Induction of Nitric Oxide Synthase and Dual Effects of Nitric Oxide and Cyclooxygenase Products in Regulation of Arterial Contraction in Human Septic Shock

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Background—The role of endogenous nitric oxide (NO) and cyclooxygenase metabolites was investigated in contractile responses of small omental arteries from patients with hyperdynamic septic shock.

Methods and Results—Expression of inducible NO synthase (immunostaining) and a high but variable level of NO production (NO spin trapping) was detected in arteries from patients with septic shock. In these vessels, ex vivo contractile responses to the thromboxane A₂ analogue U46619 and to low concentrations of norepinephrine (NE) (up to 10 μmol/L) were not significantly different from controls. However, higher concentrations of NE caused pronounced fading of contraction in septic but not in nonseptic arteries. Exposure to either the NO synthase inhibitor N⁵-nitro-L-arginine methyl ester or the cyclooxygenase inhibitor indomethacin had no effect in control vessels. However, both inhibitors increased the response to the contractile effects of the 2 agonists only in patients with septic shock. In contrast to N⁵-nitro-L-arginine methyl ester, which decreased the threshold concentration of the fading effect of NE, indomethacin abolished this effect in arteries from septic patients.

Conclusions—These results provide direct evidence for the induction of NO synthase in small arteries from patients with septic shock. They suggest that in these arteries, increased production of NO, in conjunction with vasodilatory cyclooxygenase metabolites, contributes to counteract hyperreactivity to agonists and decreases the cyclooxygenase product–mediated pronounced fading of contraction caused by a high concentration of NE. (Circulation. 1999;100:107-112.)

Key Words: shock ■ nitric oxide synthase ■ prostaglandins ■ arteries ■ vasoconstriction

During severe sepsis, the cardiovascular system adopts a high cardiac output–low peripheral resistance hemodynamic profile whose vascular component involves dilatation of resistance arteries.¹ The increased cardiac output and peripheral dilatation may improve tissue perfusion and probably contribute to protection against multivisceral injury caused by sepsis. However, a prolonged or excessive drop in peripheral resistance may also cause progressive hypotension, refractory to catecholamines, and contribute to life-threatening cardiovascular failure.² From data collected in endotoxin-treated animals, it has been suggested that this phenomenon is related to an overproduction of nitric oxide (NO) in vascular tissue through the induction of type II NO synthase (iNOS) activity (for review, see Reference 3). Indirect evidence based on the effects of NOS inhibitors also suggests a role for NO in humans demonstrating septic shock,⁴,⁵ but direct evidence for the induction of iNOS in vessels from patients with septic shock is still lacking.

Therefore, the present study was designed to look for iNOS expression and NO production in human small arteries and to assess the role of NO in vascular reactivity during severe sepsis. Because cyclooxygenase metabolites are also produced in sepsis, involvement of these products in the regulation of contraction was also investigated. At stake is a better understanding of the mechanisms of low peripheral resistance during severe infection and a more exact assessment of the actual need for an NOS inhibitor to treat these patients.

Methods

Patients and Arteries

Omental arteries were isolated from human omentum harvested in patients undergoing an abdominal laparotomy for either peritonitis (n=8) or nonseptic surgery (n=7). The protocol was approved by our institutional ethics committee.

