Nitrogen Oxide Levels in Patients After Trauma and During Sepsis

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The mediators responsible for maintenance of the hyperdynamic state and the low systemic vascular resistance (SVR) observed in sepsis have not been elucidated. Nitric oxide (·N = O) is a mediator with numerous functions, including regulation of vascular tone and a role in macrophage-mediated cytostasis and microbiostasis. Thirty-nine critically ill trauma and septic patients were studied to determine the relationship between ·N = O production and the hyperdynamic state. High plasma levels of NO_2^-/NO_3^- (the stable end products of ·N = O) were observed in septic patients ($p < 0.02$). Low SVR and high endotoxin levels were associated with high NO_2^-/NO_3^- values ($p = 0.029$, $p = 0.002$). Changes in ·N = O levels may mediate the vasodilation seen in sepsis. Low NO_2^-/NO_3^- levels were observed in trauma patients ($p < 0.001$) and remained low even in the presence of sepsis ($p = 0.001$).

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ALTHOUGH NITRATES AND nitrites (NO_2^-/NO_3^-) have been measured in human body fluids for over 70 years,[1] it has been only during the past 10 years that endogenous sources of NO_2^-/NO_3^- have been appreciated.[2] It is now known that an important source of NO_2^-/NO_3^- is through the conversion of L-arginine to L-citrulline and nitric oxide (·N = O). Animal studies have shown that endothelial cells,[3,4] macrophages,[5-7] cerebellar neurons,[8,9] neutrophils,[10,11] Kupffer cells,[12] and hepatocytes[13,14] produce ·N = O when stimulated. Endotoxin and cytokines such as interferon (IFN) and tumor necrosis factor (TNF) have been shown to stimulate ·N = O production by endothelial cells,[15,16] macrophages,[17,18] Kupffer cells,[12] and hepatocytes.[19] Studies in both animals[20,21] and humans[22] using the inhibitor of ·N = O synthesis, N^G monomethyl-L-arginine (NMA), suggest that ·N = O functions as a vasodilator *in vivo*. Evidence now exists that ·N = O also may participate in signal transduction in the cerebellum[9,23] as well as messenger functions in neutrophils.[24] Larger quantities of ·N = O appear to inhibit cellular respiration[6,25] and proliferation, and may be important in macrophage-mediated tumor cell cytostasis and microbiostasis.[26]

Because endotoxin and inflammatory cytokines can act as signals for increased ·N = O production, it is possible that disease processes associated with increased endotoxin or cytokine levels may be associated with increased ·N = O production, and therefore elevated levels of NO_2^-/NO_3^-, the stable end products of ·N = O biosynthesis. Whether increased ·N = O production mediates abnormal hemodynamic and metabolic responses during disease processes has not been reported. Increased ·N = O production, by inducing vasodilation, could explain some of the characteristic hemodynamic changes seen in sepsis, such as a decrease in systemic vascular resistance. Conversely patients with vasoconstriction (*e.g.*, hypotensive trauma patients) should theoretically produce less ·N = O. To determine if nitrogen oxide production was altered in patients with sepsis or after hemorrhagic shock, circulating levels of the stable metabolites of nitrogen oxide, NO_2^-/NO_3^-, were measured in patients admitted to intensive care units with sepsis or after trauma. Changes in NO_2^-/NO_3^- levels were correlated with hemodynamic alterations as well as with circulating factors known to enhance ·N = O production.

Methods

Patient Population

Critically ill patients admitted to the surgical or trauma intensive care units at Presbyterian University Hospital

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of Pittsburgh were followed during their stay in the intensive care units. A total of 39 patients was divided into three groups based on their diagnoses and clinical course. Group I consisted of 17 general surgery patients (12 men, 5 women), with an average age 60 ± 3 years, who were clinically septic or had severe inflammatory processes at the time of admission (Table 1). Group II was composed of 14 trauma patients (10 men, 4 women) who had an uneventful recovery after injury. The average age of this group was 45 ± 5 years. Eight trauma patients (6 men, 2 women) who became clinically septic during their intensive care unit stay constituted group III. Average age was 47 ± 9 years. Age differences were significant between groups I and II ($p \approx 0.049$), but not between groups I and III or groups II and III. Average injury severity scores (ISS) were 27 ± 3 and 28 ± 6 for groups II and III, respectively. Blood samples were collected from 14 normal volunteers as controls for plasma NO_2^-/NO_3^- levels.

