288 Blood Gas System; Ciba-Corning, Fernwald, Germany). Furthermore, hemoglobin values and hematocrit, as well as leukocyte and differential blood counts, were obtained. The PMN burst and phagocytosis activity, and serum lactate and nitrite and nitrate (NO_x) concentrations were determined according to the experimental protocol.

L-NAME

L-NAME (8) was purchased from Sigma-Aldrich (Taufkirchen, Germany). For intravenous administration, L-NAME was diluted in sterile saline and buffered with $NaHCO_3$. The final concentration of L-NAME was 1 mg/mL (pH 7.5).

Bacterial inoculum

An encapsulated, serum-resistant, nonhemolytic strain of *E. coli* (O111) with a smooth LPS phenotype, freshly isolated from the blood of a patient with sepsis, was cultured on blood agar plates. After 10 h of incubation at 37°C, the colonies were harvested and homogenized by vortexing in tryptic soy broth adjusted to a density of 10^8 CFU/mL, and frozen in aliquots at −70°C until use. The amount of 10^8 CFU *E. coli* used in the current study was based on pilot experiments providing a reproducible clearance rate and organ distribution without inducing severe hemodynamic changes that might influence clearance function by tissue hypoperfusion.

Experimental protocol

After a 30-min period of stable hemodynamics, blood samples were collected for baseline measurements, and 28 animals were randomly assigned to a NOSI group, receiving a 5 mL/kg/h L-NAME infusion of 5 mg/kg/h or to a control group only receiving 5 mL/kg/h NaCl 0.9% for 180 min. L-NAME concentrations were chosen as previously described (6, 7). After an additional 30 min, a standardized amount of *E. coli* (10^8 CFU suspended in 1 mL of tryptic soy broth) was injected into the ear vein catheter of all animals.

For analysis of bacterial blood clearance, arterial blood was aseptically drawn at 1, 5, 15, 30, 45, 60, 90, 120, 150, and 180 min after bacterial injection. Blood gases, leukocyte counts, hematocrit, and hemoglobin concentrations were determined at 30-min intervals. PMN oxidative burst and phagocytosis activity were determined before, and at 5, 30, 90, and 180 min after *E. coli* injection. At the end of the experiment (after 240 min), the animals were killed with an overdose of ketamine and xylazine. Subsequently, tissue samples of liver, spleen, kidney, and lung were removed under aseptic conditions for bacterial cultures.

PMN burst activity

The extent of intracellular oxygen radical production was determined using samples of freshly drawn heparinized blood. The oxidative burst of leukocytes was determined by means of a test kit (Bursttest; Orpegen Pharma, Heidelberg, Germany). Single-cell analysis was performed by flow cytometry. The method of the quantitative assay for monitoring the oxidative burst has been described in detail in a previous study (16). In a brief outline, two assays (100 μL) of each sample were incubated (for 10 min at 37°C) with phosphate-buffered saline (PBS, negative control) or with opsonized *E. coli*, a potent stimulator for cellular production of reactive oxygen intermediates. After an oxidization step (for 10 min at 37°C) in which the nonfluorescent substrate, dihydrorhodamine 123, is taken up by the phagocytes and converted into a green fluorescent compound (rhodamine 123) through respiratory burst metabolites, the whole blood is lysed and fixed. This oxidation process is highly specific for the respiratory burst activity. To guarantee that no cell debris, dead cells, or bacteria interfere with the measurements, DNA staining was performed with propidium iodide (red fluorescence, FL-3). Fifteen thousand cells of each sample were measured with a laser flow cytometer (FACScan/ Lysis II; Becton Dickinson, Heidelberg, Germany) using blue-green excitation light (488 nm argon laser). The gain setting of the photomultiplier was adjusted to register all intensities of cellular fluorescence within the scale of 256 channel resolution (four decades). Neutrophils were identified by their characteristic size to granularity ratio. Only the selected cell population was considered for further analysis using WinMDI 2.0 (J. Trotter, Scripps Research Institute, La Jolla, CA). During data acquisition, a "live" gate was set in the red fluorescent dot plot on those events that had at least the same DNA content as the rabbit diploid cells. Only the mean intracellular rhodamine 123 fluorescence of these cells, representing PMN burst activity, was analyzed in FL-1.

