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Urinary Nitrite and Nitrate Concentrations in Patients with Idiopathic Persistent Pulmonary Hypertension of the Newborn and Effect of Extracorporeal Membrane Oxygenation

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ABSTRACT

Persistent pulmonary hypertension of the newborn (PPHN) often requires extracorporeal membrane oxygenation (ECMO), during which time pulmonary vascular resistance gradually declines. Nitric oxide (NO) is a recently recognized pulmonary vasodilator, but its role in PPHN is unknown. We tested the hypothesis that the concentrations of the urinary metabolites of NO, *i.e.* nitrite and nitrate, are reduced in patients with PPHN and increase during ECMO as the PPHN resolves. Eight newborn infants with PPHN on ECMO were studied. Daily urinary concentrations of nitrite/nitrate were measured. We found that mean urinary concentrations of nitrite/nitrate were lower in patients with PPHN than in 47 controls without pulmonary disease ($p < 0.005$). Urinary nitrite/nitrate concentration showed an initial increase after initiation of ECMO. However, a decrease to concentrations still lower than controls occurred on the day before decannulation. We conclude that intrinsic NO production is significantly lower in patients with PPHN than in controls but increases with oxygenation. We speculate that decreased urinary NO metabolite concentrations imply a role for NO deficiency in the pathogenesis of PPHN. (***Pediatr Res* 37: 31–34, 1995**)

Abbreviations

PPHN, persistent pulmonary hypertension of the newborn
ECMO, extracorporeal membrane oxygenation
NO, nitric oxide
TPN, total parental nutrition

Idiopathic PPHN is a disease of unknown etiology characterized by the combination of pulmonary hypertension, right-to-left shunt, and a structurally normal heart. It tends to occur in term or postterm infants. The therapy for this disease includes oxygen, hyperventilation, vasodilator drugs to increase pulmonary blood flow, and, in extreme circumstances, ECMO (1). During ECMO, the underlying pathophysiology of the elevated pulmonary pressure usually resolves.

The NO radical appears to be important as an autocrine-paracrine regulator in many systems including endothelium, vascular smooth muscle, macrophages, and platelets (2). The amino acid L-arginine and molecular oxygen are cosubstrates for the enzyme NO synthase, which yields stoichiometric amounts of L-citrulline and NO (2). NO vasodilates the pulmonary vasculature, and a developmentally regulated increase in pulmonary endothelial NO production during late gestation and the early neonatal period has been shown in a lamb model (3). A significant increase in lobar pulmonary arterial pressure can be achieved by treatment with $N\omega$-nitro-L-arginine methyl ester, an NO synthesis inhibitor in the cat (4). These findings are consistent with endogenous NO synthesis acting as a mediator of pulmonary arterial resistance. Inhaled NO has been recently used for efficient selective treatment of pulmonary hypertension in newborn infants (5, 6). Inhaled NO diffuses into pulmonary smooth muscle cells, activating guanylate cyclase, which results in activation of protein kinases and smooth muscle relaxation (5). Previous animal studies have shown that inhalation of 20–160 ppm of NO could resolve induced hypoxic pulmonary hypertension (7, 8).

Because inhaled NO is an experimental drug, many centers still use ECMO as a rescue measure for infants with PPHN in whom the other therapeutic measures have failed. While infants are receiving ECMO, intrinsic pulmonary mechanisms overcome the pulmonary hypertension, resulting in "spontaneous" vasodilatation (9). The nature of these mechanisms is unclear. We hypothesized that endogenous NO production, as measured by urinary NO metabolites (nitrites and nitrates), would *1*) be significantly reduced in patients with idiopathic

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pulmonary hypertension of the newborn and 2) would increase in temporal relation to the clinical improvement of the patients when treated with ECMO. If these hypotheses prove true, NO deficiency may play a role in the pathogenesis of PPHN.