Collection of Clinical Data

Daily assessment of the patients was performed, and the relevant patient data were recorded. Evaluation of severity of disease was done by an initial measurement of the APACHE II score, and then followed by daily acute physiology score evaluations. Trauma scores and injury severity scores were recorded for the trauma patients. The presence of sepsis was defined using a previously validated score (Table 2).[27] Invasive hemodynamic monitoring was used according to the clinical indications in the patients, and when present, Swan-Ganz catheter readings were collected.

Collection of Blood Samples

Blood samples were obtained through indwelling catheters and aliquoted into vacutainer tubes, some supplemented with heparin (Vacutainer tubes, Beckton Dickinson, Rutherford, NJ). Plasma or serum was obtained by centrifugation at 3000 rpm for 10 minutes at 4 C and stored at −70 C until used for the different assays.

TABLE 1. *Clinical Diagnoses in Patients With Septic or Inflammatory Processes (Group I)*

Patient Age (yr)	Diagnosis	Outcome
69	Pancreatic abscess	Died
41	Gangrenous cholecystitis	Lived
76	Perforated duodenal ulcer	Lived
67	Sepsis of unknown etiology	Died
39	Perforated cecal volvulus	Died
62	Perforated sigmoid diverticulitis	Lived
50	Gangrene, abdominal wall	Lived
67	Acute pancreatitis	Lived
67	Perforated small bowel obstruction	Died
58	Small bowel obstruction	Died
88	Perforated sigmoid diverticulitis	Lived
36	Perforated sigmoid ulcer	Lived
72	Perforated colon cancer	Died
45	Candida sepsis	Died
52	Herpes meningoencephalitis	Lived
71	Abdominal wall cellulitis	Lived
72	Gastric cancer	Lived

TABLE 2. *Clinical Criteria for the Diagnosis of Sepsis (27)*

Major	Minor
Systolic blood pressure < 90 mm Hg	Altered sensorium
	Tachypnea, respiratory rate > 24/min, OR
Presence of an obvious primary site	Hypocapnia, $Paco_2$ < 35 mm Hg
	Tachycardia, heart rate > 109/min
Positive blood culture	Temperature > 38.9 C or < 35.6 C
	White blood cell count > 15,000/mm^3 or < 3500/mm^3
	Platelets < 100,000/mm^3

Diagnosis of sepsis is defined as the presence of five of the above criteria, including at least one of the major criteria.

NO_2^-/NO_3^- Assay

Plasma samples (200 μL) were diluted 1:5 with deionized water and deproteinized with 50 μL 30% $ZnSO_4$. NO_2^-/NO_3^- concentration was measured using an automated procedure based on the Griess reaction.[28] Briefly, the samples were passed through a column containing copper-coated cadmium, which reduced all NO_3^- to NO_2^-. The NO_2^- then was detected when it reacted with Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride, 2.5% H3PO4). Absorbance was measured at 546 nm using a spectrophotometer. Although very little or no NO_2^- is found in serum,[29] we did not attempt to differentiate between NO_2^- and NO_3^- amounts, and therefore report our results as NO_2^-/NO_3^-.

Endotoxin Assay

Endotoxin was measured using a chromogenic modification of the limulus amebocyte lysate assay (Whittaker M.A. Bioproducts, Walkersville, MD). Briefly, heparinized plasma samples were diluted 1:10 and then heat inactivated at 75 C for 10 minutes. Fifty microliters limulus amebocyte lysate was added to 50 μL sample for 10 minutes at 37 C. Chromogenic substrate then was added for 6 minutes. Reaction then was stopped with 25% acetic acid, and spectrophotometric absorbance was measured at 405 nm.

Amino Acid Analysis

Amino acid determinations were performed on sodium heparin-preserved plasma after deproteinization with 15% sulpho-salicylic acid, and measurements were done using a Beckman model 6300 dedicated amino acid analyzer (Beckman Instruments, Inc., Palo Alto, CA).

Interferon Gamma Assay

Interferon gamma levels were measured in serum using an enzyme-linked immunosorbent assay (Amgen Biologicals, CA).

Statistics

Data analysis included parametric and nonparametric t test (Mann-Whitney) analysis, and nonparametric analysis of variance (Kruskal-Wallis). Data was considered statistically significant at $p < 0.05$ level. Data are expressed as mean ± standard error of the mean.

