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Effects of Early Neuronal and Delayed Inducible Nitric Oxide Synthase Blockade on Cardiovascular, Renal, and Hepatic Function in Ovine Sepsis

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ABSTRACT

Background: Recent evidence suggests that nitric oxide produced *via* the neuronal nitric oxide synthase is involved mainly in the early response to sepsis, whereas nitric oxide derived from the inducible nitric oxide synthase is responsible during the later phase. We hypothesized that early neuronal and delayed inducible nitric oxide synthase blockade attenuates multiple organ dysfunctions during sepsis.

Methods: Sheep were randomly allocated to sham-injured, nontreated animals (n = 6); injured (48 breaths of cotton smoke and instillation of *Pseudomonas aeruginosa* into the lungs), nontreated animals (n = 7); and injured animals treated with a neuronal nitric oxide synthase inhibitor from 1 to 12 h and an inducible nitric oxide synthase inhibitor from 12 to 24 h postinjury (n = 6).

Results: The injury induced arterial hypotension, vascular leakage, myocardial depression, and signs of renal and hepatic dysfunctions. The treatment significantly attenuated, but did not fully prevent, the decreases in mean arterial pressure and left ventricular stroke work index. Although the elevation of creatinine levels was partially prevented, the decreases in urine output and creatinine clearance were not affected. The injury-related increases in bilirubin levels, international normalized ratio, and lipid peroxidation in liver tissue were significantly attenuated. Although plasma nitrite/nitrate levels were significantly increased *versus* baseline from 12–24 h in controls, plasma nitrite/nitrate levels were not increased in treated animals.

Conclusions: The combination treatment shows potential benefit on sepsis-related arterial hypotension and surrogate parameters of organ dysfunctions in sheep. It may be crucial to identify the time course of expression and activation of different nitric oxide synthase isoforms in future investigations.

What We Already Know about This Topic

- Excessive production of nitric oxide by nitric oxide synthase (NOS) may play a critical role in the pathogenesis of vasodilatory shock. Nitric oxide production may come primarily from neuronal NOS (nNOS) in the early response to bacterial challenge and from inducible NOS (iNOS) in the later phase.

What This Article Tells Us That Is New

- In an ovine sepsis model, early nNOS blockade and subsequent iNOS blockade show potential benefit with regard to systemic arterial hypotension and surrogate parameters of organ dysfunction.

SEVERE sepsis and septic shock are leading causes of morbidity and mortality among critically ill patients.[1] Of the many aspects in the pathophysiology of sepsis, development of progressive cardiovascular failure caused by excessive

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vasodilation and vascular hyporesponsiveness to catecholamines represents a major clinical problem.[2] The hemodynamic changes during sepsis are further characterized by a maldistribution of systemic and microvascular blood flow, resulting in an impairment of organ blood flow and multiple organ dysfunctions.[3]

Excessive production of nitric oxide by nitric oxide synthase (NOS) is believed to play a critical role in the pathogenesis of vasodilatory shock.[2] Previous investigations demonstrated that excessive nitric oxide may further exert cytotoxic effects by reacting with superoxide radicals from activated neutrophils, thereby yielding reactive oxygen species and possibly contributing to multiple organ damage.[4,5] In this regard, pharmacological inhibition of NOS in different sepsis models revealed beneficial effects of this approach on various outcome variables.[6–8] However, the clinical relevance of these findings remains questionable, especially since a phase III trial demonstrated an increase in mortality among patients with septic shock who were treated with a nonselective NOS inhibitor.[9] Notably, recent evidence suggests that nitric oxide production *via* the neuronal NOS is mainly involved in the early response to bacterial challenge, whereas nitric oxide derived from the inducible NOS is responsible during the later phase of the disease process.[6–8] Thus, selective inhibition of different NOS isoforms at different time points may be superior to nonselective NOS blockade. In this regard, a previous study reported potential benefit of this approach on pulmonary morbidity following acute lung injury and sepsis.[10]

In the present study, we tested the hypothesis that early inhibition of neuronal NOS and subsequent inhibition of inducible NOS attenuates cardiovascular, hepatic, and renal dysfunctions in an established ovine model of sepsis.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston, Texas, and conducted in compliance with the guidelines of the National Institutes of Health and the American Physiologic Society for the care and use of laboratory animals.

Animal Model

The ovine sepsis model of smoke inhalation injury and instillation of live *Pseudomonas aeruginosa* into the lungs has been previously described in detail.[11,12] In brief, 19 adult female sheep (body weight, 31 ± 9 kg) were surgically prepared under general anesthesia for chronic study with a femoral artery catheter, a pulmonary artery catheter, and a left atrial catheter. After a recovery period of 5–7 days, the animals received general anesthesia, and inhalation injury with 48 breaths of cotton smoke (less than 40°C) was performed using a modified bee smoker. Afterward, a stock solution of live *P. aeruginosa* ($2–5 \times 10^{11}$ colony-forming units, from a burn patient at Brooke Army Medical Center, San Antonio, Texas) suspended in 30 ml of 0.9% NaCl solution was instilled into the right middle and lower lobes and left lower lobe of the lung (10 ml each). The animals assigned to the sham-injured group were subjected to an identical procedure, but 48 breaths of room air instead of smoke were applied, and 30 ml of saline instead of bacterial suspension were instilled. Anesthesia was then discontinued, and the sheep were allowed to awaken.