METHODS

Patients with a diagnosis of idiopathic pulmonary hypertension who were treated by ECMO ($n = 8$, gestational age 37–40 wk, postnatal age 0–2 d) were enrolled in the study. Approval of the Children's Hospital Medical Center Institutional Review Board for Human Investigations was granted. Daily urine samples were collected beginning on the first day of ECMO and continued until ECMO therapy was discontinued. In the first three patients, spot morning urine samples were collected and kept at −20°C until the assay was performed. In the other five patients, 24-h urine collections were performed and the samples were stored at −20°C. The urine samples were assayed for total nitrite and nitrate concentrations using the Greiss reagent after reduction of nitrate to nitrite by passage over a copper-plated cadmium column using a modified HPLC method (10). All urine samples were diluted 1:20 in distilled water and 1 mL was injected into the column. Standards were sodium nitrate in distilled water (0.5–100 μmol/L) and blank was distilled water. The assay had an intraassay coefficient of variation of 3.4% at 8 μmol/L and response was linear over the standard curve. Comparison of absorbance measurements generated by standard curves of sodium nitrite and sodium nitrate when each was passed over the cadmium column showed that conversion of nitrate to nitrite was 100%. The relationship of absorbance measurements from nitrate with those from nitrite (both 0–100 μmol/L) was $y = 1.015x - 3.25$ ($r = 0.999$). Serial dilutions of urine in distilled water showed parallelism to the standard curve when assayed. When nitrate (0–100 μmol/L) was added to urine, the relationship of the measured concentration (y) to the expected concentration (x) was $y = 0.82x - 4.27$ ($r = 0.999$).

Urinary creatinine was also measured in the samples from the last five patients using an autoanalyzer method (VP Super-Systems, Abbott Laboratories, Irving, TX). The total nitrite/nitrate concentrations were corrected for renal function by dividing by the urinary creatinine concentration.

To establish control values, 47 spot urine samples were obtained from neonatal intensive care unit patients of various gestational ages (25–40 wk), birth weights (595–2944 g), and postnatal ages (3–54 d). These patients were preterm and term infants who were not suffering any respiratory disease or diarrhea at the time of urine collection because these conditions were reported to alter urinary nitrite/nitrate concentrations (11). These urine samples were assayed for nitrite/nitrate and creatinine concentrations using the same methods.

All patients were receiving TPN. The control group was receiving either formula or TPN. TPN fluid was assayed for nitrite/nitrate using the modified HPLC method. Concentrations were below the limit of detection for the method (<0.5 μmol/L). There was no difference in urinary nitrite/nitrate concentrations between control subjects receiving formula and those receiving TPN.

Statistical analysis included a nonpaired t test and regression analysis by least square means.

RESULTS

To accumulate data on the normal nitrite/nitrate concentrations in the urine of preterm infants, 47 spot samples were obtained from other babies in the nursery. The combined nitrite/nitrate concentrations of these urine samples were 188.1 ± 117.0 μmol/L (mean ± SD) with a range of 42.4–548.0 μmol/L. When these results were corrected for urinary creatinine, the combined nitrite/nitrate concentrations were 241.5 ± 437.9 μmol/mg creatinine with a range of 16.4–2490 μmol/mg creatinine. There was no correlation of urine nitrite/nitrate with gestational age ($r = 0.01$), postnatal age ($r = 0.02$), or birth weight ($r = 0.07$).

Inasmuch as there was no correlation between nitrite/nitrate concentrations in the control patients and gestational age, postnatal age, or birth weight, we have used the control patient population as one group. We compared the mean urinary nitrite/nitrate concentration of the ECMO patients on the day on which they were placed on ECMO bypass (88.5 ± 43.5 μmol/L, range 18.9–180.7 μmol/L, $n = 8$) with that of the control patients (Fig. 1). However, because the distribution of nitrite/nitrate concentrations in the ECMO population was not expected to be normal, a log transformation was used. The log concentration of corrected urinary nitrite/nitrate concentrations in the ECMO patients (0.9 ± 0.3) was significantly lower than that of the control patients (1.1 ± 0.3, $p = 0.01$) by nonpaired t test. The urinary concentration of nitrite/nitrate on the last day of ECMO treatment (91.2 ± 40.8 μmol/L) was still significantly lower ($p = 0.03$) than that of the control group using untransformed data and after log transformation of the concentrations ($p < 0.005$).