Results

Plasma NO_2^-/NO_3^- levels in the control group averaged 28.9 μmol/L ± 3.6 (n = 14) and ranged from 16 to 62 μmol/L. These values are similar to other reports.[29] The average for the samples obtained from group I (septic patients, n = 72 samples) was 63.1 ± 6.5 μmol/L, which was significantly higher than normal ($p < 0.02$). Samples obtained while the patients were clinically septic were even higher (72.6 ± 9.9 μmol/L) ($p < 0.03$) (Fig. 1). In contrast the average plasma NO_2^-/NO_3^- levels for the samples obtained from group II (trauma patients, n = 23 samples) averaged 12.8 ± 1.5 μmol/L, significantly lower than the control group ($p < 0.001$) (Fig. 2). Average values for trauma patients who developed sepsis (group III, n = 40 samples) were also lower than normal (15.6 ± 1.9, $p = 0.001$) and did not increase when the patients were septic, as based on clinical criteria as listed in Table 2 (15.5 ± 3.6 μmol/L, n = 14) (Fig. 3).

During their intensive care unit (ICU) stay, the patients' average daily Acute Physiology Scores (APS) were 16 ± 1, 10 ± 1, and 10 ± 1 for groups I, II, and III, respectively, and were found to be significantly higher in group I than in groups II and III ($p < 0.0001$). Average NO_2^-/NO_3^- levels were 27.7 ± 2.6 μmol/L in patients with APS ≤ 12. Average NO_2^-/NO_3^- levels increased to 55.5 ± 7.6 μmol/L, with an APS of 13 to 18 ($p = 0.003$) and 67.01 ± 19.3, with an APS > 18 ($p = 0.008$). APACHE II scores at time of admission to the ICU were 19 ± 2 for group I, 12 ± 2 for group II, and 16 ± 2 for group III, and were not significantly different between any of the groups.

To determine if increased circulating nitrogen oxide levels were associated with measurable changes in vascular

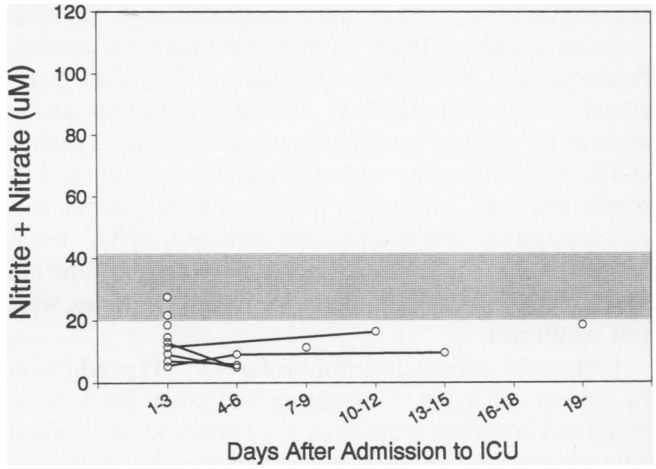

FIG. 2. Plasma $NO_2^-NO_3^-$ values in patients with nonseptic trauma. Patients with trauma showed decreased plasma $NO_2^-NO_3^-$ levels. The shaded area represents average plasma $NO_2^-NO_3^-$ values ± 1 SD in the control population.

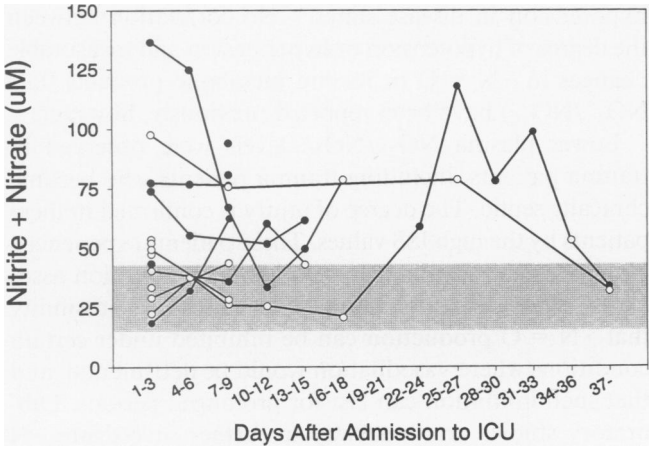

FIG. 1. Plasma NO_2^-/NO_3^- values in septic patients in the intensive care unit. Patients with sepsis or severe inflammatory processes had significant increases in $NO_2^-NO_3^-$ plasma values. During septic episodes (●), $NO_2^-NO_3^-$ value was higher ($p = 0.003$) than during nonseptic episodes (○). The shaded area represents $NO_2^-NO_3^-$ plasma values ± 1 SD in the control population.