PMN phagocytosis activity

A test kit (Phagotest; Orpegen Pharma) was used to evaluate the phagocytic activity of leukocytes in whole blood. Single-cell analysis was performed by flow cytometry. The quantitative method of assaying phagocytic activity has been previously described in detail (17). Two assays of each sample (2 × 100 μL) were incubated with fluorescein isothiocyanate (FITC)-marked opsonized *E. coli* for 10 min at 0°C (negative control) or at 37°C to allow phagocytosis of bacteria. To exclude extracellular bacteria from measurement, they were quenched with a staining solution. Further steps such as lysis, fixation, DNA staining, and data acquisition by flow cytometer were performed in the same manner as in the burst assay. The phagocytosis activity of leukocytes was determined by the content of FITC-marked *E. coli* in the phagocytic cells, expressed as mean channel fluorescence (FL-1) per cell.

Quantitative microbiology

Blood and tissue samples were cooled and assayed in duplicate at the end of the experiment. After incubation of the cultures at 37°C for 24 h, CFU of *E. coli* were counted. The final bacterial concentration was calculated as the numbers of colonies per milliliter of blood, or as colonies per gram of tissue, respectively.

Blood cultures

Blood samples were serially diluted in sterile saline. One hundred microliters each of whole blood and of blood in the various states of dilution were plated onto cysteine lactose electrolyte-deficient agar plates according to Sandys (18).

Organ cultures

Aseptically collected organs, liver, spleen, lung, and kidney were weighed, and 0.8-2 g of each organ was homogenized (Ultra-Turrax; IKA, Staufen, Germany) in 3 mL of sterile saline. Serial dilutions of tissue suspension (100 μL) were plated onto cysteine lactose electrolyte-deficient agar plates.

Detection of endotoxin

To take into account plasma-related factors that interfere with the *Limulus* amebocyte lysate-endotoxin reaction and considering the fact that each plasma sample when spiked with endotoxin follows a different slope in the standard curve, an automated, kinetic, turbidimetric *Limulus* amebocyte lysate microtiter test with individual internal standardization was used as described previously (16). The sensitivity of this assay is 0.1 pg/mL.

Measurement of nitrite and nitrate (NO_x)

A commercially available colorimetric test kit was used for the determination of total NO_x concentration in a two-step process (Cayman Chemicals, Ann Arbor, MI). In the first step, nitrate was converted into nitrite using nitrate reductase. As a second step, Greiss reagent (19) was added, which converts nitrite into a deep purple azo compound. A photometric measurement (AR 2001; Anthos, Krefeld, Germany) of the light absorption at 540 nm due to this azo chromophore determines the NO_2 concentration. Azide, ascorbic acid, dithiothreitol, and mercaptoethanol interfere with the color development in concentrations of 100 μM and phosphate >50 mM interferes with the conversion of nitrite to nitrate.

Lactate measurement

Lactate concentration was measured enzymatically by means of an automatic amperometric system (LACT MPR1; Boehringer, Mannheim, Germany) according to the manufacturer's instructions (Analyser ESAT 6661 Lactat; Eppendorf, Hamburg, Germany) in the Institute for Clinical Chemistry (University Hospital, Mannheim, Germany).

Statistical analysis

Data are presented as arithmetic means ± SD. The logarithm of bacterial counts was used for statistical comparison. Single measurements between the groups were compared with unpaired Student two-sided *t* test. Between-group differences after repeated measurements were tested by general linear model (GLM) statistics, according to a two-way analysis of variance (ANOVA). Survival was calculated with Kaplan-Meier procedure and log-rank test. Significance was accepted at $P < 0.05$. Statistical analysis was performed with SPSS software for MS Windows (Release 10.0.7; SPSS Inc., Chicago, IL).