Experimental Protocol

The sepsis experiments were performed pairwise to correct for possible variations in the bacterial suspension. After injury, the animals were randomized by flipping a coin. The following three groups were studied: (1) sham-injured, nontreated animals (sham, n = 6), (2) injured, nontreated animals (control, n = 7), 3) injured animals treated with the specific neuronal NOS inhibitor 7-nitroindazole (7-NI; 1 $mg \cdot kg^{-1} \cdot h^{-1}$; Sigma–Aldrich, St. Louis, MO)[6] from 1 to 12 h postinjury and with the specific inducible NOS inhibitor BBS-2 (100 $\mu g \cdot kg^{-1} \cdot h^{-1}$; Berlex, Richmond, CA)[7] from 12 to 24 h postinjury (treatment, n = 6). The animals of the sham and control groups received only the vehicle. The investigators were blinded to the group assignment. The selectivity of BBS-2 for the inducible NOS is 620- and 1,500-fold higher *versus* neuronal and endothelial NOS, respectively.[13] The chosen dosage of 7-NI has been established in previous studies using the same and a similar animal model.[6,14,15] It has previously been demonstrated that infusion of 7-NI in sheep at a dose of 1 $mg \cdot kg^{-1} \cdot h^{-1}$ effectively suppresses plasma nitrate/nitrite levels[6,14,15] and establishes plasma concentrations of 7-NI[15] far below the IC_{50} reported for endothelial and inducible NOS.[16] All sheep were mechanically ventilated (Servo Ventilator 900C; Siemens, Elema, Sweden) with a tidal volume of 12–15 ml/kg and a positive-end expiratory pressure of 5 cm H_2O during the entire study period of 24 h. The fraction of inspired oxygen was set at 1.0 for the first 3 h postinjury and was then adjusted to maintain sufficient oxygenation (arterial oxygen saturation more than 90%, partial arterial oxygen pressure 80–100 mmHg) whenever possible. The respiratory rate was initially set at 20 breaths/min and then adjusted according to blood gas analyses to maintain the partial arterial carbon dioxide pressure within 5 mmHg of the baseline value. All animals were fluid resuscitated, initially started with an infusion rate of 2 $ml \cdot kg^{-1} \cdot h^{-1}$ lactated Ringer's solution and adjusted to maintain hematocrit at baseline values ± 3. During the study period, all animals had free access to food, but not water. After completion of the experiments, the animals were deeply anesthetized with ketamine and xylazine and received a lethal intravenous dose of saturated potassium chloride.

Hemodynamic Measurements

Mean arterial pressure (MAP), left atrial pressure, central venous pressure, and heart rate were measured using pressure transducers (model PX3X3; Baxter Edwards Critical Care

Division, Irvine, CA) which were connected to a hemodynamic monitor (model 7830A; Hewlett Packard, Santa Clara, CA). Cardiac output was determined with the thermodilution method. Left and right ventricular stroke work index (LVSWI and RVSWI), stroke volume index, systemic vascular resistance index, and cardiac index were calculated using standard equations. Systemic perfusion pressure was calculated as MAP minus central venous pressure.

Blood Analysis

Arterial blood was withdrawn from the femoral artery catheter and cultured to test for bacteremia (Septi-check; Becton Dickinson, Sparks, MD). Blood gases were determined using a blood gas analyzer (Synthesis 15; Instrumentation Laboratories, Lexington, MA). Plasma protein concentrations were measured using a refractometer (National Instrument, Baltimore, MD). Plasma oncotic pressures were determined through a semipermeable membrane in a colloid osmometer (Model 4420; Wescor, Logan, UT). Blood cell count was performed with a Hemavet 850 (Drew Scientific, Oxford, CT) adjusted for sheep blood. In addition, blood was centrifuged and plasma and serum samples frozen at −80°C for the determination of serum aspartate aminotransferase, alanine aminotransferases, γ-glutamyl transpeptidase, lipase, bilirubin, creatinine, and creatinine kinase (Vitros 5,1 FS; Ortho Clinical Diagnostics, Rochester, NY) as well as osmolality (Advanced Model 3300 Micro-Osmometer; Advanced Instruments, Norwood, MA). Creatinine clearance (ml/min) was estimated by the formula: urine creatinine concentration (mg/dl) × urine volume (ml)/serum creatinine concentration (mg/dl) × collection time (min). Nitric oxide levels were evaluated by measuring the intermediate and end products using a Cayman nitrate/nitrite colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI).