The septic patients (age range 28 to 71 years) demonstrated a diffuse perforative peritonitis (appendicitis n=3; colon n=4; rectum...
with parietal gangrene \( n = 1 \) with a systemic inflammatory response syndrome that lasted for >6 hours: fever (range 38.2°C to 39.7°C), respiratory failure requiring mechanical ventilation, and hyperdynamic circulatory failure. The patients were infused with a combination of a \( \beta \)-lactam antibiotic and an aminoglycoside and were given conventional intensive care for septic patients, including dobutamine (range 10 to 15 \( \mu \)g \( \cdot \) kg \( \cdot \) min \( ^{-1} \)) and norepinephrine (NE; range 3 to 15 \( \mu \)g \( \cdot \) kg \( \cdot \) min \( ^{-1} \)), after plasma volume expansion with colloids up to a pulmonary artery wedge pressure of 10 mm Hg. Hemodynamic parameters before and 5 hours after the onset of catecholamine infusion, respectively, were as follows: mean arterial pressure 61 ± 27 and 88 ± 37 mm Hg, heart rate 145 ± 25 and 138 ± 10 bpm, cardiac index 4.64 ± 1.02 and 4.09 ± 1.3 L \( \cdot \) min \( ^{-1} \) \( \cdot \) m \( ^{-2} \), peripheral resistance 1054 ± 312 and 1374 ± 241 dyne \( \cdot \) s \( \cdot \) cm \( ^{-5} \) \( \cdot \) m \( ^{-2} \). The patients involved in the control population (age range 36 to 61 years) required planned nonseptic surgery: large-bowel resection for limited cancer (\( n = 4 \)), cholecystectomy (\( n = 2 \)), and an intestinal bypass (\( n = 1 \)). None of them had fever or hypothermia or any evidence of parameters that were suspected to be the result of sepsis or underlying comorbidities. None was given any cardiovascular therapy, but each patient was infused with ceftriaxone (2 g before the surgical procedure) to prevent perioperative life-threatening sepsis. In both groups, anesthesia was induced with alfentanil or sufentanil combined with midazolam.

Macroscopically normal segments of arteries cleaned of fat and connective tissue were collected into cold physiological salt solution (PSS; in mmol/L): NaCl 119, KCl 4.7, KH\(_2\)PO\(_4\) 0.4, NaHCO\(_3\) 15, MgSO\(_4\) 1.17, CaCl\(_2\) 2.5, glucose 5.5.

### iNOS Staining and Confocal Microscopy Imaging

Segments (2 mm in length) of omental arteries were incubated in a bath containing PSS kept at 37°C and continuously gassed with a mixture of 95% O\(_2\) -5% CO\(_2\). Tissue segments were incubated (30 minutes at room temperature) in 200 µL of nonspecific site-blocking buffer (5% [vol/wt] nonfat dry milk in Dulbecco’s modified PBS without Ca\(^{2+}\) and Mg\(^{2+}\), pH 7.2). After 3 washes in 200 µL of PBS, tissue segments were further incubated for 10 minutes at 37°C in 150 µL of monoclonal murine macrophage anti-iNOS antibody (Transduction Laboratories) diluted 1:50 in incubation buffer A (1% [vol/wt] nonfat dry milk, 0.5% [vol/wt] Triton in PBS). Three more washes in PBS were followed by an incubation (10 minutes at 37°C) in 150 µL of a solution of goat anti-mouse IgG rhodamine (TRITC) conjugated antibody diluted 1:100 in incubation buffer A. For negative controls, the only difference was that the vessels were only incubated with the secondary fluorescence-labeled antibody. After 4 washes in PBS followed by fixation in 3.7% (vol/vol) paraformaldehyde-PBS (4°C), tissue segments of 2 mm length were opened and mounted on glass slides.

Sequential through-focus images of labeled vessels were acquired on a Zeiss laser scanning microscope (LSM 410 invert) equipped with a Neofluar oil immersion lens (40, numerical aperture 1.4). The TRITC was excited with the He/Ne laser line 543-nm beam. The emission signal was recorded with a Zeiss 515-565-nm filter (fluorescein emission) or with a long-pass 595-nm filter (rhodamine signal). Nonspecific fluorescence was measured by incubation of the vessels with the secondary fluorescence-labeled antibodies, and this value was then subtracted from all images. Each sample was subjected to optically serial sectioning, offering images in the X-Y and X-Z planes. Each optical section was averaged 8 times, and the settings between control and treated samples were not modified.