RESULTS

Baseline measurements of mean arterial blood pressure were 53 ± 13 mmHg (control) versus 53 ± 11 mmHg (L-NAME) and did not differ among the groups. Continuous infusion of 5 mg/kg/h L-NAME significantly increased systemic blood pressure (Fig. 1) compared with controls ($P = 0.013$). This effect was most pronounced after onset of L-NAME infusion. However, blood pressure progressively decreased in both groups. The observation of increased blood pressure in the L-NAME group was paralleled with significantly reduced levels of the stable products of NO_x (Fig. 2, baseline 35.9 ± 10.7 μmol/L). Nevertheless, the cumulative mortality in the L-NAME group was 40% at the end of the 4-h observation period, whereas all control animals survived (Fig. 3). The difference between groups reached statistical significance ($P = 0.015$).

Bacterial clearance was significantly delayed during the entire experiment in the NOSI group (Fig. 4A) showing higher *E. coli* blood concentrations ($P < 0.001$). These findings correlated with a significant augmentation of all organ *E. coli* findings (Fig. 4B, $P = 0.002$-0.035).

PMN phagocytosis activity was notably reduced by L-NAME treatment during the experimental observation (Fig. 5A). However, this difference was only suggestive of statistical significance at $P = 0.062$. Neutrophil burst, on the other hand, was amplified by NOS inhibition (Fig. 5B, $P = 0.008$).

Before bacterial inoculation, serum endotoxin levels were below the detection limit. After *E. coli* injection in controls, serum LPS levels of 745 ± 633 ng/mL were detected (difference from baseline in percentage, see Fig. 5C).

In the course of the experiment, rising LPS values were noted in the L-NAME group. This difference was not evident after 180 min, and it never reached statistical significance.

Further parameters, such as lactate, blood gases, pH, bicarbonate, and SaO_2, showed no statistically significant between-group differences (Table 1).

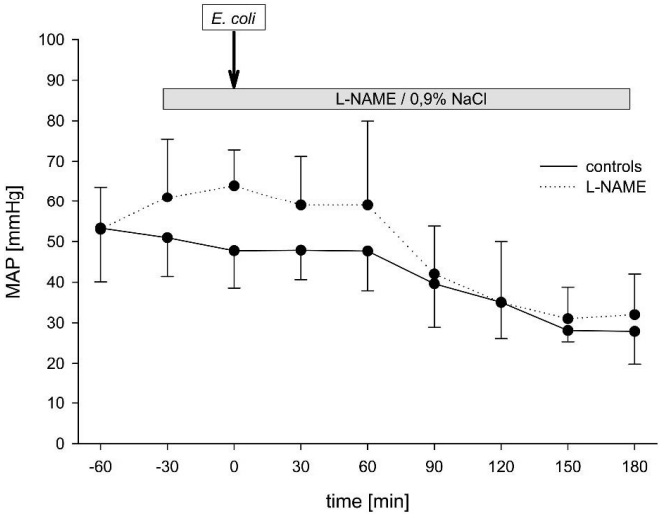

FIG. 1. **Mean arterial blood pressure.** Statistical significance of L-NAME versus controls. $P = 0.013$ (GLM).