To assess whether there was an increase of urinary nitrite/nitrate concentrations over time while the patients were on ECMO and clinically improving, we plotted the urinary con-

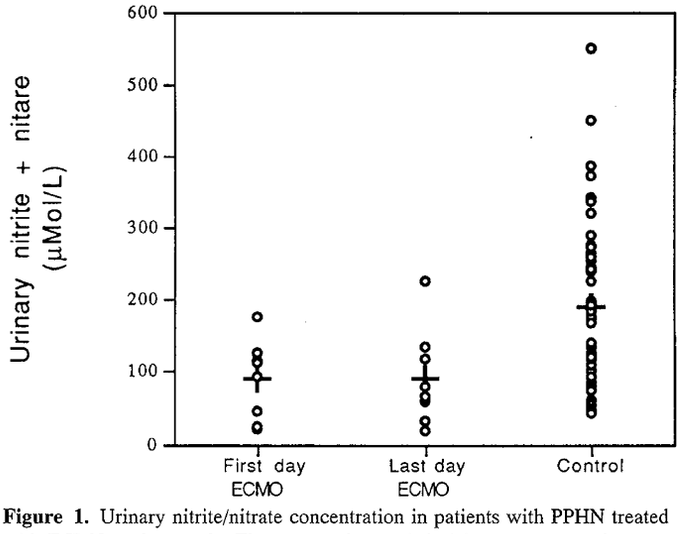

Figure 1. Urinary nitrite/nitrate concentration in patients with PPHN treated with ECMO and controls. The mean urinary nitrite/nitrate concentration was significantly lower in the PPHN patients ($n = 8$) on the first and the last day on ECMO than the mean of the controls ($n = 47$, $p < 0.005$).

centrations as a function of time. The slope of the regression lines in all these patients was not significantly different from zero, indicating that there is no directly related increase over time. Figure 2 shows the apparent lack of change in urinary nitrite/nitrate concentrations over the period in those babies who were treated with ECMO. The urinary concentrations of nitrite and nitrate were corrected for urinary creatinine excretion to control for alterations in renal perfusion that may be occurring over time on ECMO. Figure 3 shows that in some of the infants there is an increase in urinary nitrate/nitrite per mg creatinine beginning 2 to 3 d after initiation of ECMO. However, as can be seen, these concentrations then decrease again.

DISCUSSION

PPHN is a major therapeutic problem, and medications for selective pulmonary vasodilation have been sought. Current medications that decrease pulmonary hypertension (*i.e.* tolazoline) are systemic vasodilators and cause severe hypotension (12). NO, a recently discovered vasodilator, diffuses from vascular endothelium, where it is formed endogenously, into the underlying smooth muscle, where it activates the soluble form of guanylate cyclase and increases intracellular concentrations of cGMP. cGMP in turn activates cGMP-dependent protein kinases and causes smooth muscle relaxation (13). Upon diffusion of the NO into intravascular space, it avidly binds to Hb and is deactivated by forming nitrosylhemoglobin (14, 15). Nitrosylhemoglobin is oxidized to methemoglobin, forming nitrite and nitrate, which are excreted in the urine (16). This rapid metabolism of NO means that inhaled NO has only a local pulmonary effect without causing systemic hypotension.

Urinary nitrite/nitrate concentrations are regarded as accurate measures of whole-body NO production (17, 18). Although dietary nitrate ingestion contributes to urinary nitrate excretion, the majority of urinary nitrate arises from endogenous synthesis (11), *i.e.* from NO production. Several situations increase urinary nitrite/nitrate concentrations including

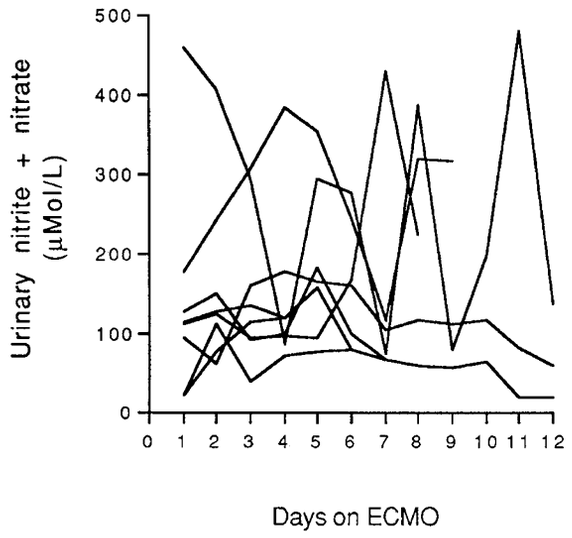

Figure 2. Urinary nitrite/nitrate concentrations in the eight PPHN patients over the time course on ECMO. No significant increase in urinary nitrite/nitrate concentrations was observed over time.