### NO Spin Trapping and Electron Paramagnetic Resonance Studies

Detection of NO production within intact tissue was performed with a previously described technique with Fe\(^{2+}\) diethyldithiocarbamate (DETC) as spin trap. Omental arteries (4 to 35 mg of wet tissue) were incubated for 60 minutes in PSS in the presence of sodium-nitro- \( L \)-arginine methyl ester (L-NAME, 300 µmol/L) or the cyclooxygenase inhibitor indomethacin (10 µmol/L).

### Expression of Results and Statistical Analysis

For construction of concentration-effect curves, tension values obtained at the peak of the response elicited after each addition of the agonist were used. Tension values were expressed as a percentage of the maximal contractile capacity of the vessels challenged with KCl (100 mmol/L) plus U46619 (1 µmol/L). Maximal responses were not significantly different in arteries from septic and nonseptic patients (9.9 ± 1.5 and 10.2 ± 0.65 mN/mm, respectively). Sensitivity to agonist is expressed as the pD\(_2\) value, where pD\(_2\) represents −log of the half-maximally effective molar concentration. ANOVA was used for statistical analysis. \( P < 0.05 \) was considered significant.

### Results

#### iNOS Staining and NO Spin Trapping

For ethical reasons, the amount of tissue obtained from patients was limited and did not allow measurement of iNOS and NO production in arteries from the same patients. Marked iNOS immunostaining was found in intimal and medial layers of arteries from all 3 septic patients studied. In addition, the adventitial layer was stained in 2 of them, as illustrated in Figure 1 (E through H). By contrast, no iNOS immunostaining could be detected in arteries from 2 control patients (not shown). Only weak iNOS immunostaining was present in the intimal layer, but not in the medial and adventitial layers, of the third control artery (Figure 1, A through D).

Despite the small size of samples and the low sensitivity of the technique, NO production was detected by EPR spectroscopy in 2 of 4 studied arteries from septic patients (21 and 15 pmol \( \cdot \) sample \(^{-1} \) \( \cdot \) h \(^{-1} \)) and 19 pmol \( \cdot \) sample \(^{-1} \) \( \cdot \) h \(^{-1} \)). By contrast, NO spin trapping could be detected in only 1 of 6 arteries from nonseptic patients (9 pmol \( \cdot \) sample \(^{-1} \) \( \cdot \) h \(^{-1} \)).

### Contraction Experiments

After addition of each dose of NE or U46619, tension rapidly increased to a constant plateau level in control vessels. However, in arteries from septic shock patients, the responses to NE were less stable than in controls, and increasing the NE concentration from 10 to up to 30 µmol/L caused a rapid and...
pronounced decline of tension in arteries from septic patients but not in those from controls. The concentration-effect curves describing the mean plateau (or peak) increase in tension reached after each addition of agonist are shown in Figure 2. No difference in the increases in tension produced by NE (up to 10 μmol/L) and U46619 was observed between arteries from nonseptic and septic patients. Addition of L-arginine (300 μmol/L) when the maximal responses to the 2 agonists were reached did not cause any change in tension, either in arteries from patients with septic shock or in those from control patients (not shown). In separate experiments, cocaine (3 μmol/L) did not change significantly the responses to NE in arteries from either control (n=6) or septic shock patients (n=7) (not shown), indicating that neuronal uptake

Figure 1. Confocal images of iNOS. iNOS labeling in segments of small omental artery taken from 1 control patient (A, B, C, and D) and 1 patient with septic shock (E, F, G, and H) using confocal microscopy. Each vascular segment was subjected to optically serial sections in X-Z (A and E; arrows indicate intimal side of vessel) and X-Y planes in different layers of labeling (B and F, intima; C and G, media; D and H, adventitia). Bars=50 μm.
of NE did not influence the response to NE under the experimental condition.