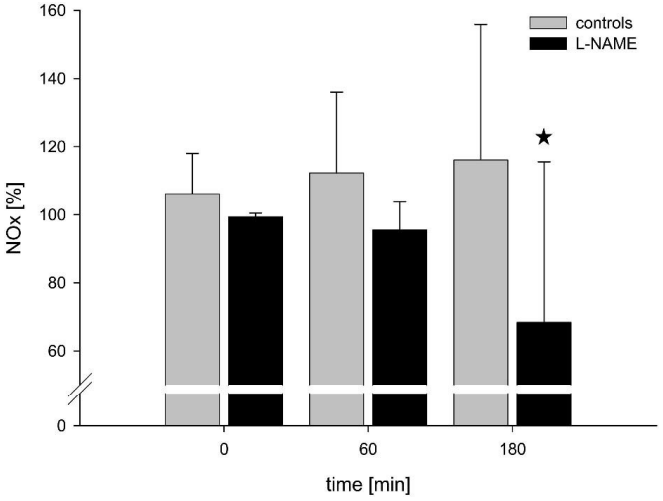

FIG. 2. **Nitrite and nitrate levels (NO_x ± SD) in percentage of baseline measurements before L-NAME application.** *$P < 0.05$ (*t* test with T_0 as a covariate).

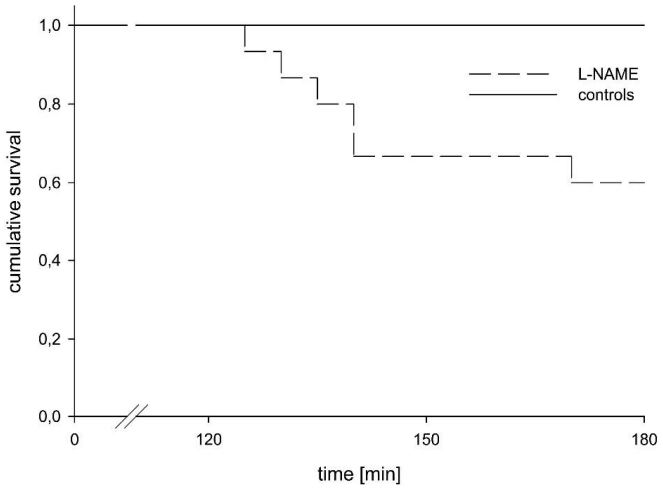

FIG. 3. **Survival analysis in the L-NAME group (5 mg/kg/h) after injection of 10^8 CFU *E. coli*.** $P = 0.015$ (Kaplan-Meier, log-rank test).

DISCUSSION

The clinical use of NOSI as a therapeutic option in septic shock is at present being discussed controversially. Blood pressure-augmenting effects are accompanied by a reduction in CO and DO_2 (2, 3), possibly due to a relative decrease in cardiac preload (20), resulting in an impaired tissue perfusion (21). In animal models, impaired liver perfusion (22–24), increased thrombocyte aggregation (25), and leukocyte adhesion (26) as well as increased mortality (7) were observed with NOSI. After a phase II study with 312 patients showed better recovery from septic shock within 72 h (27), a phase III study with 797 patients had to be discontinued due to higher mortality rates in the NOSI group (28). Although titrated NOSI therapy may show beneficial effects in sepsis hemodynamics (3), *in vivo* effects on the cellular immune system are still unclear. Only recently were the final results of the multiple-center study on NOS inhibition with 546C88 published (29–31). The exact reason for the increased mortality in the treatment group still remains unclear. Bakker et al. (29) report that ". . . no apparent