Tissue Analysis

After completion of the 24-h experiments, representative transmural tissue samples were obtained from the heart and liver. Tissues were immediately shock frozen in liquid nitrogen for the measurement of tissue malondialdehyde. Liver and heart tissue malondialdehyde formation, an index of lipid peroxidation, was quantified with a commercially available assay (Northwest Life Science Specialties, Vancouver, WA) and expressed as malondialdehyde per mg protein, measured by a commercially available assay (Fluka BioChemika, Buchs, Switzerland).

Statistical Analysis

σ Stat 3.1 software (Systat Software, Inc., San Jose, CA) was used for statistical analyses. All values are expressed as means ± SEM. To ensure reasonable normality, we tested whether the means and standard deviations per group and time were uncorrelated for each variable analyzed, and log transformation was used to stabilize the standard deviations when required. A two-way analysis of variance (ANOVA) for repeated measurements with appropriate Student-Newman-Keuls *post hoc* comparisons was used to compare differences within and between groups. One-way ANOVA was used to compare all groups when measurements were made at only one time period, and the Newman-Keuls procedure was used for *post hoc* pairwise comparisons to adjust for multiplicity. A value of $P < 0.05$ was regarded as statistically significant.

Results

The peak carboxyhemoglobin concentrations were not significantly different between the control and treatment groups (75 ± 6 *vs.* 65 ± 5%; $P > 0.05$), suggesting that both groups received similar amounts of smoke. Six (86%) of the animals of the control group and four (67%) of the animals of the treatment group had positive blood cultures, indicating bacteremia. The injury was further associated with a significant decrease in leukocyte count and a transitory increase in body core temperature (table 1).

Cardiovascular Function

The injury induced significant decreases in MAP and systemic vascular resistance index with concomitant significant increases in heart rate and cardiac index (table 2). The fall in MAP was significantly attenuated by the treatment (fig. 1A). The relationship between LVSWI and left atrial pressure, as an index of myocardial contractility, is illustrated in figure 1B. After 24 h, this index showed a less-emphasized downshift and rightward shift in the treatment than in the control group, suggesting improved cardiac performance by combined neuronal and inducible NOS inhibition. There were no significant differences in stroke volume index and RVSWI among groups (data not shown). However, the injury-related decrease in LVSWI was partially inhibited by the treatment (table 2).

Renal Function and Vascular Leakage

Injury-related kidney dysfunction was indicated by increased serum creatinine levels as well as decreased urine output and creatinine clearance compared with sham animals. Although serum creatinine levels were partially attenuated in the treatment group, the decreases in urine output and creatinine clearance were not affected (table 3). Injured sheep required more fluid resuscitation than animals of the sham group, resulting in significantly increased total fluid balance (fig. 2). Despite the positive fluid balance, both hematocrit and hemoglobin tended to be higher in injured sheep (table 3). In addition, the injury was associated with progressive declines in plasma protein concentrations and colloid oncotic pressures which were both partially attenuated in the treatment group (fig. 3).

Hepatic Function

The injury was associated with signs of liver dysfunction as indicated by significant increases in serum bilirubin concentrations and international normalized ratio in the control group. These changes were significantly attenuated in the treatment group (table 3). Serum aspartate aminotransferase similarly in-

Table 1. Changes in Body Core Temperature, White Blood Cell Count, and Metabolic Status