Neither the NOS inhibitor L-NAME (300 μmol/L) or the cyclooxygenase inhibitor indomethacin (10 μmol/L) had a significant effect on contractile responses in arteries from nonseptic patients (Figure 2, A and B). However, they both enhanced contractile responses in arteries from patients with sepsis. In these vessels, L-NAME produced a significant leftward shift of the concentration-effect curves of the 2 agonists (Figure 2, C and D). Furthermore, both the ascending and descending components of the biphasic concentration-effect curve to NE were displaced to the left by L-NAME, with an increase of NE above 3 μmol/L causing pronounced vasorelaxation. Indomethacin increased sensitivity to U46619 but not to NE in arteries from septic patients (Figure 2, C and D). In addition, it markedly enhanced the maximal responses to the 2 agonists in these arteries. By contrast with the effect of L-NAME, indomethacin abolished the fading response to the highest concentration of NE.

The endothelium-dependent relaxation produced by bradykinin was not significantly different in arteries from either group (not shown): the pD₂ values for NE (A and C) and U46619 (B and D) on contraction of small omental arteries from control (A and B) and septic patients (C and D) in absence (C) and presence of either L-NAME (300 μmol/L) (●) or indomethacin (10 μmol/L) (▲). Vertical bars indicate SEM; n=3 to 7. In the case of NE, pD₂ values were calculated from ascending part of curve in vessels from septic patients. *P<0.05, **P<0.01 compared with experiments without inhibitors (comparing all concentration-response curves).

The above results provide direct evidence for iNOS expression and NO production in small arteries from septic shock patients. They also shed new light on the involvement of NO and vasodilatory metabolites of cyclooxygenase in regulating contractile responses of these arteries to agonists. Of particular interest is the finding that the NOS inhibitor L-NAME enhanced the fading effect of high NE concentration, which was abolished by the cyclooxygenase inhibitor indomethacin.
Immunostaining experiments showed that iNOS was expressed in the 3 tunicae of the vessels from the septic shock patients studied (the intimal, medial, and adventitial layers were more or less labeled, although immunostaining was predominant in the intima and, in 2 of 3 patients, in the adventitia). Despite its relatively low sensitivity, EPR spectroscopy affords the unique possibility of directly monitoring tissue NO production. It enabled the detection of production of NO over the limit of detection of the technique (6 to 8 pmol/h) in 2 of 4 small segments of arteries from septic patients; these data show very high NO production in at least some of these vessels but also individual variations. All these patients had surgery at least 6 hours after the onset of clinical symptoms and experienced a hyperdynamic circulatory state. Additional EPR and iNOS immunostaining experiments are needed to assess more precisely the localization of iNOS, NO production, and their individual variations in the arteries from these patients. However, the finding that L-NAME significantly enhanced sensitivity to both NE and U46619 supports the conclusion that NO overproduction was associated with iNOS induction in arteries from septic patients. With the use of a different experimental protocol, it was recently reported that L-NAME also prevented the progressive decline in responses to repeated bolus injection of NE (1 μmol/L) in small omental arteries from septic patients, whereas no such phenomenon was seen in controls. Although L-NAME can inhibit nonselectively the 3 NO synthases, it did not modify contractile responses in control small omental arteries. This is consistent with previous reports showing that endothelium-derived NO does not have a major role in these arteries. In the present study, the mean increase in sensitivity to agonists produced by L-NAME was nevertheless moderate in arteries from septic patients. Although the maximal relaxing effect of the NO donor was not impaired in these arteries, it cannot be excluded that the biological activity of endogenous NO and generation of a paramagnetic reaction complex with the spin trap were partially blunted in these vessels, because O$_2^-$ production is enhanced in sepsis.

The presence of iNOS staining in the intimal layer of the small omental artery from 1 control patient and of significant NO production in an artery from another patient is noteworthy. Obviously, many causes other than sepsis, including cancer, may induce iNOS in the blood vessels of patients. However, these patients presented no sign of systemic inflammatory syndrome and none of the hemodynamic features of hyperdynamic circulatory failure. The induction of iNOS activity and NO overproduction may not be sufficient by itself to cause unrelenting hypotension, because many other mechanisms may be involved in the circulating failure associated with sepsis.