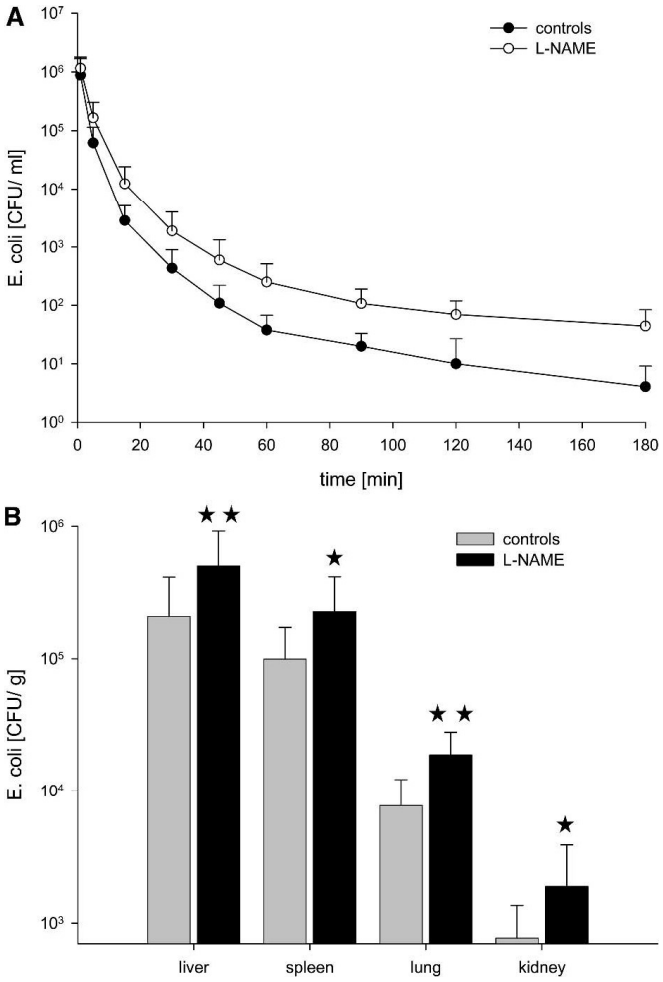

FIG. 4. **(A) *E. coli* blood clearance (mean ± SD) of controls and animals after L-NAME application (5 mg/kg/h) after injection of 10^8 CFU *E. coli*.** $P_{total} < 0.001$ (GLM). (B) *E. coli* organ culture findings (mean ± SD) 3 h after injection of 10^8 CFU *E. coli* in controls and after L-NAME application (5 mg/kg/h). $^{**}P < 0.01$, $^{*}P < 0.05$ (*t* test).