	Time after Injury					
	0 h	3 h	6 h	12 h	18 h	24 h
BCT, °C						
Sham	39.3 ± 0.1	39.9 ± 0.1	39.6 ± 0.1	39.6 ± 0.1	39.8 ± 0.1	40.1 ± 0.1
Control	39.4 ± 0.1	40.8 ± 0.2*	40.9 ± 0.2*†	40.2 ± 0.4	39.5 ± 0.4	39.0 ± 0.5
Treatment	39.3 ± 0.1	40.8 ± 0.1*	41.2 ± 0.2*†	40.8 ± 0.2*	40.0 ± 0.6*	39.5 ± 0.8
WBC, $K \cdot \mu l^{-1}$						
Sham	4.8 ± 0.5			6.6 ± 0.6		7.0 ± 0.4
Control	6.6 ± 1.1			1.6 ± 0.3*†		1.4 ± 0.2*†
Treatment	6.0 ± 0.4			2.3 ± 0.6*†		2.7 ± 0.8*†
pH, $-\log_{10}[H^+]$						
Sham	7.51 ± 0.01	7.56 ± 0.02	7.56 ± 0.03	7.49 ± 0.02	7.49 ± 0.01	7.48 ± 0.02
Control	7.49 ± 0.01	7.59 ± 0.04	7.54 ± 0.03	7.46 ± 0.02	7.37 ± 0.03*†	7.32 ± 0.07*†
Treatment	7.49 ± 0.01	7.56 ± 0.01	7.54 ± 0.05	7.51 ± 0.02	7.50 ± 0.03‡	7.50 ± 0.01‡
Lactate, mmol/l						
Sham	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
Control	0.6 ± 0.1	2.0 ± 0.2	2.4 ± 0.3	3.3 ± 0.7	5.8 ± 1.3*†	8.2 ± 1.7*†
Treatment	0.5 ± 0.1	2.7 ± 1.3	2.6 ± 0.8	3.1 ± 0.6	3.0 ± 0.9	2.8 ± 0.8‡
Svo_2, %						
Sham	66 ± 4	77 ± 2	73 ± 3	72 ± 3	73 ± 3	73 ± 3
Control	61 ± 2	72 ± 3*	73 ± 4	61 ± 4	60 ± 4	54 ± 6†
Treatment	61 ± 2	77 ± 2*	72 ± 2	72 ± 4	70 ± 3	69 ± 5
Sao_2, %						
Sham	94 ± 0	97 ± 0	96 ± 0	95 ± 0	96 ± 1	96 ± 0
Control	94 ± 0	95 ± 1	94 ± 1	83 ± 4*†	84 ± 5*†	75 ± 6*†
Treatment	93 ± 0	96 ± 0	94 ± 0	91 ± 2‡	92 ± 1‡	90 ± 3‡
O_2-ER, %						
Sham	30 ± 4	28 ± 2	29 ± 2	29 ± 2	29 ± 1	28 ± 1
Control	35 ± 2	27 ± 2	24 ± 4	27 ± 2	29 ± 3	29 ± 4
Treatment	35 ± 2	24 ± 2	23 ± 2	21 ± 4	24 ± 3	25 ± 4
Do_2I, $ml \cdot min^{-1} \cdot m^{-2}$						
Sham	705 ± 48	805 ± 44	660 ± 47	621 ± 49	541 ± 21	591 ± 21
Control	750 ± 40	824 ± 76	981 ± 73†	801 ± 81†	955 ± 95†	824 ± 64†
Treatment	654 ± 79	873 ± 61*	921 ± 77*†	946 ± 103*†	1,053 ± 119*†	885 ± 113*†

Data are expressed as mean ± SEM.

* $P < 0.05$ *vs.* 0 h within group; † $P < 0.05$ *vs.* sham; ‡ $P < 0.05$ *vs.* control.

BCT = body core temperature; Do_2I = oxygen delivery index; O_2-ER = oxygen extraction rate; pH = potentia hydrogenii; Sao_2 = arterial oxygen saturation; Svo_2 = mixed-venous oxygen saturation; WBC = white blood cell count.

creased in both injured groups as compared with the sham group. There were no significant differences in alanine aminotransferases, lipase, or γ-glutamyl transpeptidase between groups (data not shown).

Metabolic Changes

The injury was related to severe signs of metabolic disturbance as evidenced by a significant decrease in pH and a concomitant increase in lactate levels in the control group. These alterations were attenuated in the treatment group (table 1).

Nitric Oxide and Oxidative Stress

Plasma nitrite/nitrate levels gradually increased in the control group and were significantly higher than in the sham group from 12 to 24 h. Plasma nitrite/nitrate levels were not significantly increased in the treatment group (fig. 4). Malondialdehyde contents in liver tissue were significantly increased by the injury. In treated animals, the increase in liver malondialdehyde was significantly attenuated (fig. 5). Malondialdehyde contents in heart tissue were equally increased in both injured groups (data not shown).

Discussion

The present study demonstrates that a combination therapy consisting of early, selective inhibition of neuronal NOS and subsequent specific blockade of inducible NOS effectively suppressed plasma nitrite/nitrate levels in sheep with acute lung injury and sepsis. The combination treatment further attenuated, but did not fully prevent, the sepsis-related decrease in MAP and deteriorations of surrogate parameters of hepatic function in this sepsis model.

The employed animal model of pulmonary sepsis induced by smoke inhalation following instillation of *P. aeruginosa* bacteria into the lungs mimics a common clinical setting in burn patients with smoke inhalation injury.[17] In the current investigation, the double hit injury was associated with bacteremia and signs of systemic inflammation, including increased body temperature and decreased leukocyte counts.