The finding that contractile responses of small omental arteries from septic patients were not significantly changed ex vivo (except in the presence of a high NE concentration) is intriguing because the arteries were removed from patients whose peripheral resistance and blood pressure were dramatically reduced (see Methods). Contraction experiments performed in the presence of either L-NAME or indomethacin unmasked enhanced responses of arteries from septic patients to U46619 and to low concentrations of NE. The mechanisms of this hyperreactivity are unknown. Obviously, they do not involve cyclooxygenase products. An increase in intracellular Ca$^{2+}$ might be involved, as was previously found in small arteries from endotoxin-treated rats. It seems that the role of overproduction of NO and, to a larger extent, of vasodilatory products of cyclooxygenase is to counteract the vascular hyperreactivity associated with sepsis. The results obtained with L-NAME are consistent with those recently reported by Avontuur et al showing that inhibitors of NO synthesis unmasked a tonic pressor response to endothelin-1 in human septic shock.

Increasing the concentration of NE from 10 to 30 μmol/L dramatically reduced contraction (by ∼50%) in arteries from septic patients. In agreement with previous findings in the same vessels, this did not occur in arteries from nonseptic patients. Rapid fading or desensitization of responses to high concentrations of a variety of vasoconstrictor agonists has been described frequently. Our monitoring of hemodynamic parameters showed that blood pressure and peripheral resistance, although partially restored, remained low, with no sign of further desensitization for several hours during catecholamine infusion. It is well established that the circulating level of NE increases markedly during sepsis. Furthermore, additional doses of catecholamines were injected in septic patients. Therefore, it is possible that the NE concentration reached a level at which noradrenergic vasoconstriction was impaired in these patients.

The results obtained with indomethacin and L-NAME suggest that the fading response to high concentrations of NE involved cyclooxygenase products and was enhanced by endogenous NO in arteries from septic patients. The mechanisms by which NO might oppose the fading of contraction caused by high concentrations of NE, whereas it reduces contraction produced by lower concentrations of NE or by other agonists, warrant further investigation. There are conflicting reports in the literature on the possible cross talk between NOS and cyclooxygenase metabolites in tissues exposed to endotoxin or cytokines. The interactions between the 2 systems are probably extremely complex. However, the fact that L-NAME increased the sensitivity of isolated arteries from septic patients not only to the vasoconstrictor effect but also to the fading effect of high concentrations of NE may be important for the use of NOS inhibitors in sepsis if the same effect occurs in vivo. The rationale for the apparently opposite effects of NO on responses to low and high concentrations of NE would be to keep vasoconstriction to NE within normal limits up to the concentration of 10 μmol/L in septic patients.

In conclusion, the present study provides direct evidence for iNOS expression and large but variable NO production in omental small arteries from patients in the hyperdynamic phase of septic shock. It suggests that the role of NO in patients with severe sepsis might be not only to act with cyclooxygenase metabolites to counteract hyperreactivity to endogenous vasoconstrictor agonists but also to increase the threshold concentration at which enhanced production of cyclooxygenase metabolites causes a fading response to NE. This dual effect and the involvement of cyclooxygenase metabolites may be important in evaluating the potential
interest of blocking iNOS, cyclooxygenase, or both in the treatment of human septic shock.

Acknowledgments
This work was supported by the European Union Grant Biomed II and by Délegation à la Recherche Clinique des Hôpitaux Universitaires de Strasbourg. The authors are grateful to the surgeons of the University Hospital of Hautepierre at Strasbourg for providing the human omentum and to Dr R. Wadsworth for carefully reading the manuscript.

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Circulation. 1999;100:107-112
doi: 10.1161/01.CIR.100.2.107
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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