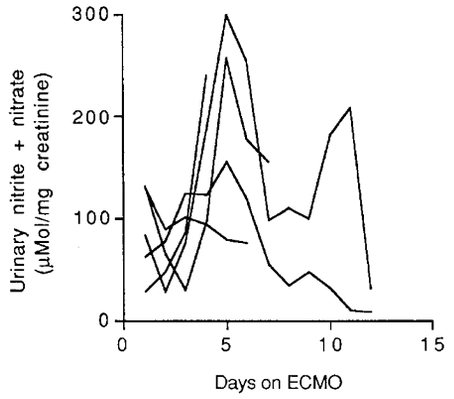

Figure 3. Twenty-four-h urinary nitrite/nitrate concentrations corrected for urinary creatinine, in five PPHN patients over the time course on ECMO.

diarrhea, infection, endotoxemia, exercise, and L-arginine infusion (11). Moreover, L-arginine antagonists decrease urinary nitrite excretion. Although we were unable to control for diet during our study, all infants received TPN and we were unable to detect nitrite/nitrate in this fluid. Therefore, we believe that urinary nitrate excretion reflects endogenous NO synthesis.

The pathogenesis of PPHN is obscure. Immaturity of the normal mechanisms that decrease pulmonary vascular resistance after birth have been implicated as the major cause of PPHN (9). An imbalance of arachidonic acid metabolites, mainly prostacyclin and leukotriene C_4 and D_4 has been suggested as participating in the pathogenesis of PPHN (19, 20). The recent demonstration of a regulated increase in pulmonary endothelial NO synthesis in late gestation and early neonatal life of the lamb (3) suggests that a deficiency of NO may also have a role. Decreased synthesis of NO may have a role in hypoxemic patients with PPHN, because the amino acid L-arginine and oxygen are cosubstrates for the production of NO. Moreover, NO-mediated vasodilation of rabbit pulmonary artery is inhibited by decreased oxygen tension (21).

NO was only recently discovered to mediate vasodilation (2). Despite its value as a therapeutic agent, its role in the pathogenesis of PPHN is still unknown. Our results show that the mean urinary concentrations of NO metabolites, and hence endogenous NO synthesis, are apparently significantly lower in patients who suffer from PPHN than the mean urinary concentrations of control patients. Six of eight patients showed an initial increase in urinary nitrite/nitrate concentrations when placed on ECMO (Fig. 2). Although each infant received 2 U of adult blood at the commencement of ECMO and then more blood was transfused as necessary, it is unlikely that this increased urinary nitrite/nitrate arises from this blood because the increase takes 2–3 d to occur (Fig. 2). Moreover, previous studies have demonstrated a 52% decrease in urinary nitrite/nitrate on the day of transfusion when preterm infants received transfusions (18). Therefore, the increase in urinary nitrite/nitrate may indicate an increase in endogenous NO production that would be consistent with either pulmonary vasodilatation and improved oxygenation or improved oxygenation resulting from the ECMO. Interestingly, an as yet unexplained decline in the urinary nitrite/nitrate concentrations followed, as these infants became ready for discontinuation of ECMO support.

However, at this time, presumably the flow rate and hence the oxygen received from ECMO was being reduced, which may have led to the decrease in NO synthesis. Indeed, when weaned from ECMO, these infants still had significantly lower urinary concentrations of nitrite/nitrate than the controls despite resolution of PPHN.

From these findings, we suggest that there is decreased endogenous production of NO in the PPHN infant. These studies *per se* cannot establish whether the decreased oxygenation secondary to PPHN is responsible for decreased NO synthesis or whether this decreased urinary nitrate represents a failure of the intrinsic mechanisms that normally lead to a developmentally regulated increase in NO production through the late stages of gestation and the early neonatal period (3) and that result in PPHN. However, increasing oxygenation from ECMO or from resolution of PPHN does appear to be associated with increased NO synthesis. NO production may be a key factor in the production of the normal pulmonary vasodilatation that occurs after birth.

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