Table 2. Changes in Hemodynamic Variables

	Time after Injury					
	0 h	3 h	6 h	12 h	18 h	24 h
MAP, mmHg						
Sham	99 ± 2	113 ± 4*	115 ± 2*	111 ± 3	109 ± 2	106 ± 3
Control	98 ± 3	101 ± 4†	89 ± 3*†	76 ± 3*†	67 ± 4*†	61 ± 3*†
Treatment	94 ± 3	98 ± 5†	94 ± 5†	72 ± 3*†	74 ± 6*†	79 ± 5*†‡
SVRI, dyne · s · cm^{-5} · m^{-2}						
Sham	1,406 ± 83	1,504 ± 127	1,644 ± 75	1,630 ± 95	1,722 ± 87	1,669 ± 88
Control	1,350 ± 81	1,449 ± 235	988 ± 84*†	812 ± 84*†	569 ± 78*†	490 ± 62*†
Treatment	1,351 ± 151	1,103 ± 92*†	987 ± 84*†	648 ± 49*†	596 ± 113*†	723 ± 122*†
HR, beats · min^{-1}						
Sham	97 ± 4	110 ± 4	103 ± 4	104 ± 5	102 ± 7	105 ± 6
Control	101 ± 3	143 ± 6*†	151 ± 7*†	141 ± 5*†	148 ± 13*†	138 ± 8*†
Treatment	91 ± 5	116 ± 7*‡	134 ± 6*†	136 ± 4*†	133 ± 8*†	131 ± 8*†
CI, L · min^{-1} · m^{-2}						
Sham	5.3 ± 0.3	5.6 ± 0.4	5.1 ± 0.2	5.0 ± 0.3	4.6 ± 0.2	4.6 ± 0.2
Control	5.6 ± 0.2	5.8 ± 0.7	6.8 ± 0.4	6.9 ± 0.7†	8.4 ± 0.9*†	8.3 ± 0.8*†
Treatment	5.6 ± 0.7	6.7 ± 0.4	7.1 ± 0.6*	7.7 ± 0.7*†	8.7 ± 0.6*†	7.9 ± 0.9*†
LVSWI, g · m^{-1} · m^{-2}						
Sham	69 ± 6	71 ± 5	70 ± 4	66 ± 7	62 ± 8	58 ± 5
Control	69 ± 3	51 ± 5*†	48 ± 3*†	41 ± 5*†	40 ± 4*†	36 ± 3*†
Treatment	72 ± 7	69 ± 4‡	60 ± 5	46 ± 5*†	54 ± 6*	52 ± 6*‡

Data are expressed as mean ± SEM.

* $P < 0.05$ *vs.* 0 h within group; † $P < 0.05$ *vs.* sham; ‡ $P < 0.05$ *vs.* control.

CI = cardiac index; HR = heart rate; LVSWI = left ventricular stroke work index; MAP = mean arterial pressure; SVRI = systemic vascular resistance index.

The injury further led to a profound drop in MAP and a concomitant increase in cardiac index as frequently seen in patients with sepsis.[2] In addition, untreated control animals developed signs of multiple organ failure, *i.e.*, surrogate parameters of myocardial, renal, and hepatic dysfunction, as well as vascular leakage and metabolic disturbances.

The endogenous vasodilator nitric oxide is generated from L-arginine through catalysis by three different genetic isoforms of NOS. In contrast to the constitutively synthesized isoenzymes endothelial (NOS −3) and neuronal NOS (NOS −1), inducible NOS (NOS −2) is up-regulated by diverse stress stimuli such as oxidative burst and systemic inflammation.[18] Cytokine-induced stimulation of inducible NOS is known to be a significant contributor to sepsis-related vasodilation *via* excessive production of nitric oxide and its second messenger cyclic guanosine monophosphate[19] and activation of potassium channels in vascular smooth muscles.[20,21] The amount of nitric oxide production within the vascular system may vary at different anatomical sites, resulting in different degrees of vasodilation. Consequently, underperfusion of metabolically active tissue and overperfusion of metabolically inactive tissues may occur,[22] probably contributing to tissue hypoxia and lactic acidosis.[23] However, increased lactate levels during septic shock may not only be a marker of tissue hypoxia, but may also result from aerobic glycolysis through Na^+K^+ ATPase stimulation in skeletal muscles.[24]

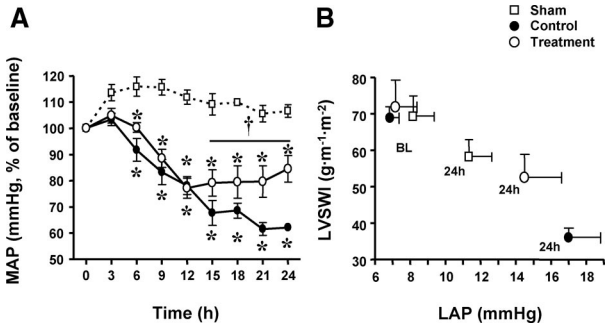

Fig. 1. Impact of early neuronal and delayed inducible nitric oxide synthase inhibitor treatment on mean arterial pressure (MAP) (*A*) and the relationship of left ventricular stroke work index (LVSWI) and left atrial pressure as an indicator of myocardial contractility (*B*) in sheep with pulmonary sepsis. Data of LVSWI and left atrial pressure are expressed as mean ± SEM. MAP is presented as % of baseline values (for mean ± SEM see table 2). * $P < 0.05$ *versus* sham; † $P < 0.05$ *versus* control.

Large amounts of nitric oxide may in addition exert potential cytotoxic effects by reacting with superoxide radicals, yielding reactive nitrogen and oxygen species such as peroxynitrite. Peroxynitrite, in turn, may exert a deleterious influence by oxidizing and/or nitrosating various other molecules,[4,5] including enhanced lipid peroxidation. This is supported by the current finding that the injury-related significant elevation in plasma nitrite/nitrate levels was associated with increased markers of lipid peroxidation in liver tissue.