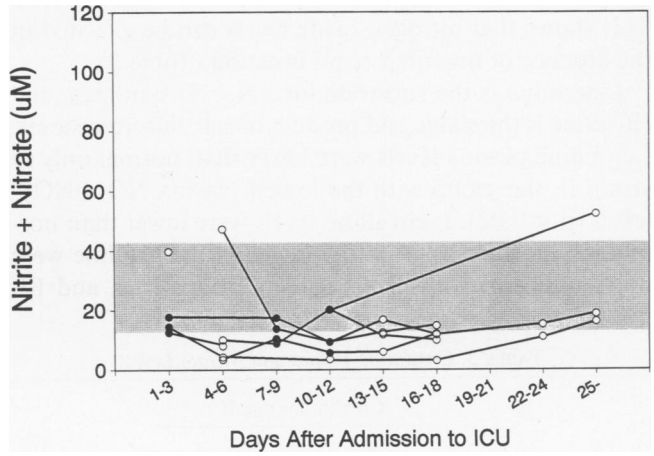

FIG. 3. Plasma nitrate values in patients who had trauma and sepsis. These patients had lower than normal plasma $NO_2^-NO_3^-$ values. During septic episodes (●), plasma $NO_2^-NO_3^-$ values were not increased compared with nonseptic episodes (○). The shaded are represents average plasma $NO_2^-NO_3^-$ values ± 1 SD in the control population.

tone, NO_2^-/NO_3^- levels were correlated with systemic vascular resistance index in those patients with invasive hemodynamic monitoring. Circulating NO_2^-/NO_3^- levels greater than 42 μmol/L (1 standard deviation above average for normal levels) were associated with a significantly lower systemic vascular resistance (1106 ± 119 dynes • sec • cm^{-5}/m^2) when compared with systemic vascular resistance index in patients with NO_2^-/NO_3^- levels less than 42 μmol/L (1353 ± 93 dynes • sec • cm^{-5}/m^2) at that time ($p = 0.029$). Differences in cardiac index were not significant.

Endotoxin is a potent stimulus for • N = O production by several cell types. Circulating endotoxin levels were measured in patient samples (n = 77 samples) and normal controls (n = 7 samples). Normal endotoxin levels were found to be 0.047 ± 0.006 ng/mL. Significantly increased endotoxin levels were found when NO_2^-/NO_3^- levels were high (greater than 42 μmol/L) compared with when NO_2^-/NO_3^- levels were normal or low (less than 42 μmol/L), (0.144 ng/ml ± 0.06 *vs.* 0.044 ng/mL ± 0.02) ($p = 0.002$). Interferon gamma is known to potentiate • N = O synthesis *in vitro;* therefore circulating levels of this cytokine were also measured. Low circulating interferon gamma levels were found in 3 of 11 samples analyzed in the control population, and in 13 of 48 patient samples analyzed. No correlation was found between elevated NO_2^-/NO_3^- levels and circulating levels of interferon gamma.

Nitrogen oxide is cleared primarily by the kidneys.[30] Therefore NO_2^-/NO_3^- levels were correlated with renal function to determine if the elevated nitrogen oxide levels would be explained solely on decreased renal clearance. A higher average NO_2^-/NO_3^- level was found in patients with creatinine levels higher than 2 mg/dL. However when the samples collected from patients of group I (sepsis) were divided into samples with creatinine above or below 2 mg/dL, no difference in NO_2^-/NO_3^- levels was detected. This shows that nitrogen oxide levels can be elevated in the absence of impaired renal function (Table 3).

L-arginine is the substrate for • N = O synthesis, and citrulline is the stable end product in cell culture systems. L-arginine plasma levels were lower than normal only in group II, the group with the lowest plasma NO_2^-/NO_3^- levels ($p < 0.02$). L-citrulline levels were lower than normal in all groups ($p < 0.01$). Levels of L-citrulline were not significantly different between groups I, II, and III.

TABLE 3. *Creatinine Values and Nitrate Levels*

	Creatinine (mg/dL)		
	< 2	> 2	p
All patients	29.9 ± 3.1	66.0 ± 10.3	< 0.001
Group I (sepsis)	59.2 ± 7.4	66.6 ± 10.6	NS

No correlation was found between NO_2^-/NO_3^- levels and arginine or citrulline levels (Table 4).