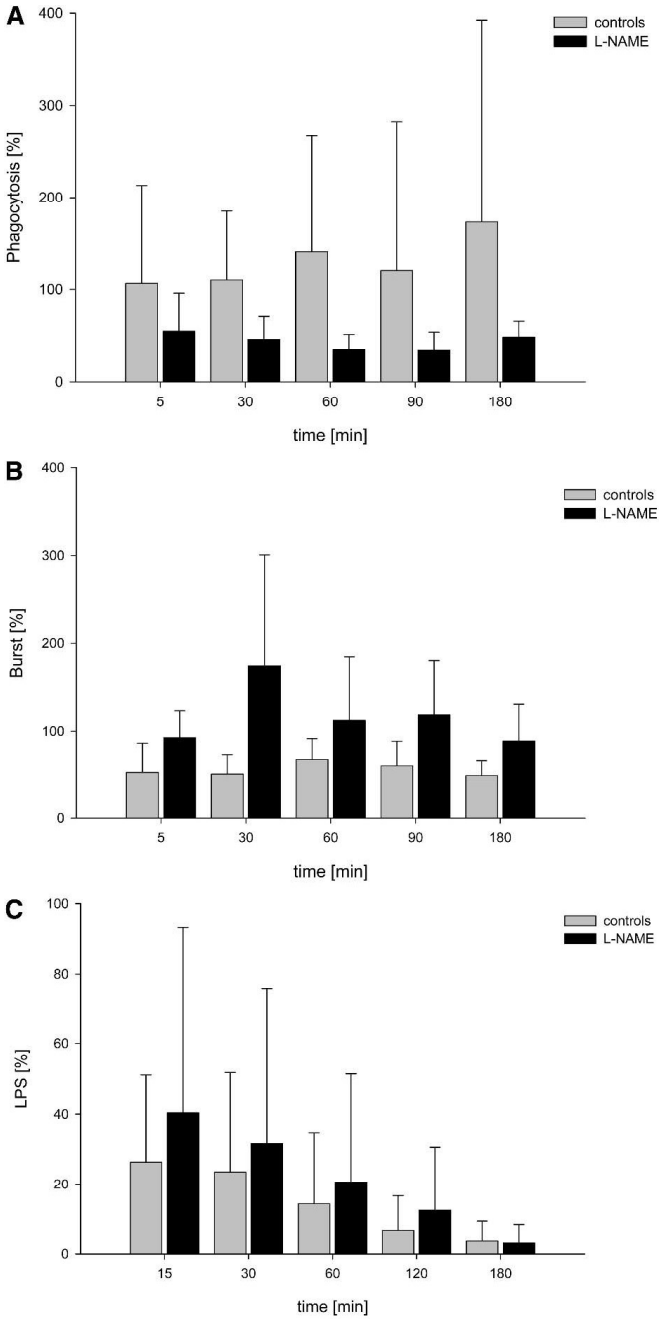

FIG. 5. **(A) PMN phagocytosis activity (mean ± SD) of FITC-marked *E. coli* in percentage of baseline measurements (–60 min) in controls and after L-NAME application (5 mg/kg/h).** $P_{total} = 0.062$ (GLM). (B) Right, Neutrophil burst activity (mean ± SD) in percentage of baseline measurements (–60 min) in controls and after L-NAME application (5 mg/kg/h). $P_{total} = 0.008$ (GLM). (C) Endotoxin (LPS) blood clearance in percentage (±SD) of measurement 1 min after injection of 10^8 CFU *E. coli* in controls and after L-NAME application.

adverse effects on non-cardiovascular organ function were found." Our study explicitly looks into the immunological aspect of systemic NOS inhibition and does find significant evidence for severe undesirable side effects.

After injury and surgical trauma, transient reticuloendothelial depression has been observed and attributed to have an essential role in the development of septic events (32). For more than 20 years, it has been suggested that a dysfunction of systemic bacterial clearance due to immunosuppression is a major contributor to sepsis and organ failure (33).

The exact role NO plays within this system of host defense has mainly been regarded from the point of view of experiments studying NO application effects: *in vitro* (34) and *in vivo* (35) studies point to a possible impairment of neutrophil function.

To improve the understanding of the role of NO and NOSI therapy on the complex pathophysiological process of reticuloendothelial and circulating phagocyte immune function, our interest was focused on a possible impairment of bacterial clearance. The injection of *E. coli* was chosen as a correlate of bacterial invasion from various compartments, e.g., the gut, urogenital tract, wounds, or catheters. The exact role of inducible NOS was not evaluated in this study. Radomski et al. (36) did not detect iNOS mRNA expression until 2 h after shock induction, reaching peak values between 6 and 12 h. Nevertheless, increased endothelial NO release has been shown immediately after endotoxin administration (37).

In the present animal model, the L-NAME concentration proved to have a significant effect on extracellular NO_X

TABLE 1. **Blood gas, acid-base, and lactate data (±SD values in parentheses) for L-NAME-treated animals (top line) and controls (bottom line, bold font). All values showed no between-group statistical significance.**