Although it has been commonly believed that constitutively produced nitric oxide is involved in various regulatory

Table 3. Changes in Surrogate Parameters of Organ Dysfunction, Diuresis, Hematocrit, and Hemoglobin

	Time after Injury					
	0 h	3 h	6 h	12 h	18 h	24 h
Serum creatinine, mg · dl^{-1}						
Sham	0.73 ± 0.04	0.79 ± 0.05	0.78 ± 0.05	0.79 ± 0.06	0.81 ± 0.06	0.82 ± 0.06
Control	0.70 ± 0.03	0.74 ± 0.04	0.80 ± 0.03	1.05 ± 0.09*	1.49 ± 0.21*†	1.73 ± 0.28*†
Treatment	0.74 ± 0.01	0.76 ± 0.03	0.85 ± 0.03	1.07 ± 0.07	1.26 ± 0.18*	1.28 ± 0.23*†‡
Creatinine clearance, ml · min^{-1}						
Sham		104 ± 32	100 ± 33	97 ± 14	84 ± 9	90 ± 18
Control		85 ± 8	100 ± 20	61 ± 14	41 ± 19	53 ± 18†
Treatment		100 ± 12	87 ± 9	60 ± 13	59 ± 17	37 ± 11†
Diuresis, ml · kg^{-1} · h^{-1}						
Sham		4.9 ± 1.0	4.4 ± 1.3	5.3 ± 1.1	4.6 ± 0.8	4.6 ± 0.9
Control		3.6 ± 0.7	3.5 ± 0.4	3.3 ± 1.2	2.7 ± 2.2	1.1 ± 0.8†
Treatment		4.8 ± 0.5	5.9 ± 0.7	4.3 ± 0.9	2.5 ± 1.2	1.0 ± 0.5†
Serum bilirubin, mg · dl^{-1}						
Sham	0.12 ± 0.02	0.15 ± 0.03	0.17 ± 0.04	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.02
Control	0.20 ± 0.03	0.16 ± 0.04	0.10 ± 0.00	0.24 ± 0.12	0.48 ± 0.19*†	0.56 ± 0.20*†
Treatment	0.14 ± 0.02	0.12 ± 0.02	0.10 ± 0.00	0.14 ± 0.04	0.22 ± 0.02‡	0.28 ± 0.07‡
International normalized ratio						
Sham	1.9 ± 0.2			1.6 ± 0.1		1.3 ± 0.1
Control	0.9 ± 0.0†			1.6 ± 0.1		4.8 ± 0.2*†
Treatment	1.2 ± 0.1			1.8 ± 0.5		2.4 ± 0.3*†‡
CK, U · l^{-1}						
Sham	52 ± 6	64 ± 5	58 ± 6	52 ± 7	48 ± 7	45 ± 8
Control	57 ± 11	78 ± 19	83 ± 12	199 ± 77	186 ± 32	363 ± 93*†
Treatment	88 ± 19	94 ± 39	136 ± 55	249 ± 104†	328 ± 134*†	257 ± 90†
CK-MB, ng · ml^{-1}						
Sham	0.8 ± 0.3	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.2
Control	0.8 ± 0.2	0.9 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	2.0 ± 0.5*†
Treatment	0.7 ± 0.2	0.8 ± 0.2	0.7 ± 0.1	0.6 ± 0.2	0.8 ± 0.2	0.9 ± 0.2‡
Hematocrit, %						
Sham	29 ± 2	29 ± 1	27 ± 2	26 ± 1	26 ± 1	26 ± 1
Control	30 ± 2	31 ± 2	31 ± 1	29 ± 1	29 ± 1	28 ± 2
Treatment	27 ± 1	28 ± 1	28 ± 2	28 ± 1	28 ± 1	28 ± 1
Hemoglobin, g · dl^{-1}						
Sham	10.1 ± 0.5	9.8 ± 0.2	9.2 ± 0.6	9.0 ± 0.7	8.5 ± 0.4*	9.1 ± 0.4
Control	10.5 ± 0.6	10.7 ± 0.6	11.0 ± 0.5†	10.2 ± 0.4	10.1 ± 0.5†	9.9 ± 0.4
Treatment	9.0 ± 0.3	9.3 ± 0.5	10.0 ± 0.6	9.7 ± 0.6	9.3 ± 0.5	9.0 ± 0.5

Data are expressed as mean ± SEM.

* $P < 0.05$ *vs.* 0 h within group; † $P < 0.05$ *vs.* sham; ‡ $P < 0.05$ *vs.* control.