Discussion

The presence of nitrogen oxides in human pathology has been of interest to investigators studying infantile methemoglobinemia[29] and the formation of carcinogenic compounds such as nitrosamines.[31] It had been assumed that exogenous sources accounted for NO_2^-/NO_3^- detected in different body fluids. Even though Mitchell in 1916 had noted excess NO_2^-/NO_3^- excretion in humans receiving low NO_2^-/NO_3^- diets, it was not until 1981 that Green et al.[2] provided convincing evidence that endogenous sources of nitrogen oxide biosynthesis existed in humans. The only known endogenous source of NO_2^- and NO_3^- production in mammalian tissues is through the conversion of L-arginine to • N = O. • N = O degrades to NO_2^- and NO_3^-, then NO_2^- is further converted to NO_3^- when it reacts with hemoglobin.[29]

In this study we examined circulating NO_2^-/NO_3^- levels as a measure of ongoing • N = O production. Increased levels were seen in general surgery patients with clinical sepsis, and this correlated with the severity of disease. High NO_2^-/NO_3^- levels were inversely related to low systemic vascular resistance. This last observation suggests that • N = O may in fact be an important mediator of increased vasodilation observed in sepsis. Vallance et al.[22] showed that infusion of the inhibitor of • N = O synthesis, N^G monomethyl-L-arginine, into the brachial arteries of humans, induced a decrease in blood flow to that limb, showing that • N = O acts as a vasodilator in humans.[22] A recent report has shown that blocking • N = O production reverses TNF-mediated hypotension in dogs, providing evidence that endogenous • N = O can mediate hypotension in disease states.[32] No correlation between the degree of hypotension or hypertension and measurable changes in • N = O or its end metabolic products (*i.e.*, NO_2^-/NO_3^-) have been reported previously, however.

Lower plasma NO_2^-/NO_3^- levels were observed in trauma patients, including trauma patients who became clinically septic. The degree of injury is confirmed in these patients by the high ISS values. These patients experienced blood loss and resultant hypovolemia, a condition associated with vasoconstriction. This raises the possibility that • N = O production can be inhibited under certain conditions where vasodilation would be detrimental, and that such inhibition can last for prolonged periods. Laboratory studies are underway to further investigate • N = O production after hypovolemic shock.

NO_2^-/NO_3^- is principally excreted through the kidneys. The fact that there is a positive correlation between impaired renal function and NO_2^-/NO_3^- levels raises the

TABLE 4. *Plasma Arginine and Citrulline Levels*

	Group I (sepsis)	Group II (trauma)	Group III (trauma and sepsis)	Normal Group
Citrulline μmol/L	28.7 ± 4.9*	11.7 ± 3.1*	10.7 ± 1.5*	48.9 ± 2.1
Arginine μmol/L	84.7 ± 9.6	57.8 ± 6.5†	93.9 ± 12.3	80.7 ± 4.6

* $p < 0.001$ compared with normal values.
† $p < 0.02$ compared with groups I or III, or normal control subjects.

question as to whether NO_2^-/NO_3^- levels are elevated because of lack of excretion and not because of excess production. The finding that samples taken in the septic group with normal renal function are elevated, however, strongly suggests that elevated NO_2^-/NO_3^- levels in this patient group can be caused by increased NO_2^-/NO_3^- production, and not only by decreased excretion.

Which cells are the main sources of $\cdot N = O$ in humans is unknown. Endotoxin injection into mice has been shown to induce macrophage NO_2^-/NO_3^- synthesis[33] and Kupffer cell[14] NO_2^-/NO_3^- synthesis in rats. *In vitro* studies have shown that endotoxin and cytokines can also stimulate endothelial cell[15,16] and hepatocyte[19] $\cdot N = O$ biosynthesis. Our data would suggest that endotoxin may be an important stimulus to increased $\cdot N = O$ synthesis in humans. The importance of other mediators both locally and systemically and the exact cells involved in human $\cdot N = O$ synthesis remain to be determined. No clear correlation between circulating substrate, L-arginine, and elevated NO_2^-/NO_3^- production could be demonstrated. Although levels of L-arginine were lower in group II, it is not known if the lower arginine levels contribute to lower NO_2^-/NO_3^- in trauma patients. *In vitro,* $\cdot N = O$ production is dependent on L-arginine concentrations; it is not known, however, if a similar strict dependence exists *in vivo.* L-citrulline levels have been shown to correlate with nitrogen oxide biosynthesis in cell culture for all cell types except hepatocytes.[13] It is likely that the failure of L-citrulline to correlate with NO_2^-/NO_3^- elevation is related to the rapid plasma clearance of this product.

In this study elevated levels of nitrogen oxide were documented in clinically septic patients. Trauma patients, even when they developed clinical sepsis, maintained nitrogen oxide levels that were lower than levels found in normal controls. An inverse correlation was found between systemic vascular resistance and nitrogen oxide levels. These data suggest that nitrogen oxide may be an important mediator in the control of vascular tone.

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