	0 min	30 min	60 min	90 min	120 min	180 min
pH	7,62 (0,07)	7,53 (0,08)	7,39 (0,11)	7,33 (0,11)	7,33 (0,10)	7,31 (0,10)
	7,60 (0,07)	**7,51 (0,07)**	**7,44 (0.07)**	**7,37 (0,07)**	**7,31 (0,07)**	**7,27 (0,08)**
pCO_2 (mmHg)	26,5 (4,0)	27,7 (4,7)	29,8 (6,4)	27,9 (6,1)	23,3 (9,3)	24,4 (7,6)
	25,8 (5,7)	**27,2 (4,7)**	**24,6 (3,9)**	**24,1 (3,7)**	**25,4 (8,8)**	**24,9 (5,3)**
pO_2 (mmHg)	168,7 (23,9)	174,7 (31,9)	173,9 (26,8)	179,3 (26,7)	181,8 (19,4)	188,5 (34,0)
	169,4 (12,5)	**178,1 (18,3)**	**175,9 (19,2)**	**180,2 (17,7)**	**173,7 (33,0)**	**176,3 (21,1)**
HCO_3 (mmol/L)	31,7 (4,6)	26,9 (4,4)	19,9 (3,3)	16,9 3,5)	14,9 (3,4)	14,8 (3,0)
	30,1 (5,2)	**25,0 (4,9)**	**20,1 (3,7)**	**17,2 (3,3)**	**15,1 (2,8)**	**13,8 (3,3)**
BE (mmol/L)	5,7 (4,8)	1,1 (4,7)	–6,2 (3,5)	–9,6 (4,2)	–12,3 (4,5)	–12,5 (3,9)
	4,3 (6,1)	**–0,4 (6,2)**	**–6,4 (4,4)**	**–9,6 (4,0)**	**–12,0 (3,7)**	**–14,0 (4,5)**
Hb (g/dL)	10,6 (1,5)	9,8 (1,6)	9,6 (1,7)	8,5 (1,7)	7,8 (1,8)	8,6 (1,2)
	11,1 (1,8)	**10,4 (1,6)**	**10,4 (1,8)**	**9,7 (1,5)**	**9,2 (1,4)**	**9,2 (1,5)**
O_2SAT (%)	99,4 (0,3)	99,2 (0,7)	99,1 (0,4)	99,1 (0,4)	99,1 (0,3)	99,2 (0,2)
	99,4 (0,1)	**99,3 (0,1)**	**99,2 (0,2)**	**99,2 (0,2)**	**98,7 (1,4)**	**99,0 (0,2)**
Hc (%)	31,1 (4,4)	28,7 (4,6)	28,2 (5,1)	25,1 (4,9)	22,8 (5,3)	25,3 (3,6)
	32,7 (5,4)	**30,5 (4,8)**	**30,6 (5,4)**	**28,5 (4,3)**	**27,0 (4,1)**	**27,2 (4,4)**
Lactate (mmol/L)	2,4 (0,9)	3,8 (2,3)	—	—	—	7,7 (3,7)
	1,9 (1,2)	**2,5 (1,0)**	—	—	—	**6,5 (3,5)**

production. A significant delay in *E. coli* blood clearance and an augmented organ colonialization in combination with a tendentially reduced PMN phagocytosis portend an interference with PMN-dependent immune mechanisms in the L-NAME treatment group. The observed significant augmentation of respiratory burst activity may at first seem to be contradictory. However, phagocytosis and respiratory burst are two different mechanisms of host defense. They may be affected independently because phagocytosis is predominantly based on cytoskeletal factors (38) and respiratory burst is more reliant on oxidases (39). Chen et al. (40) found an increase of superoxide production in isolated human PMN after N-G-monomethyl-L-arginine application (40). Therefore, an inhibition by L-NAME within these systems could lead to a compensatory upregulation of respiratory burst activity. Because the slowest step in the series of adhesion, phagocytosis, and intracellular bacterial killing by phagocyte burst sets the pace of bacterial clearance, a reduction in phagocytosis–even with augmented PMN burst–in consequence leads to a limited overall bacterial immune reaction. Supra-effective activation of neutrophil burst, on the other hand, induces host tissue damage (41), but overflow limitation of neutrophil activation, e.g., by substitutional doses of corticoids, vice versa, preserves organ function and enables an adequate immune response (19). In addition, NO is known to be an oxygen free radical scavenger (41) and inhibitor of oxygen free radical production (34). NOSI may enhance oxidative damage due to missing NO effects. As a probable consequence of enhanced organ damage, limited bacterial clearance, and hemodynamic disturbance, the mortality in the L-NAME group was significantly higher than in the control group.

In summary, we find strong evidence showing that L-NAME impairs bacterial clearance from the blood and from tissue of animals injected with *E. coli*. Thus, our results indicate a possible cause for the deleterious effect of early nonselective NOSI in critically ill patients.

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