CK = creatinine kinase; CK-MB = creatinine kinase muscle-brain isoenzyme.

physiologic processes, including neurotransmission, the regulation of vascular tone and blood flow, and myocardial function,[23,25,26] recent investigations revealed that not only inducible nitric oxide-derived nitric oxide, but also nitric oxide produced by constitutively expressed NOS isoforms may be critically involved in the pathophysiology of multiple organ dysfunctions during systemic inflammation.[6,27–30] In this context, it has been demonstrated that pharmacological inhibition of neuronal NOS, starting 1 h after the injury, significantly improved cardiopulmonary morbidity in ovine experimental sepsis.[6,27] In contrast, inducible NOS inhibition had beneficial effects only on pulmonary gas exchange, not on cardiovascular morbidity.[26] It is worth noting that neuronal NOS inhibition neither entirely blocked excessive nitric oxide production nor completely prevented cardiopulmonary dysfunction in sheep with pulmonary sepsis.[6,27] In a rat model of peritonitis-induced sepsis, Okamoto *et al.* observed that specific inhibition of inducible NOS at different time points after the injury had inconsistent effects on survival. Although inducible NOS blockade ameliorated survival when administered 12 h postinjury, the same intervention increased mortality when administered earlier.[8] Taken together, these findings may provide explanation for the failure of nonselective NOS inhibition in a recent phase III clinical trial[9] because nonspecific inhibition of NOS[9] or specific inhibition of different NOS isoforms at the wrong time points[7,8,31] may be ineffective or even detrimental.

In the present study, we hypothesized that nitric oxide production from neuronal NOS is involved mainly in the early response to sepsis in our ovine model, whereas inducible NOS -derived nitric oxide is responsible during the later phase of the disease process. This assumption has been made

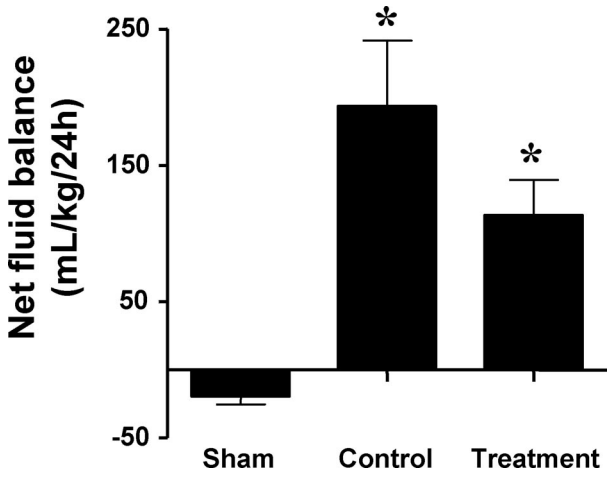

Fig. 2. Impact of early neuronal and delayed inducible nitric oxide synthase inhibitor treatment on net fluid balance in sheep with pulmonary sepsis. Data are expressed as mean ± SEM. * $P < 0.05$ *versus* sham.

because in previous investigations with the same animal model, sole neuronal NOS blockade more effectively suppressed plasma levels of stable nitric oxide metabolites during the first hours after the insults,[6] whereas inducible NOS inhibition proved more effectual during the later course.[7] The combination therapy consisting of early neuronal and delayed inducible NOS blockade completely inhibited the increase in plasma nitrite/nitrate levels of septic sheep. Plasma nitrite/nitrate levels were not significantly increased in the control groups compared with sham animals before the 12-h time points in the current study, possibly indicating that neuronal NOS did not produce large amounts of nitric oxide in the early phase after the injury. However, it appears more likely that an early and transitory increase in plasma nitrite/nitrate levels has been missed due to the limited time points of measurement. In this context, it has previously been demonstrated that increased activity and expression of neuronal NOS may occur earlier than 4 h in the paraventricular nucleus of rats subjected to lipopolysaccharide injection.[32]

The combination treatment in the present study was further associated with attenuations of injury-related decreases in MAP and load-dependent indices of myocardial contractility, as well as increased surrogate parameters of vascular leakage and hepatic dysfunction. This was linked to significant improvements of metabolic status as evidenced by the prevention of the decrease in arterial pH and less increase in arterial lactate concentrations. The beneficial effects of the treatment may, in part, be attributed to a reduction of nitric oxide-induced oxidative stress as indicated by reduced markers of lipid peroxidation in liver homogenate, whereas the attenuation of organ dysfunctions and metabolic disturbances may to some extent be explained by the improved pulmonary gas exchange and oxygenation.

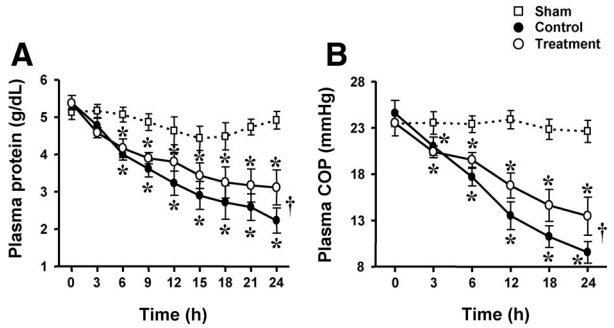

Fig. 3. Impact of early neuronal and delayed inducible nitric oxide synthase inhibitor treatment on plasma protein concentration (*A*) and plasma colloid oncotic pressure (COP) (*B*) in sheep with pulmonary sepsis. Data are expressed as mean ± SEM. * $P < 0.05$ *versus* sham; † $P < 0.05$ *versus* control.

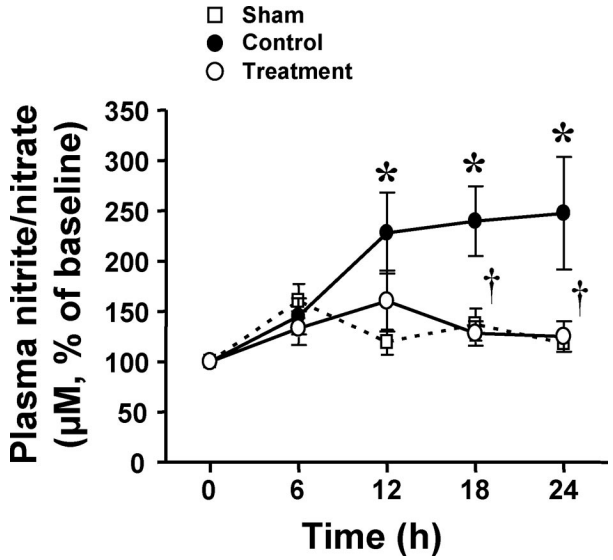

Fig. 4. Impact of early neuronal and delayed inducible nitric oxide synthase inhibitor treatment on plasma levels of stable metabolites of nitric oxide (nitrite/nitrate) in sheep with pulmonary sepsis. Data are expressed as % of baseline values. * $P < 0.05$ *versus* sham; † $P < 0.05$ *versus* control.

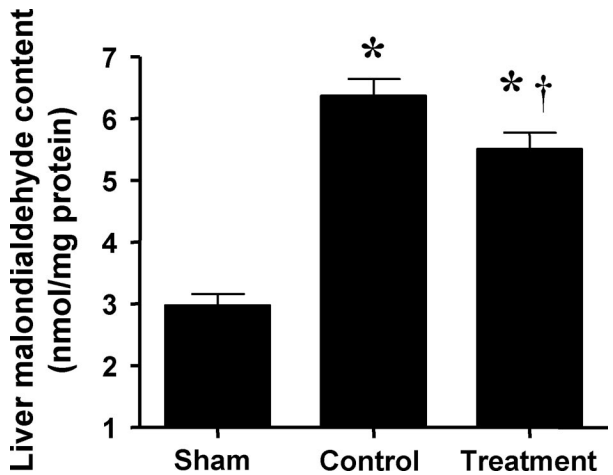

Fig. 5. Impact of early neuronal and delayed inducible nitric oxide synthase inhibitor treatment on malondialdehyde contents of liver tissue homogenates in sheep with pulmonary sepsis. Data are expressed as mean ± SEM. * $P < 0.05$ *versus* sham; † $P < 0.05$ *versus* control.

The effects of combined NOS inhibition on renal function require further evaluation in future studies. Whereas the serum creatinine concentration was slightly, but significantly, improved in the treatment group, there was no statistically significant difference in creatinine clearance between the control and treatment groups. In general, renal function was not substantially improved by the treatment.

When interpreting the current findings, it becomes obvious that, despite its beneficial impact on organ dysfunction, the combination treatment of specific NOS inhibitors is not (yet) the magic bullet of sepsis therapy. First, it needs to be considered that the combination treatment indeed entirely blocked the increase in plasma nitrite/nitrate levels, but on the other hand failed to be superior in preventing the sepsis-related drop in MAP and systemic vascular resistance index than sole blockade of neuronal NOS in a previous study using the same model.[27] Second, it is conceivable that, depending on the type of injury, different NOS isoforms are increasingly expressed and/or activated at different time points during the disease process. In this regard, the present study investigated only one possible combination of NOS blockers, and future testing of other combinations in diverse animal models may provide different results. Third, the fact that neither selective inducible nor neuronal NOS inhibition completely suppressed plasma nitrite/nitrate levels in previous studies[6,7,27] may indicate the potential involvement of endothelial NOS in the pathogenesis of sepsis-related morbidity. Unfortunately, the role of endothelial NOS-derived nitric oxide cannot be tested in the ovine sepsis model because neither selective endothelial NOS inhibitors nor gene-knocked out sheep are currently available. The use of endothelial NOS-knocked out subjects in similar sepsis models may shed some light on this issue.[33] Finally, the effects of combined NOS inhibition on survival have not been determined. The ameliorations of physiologic parameters by the treatment may not necessarily be associated with improved overall outcome.

In conclusion, the present study demonstrates that early pharmacological neuronal and subsequent inducible NOS blockade shows potential benefit on sepsis-related systemic arterial hypotension and surrogate parameters of organ dysfunctions in sheep. Before considering possible treatment strategies of patients with sepsis, however, it may be crucial to identify the time changes of the expression and activation of NOS isoforms and to define markers for the activation of different isoforms in future investigations.

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