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Inhaled Nitric Oxide Increases Urinary Nitric Oxide Metabolites and Cyclic Guanosine Monophosphate in Premature Infants: Relationship to Pulmonary Outcome

Philip L. Ballard, MD, PhD¹, Roberta L. Keller, MD¹, Dennis M. Black, PhD², David J. Durand, MD³, Jeffrey D. Merrill, MD³, Eric C. Eichenwald, MD⁴, William E. Truog, MD⁵, Mark C. Mammel, MD⁶, Robin Steinhorn, MD⁷, Rita M. Ryan, MD⁸, Sherry E. Courtney, MD⁹, Hart Horneman, BS¹, Roberta A. Ballard, MD¹, and Investigators of TOLSURF Pilot and TOLSURF

¹Department of Pediatrics, University of California, San Francisco, California

²Department of Epidemiology and Biostatistics, University of California, San Francisco, California

³Division of Neonatology, Children's Hospital and Research Center Oakland, Oakland, California

⁴Department of Pediatrics, University of Texas Medical School, Houston, Texas

⁵Department of Pediatrics, Children's Mercy Hospitals and Clinics, University of Missouri-Kansas City School of Medicine, Kansas City, Missouri

⁶Department of Pediatrics, Children's Hospital and Clinics of Minnesota, St. Paul, Minnesota

⁷Department of Pediatrics, University of California Davis Children's Hospital, Sacramento, California

⁸Department of Pediatrics, Medical University of South Carolina, Charleston, South Carolina

⁹Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas

Abstract

Objective—Inhaled nitric oxide (iNO) has been tested to prevent bronchopulmonary dysplasia (BPD) in premature infants, however, the role of cyclic guanosine monophosphate (cGMP) is not known. We hypothesized that levels of NO metabolites (NOx) and cGMP in urine, as a

Address for correspondence Philip L. Ballard, MD, PhD, Suite 150, 3333 California St, San Francisco, CA 94118, (ballardp@peds.ucsf.edu).

Note

Other contributing TOLSURF Pilot and TOLSURF investigators: Elizabeth Rogers, MD (University of California San Francisco, San Francisco CA); Anne Marie Reynolds, MD (Women and Children's Hospital of Buffalo, Buffalo, NY); Aruna Parekh, MD (Stony Brook University Hospital, Stony Brook, NY); Dennis Mayock, MD (University of Washington, Seattle, WA); Mark Hudak, MD (University of Florida College of Medicine at Jacksonville, UF Health and Wolfson Children's Hospital, Jacksonville, FL); Beatrice Stefanescu, MD, Jennifer B. Helderman, MD, T. Michael O'Shea, MD (Wake Forest School of Medicine and Forsyth Medical Center, Winston Salem, NC); Michael Georgieff, MD, Catherine M. Bendel, MD (University of Minnesota Amplatz Children's Hospital, Minneapolis, MN); Andrea Lampland, MD (Department of Pediatrics, Children's Hospital and Clinics of Minnesota, St. Paul, MN); Ellen Bendel-Stenzel, MD, Neil Mulrooney (Children's Hospitals and Clinics of Minnesota, Minneapolis, MN); Frances Koch, MD (Medical University of South Carolina, Charleston, SC); Reddy Dhanireddy, MD (University of Tennessee-Memphis, Memphis TN); Victor McKay, MD (All Children's Hospital, St Petersburg, FL); Rajan Wadhawan, MD (Florida Hospital for Children, Orlando FL); Nicolas Porta, MD (Northwestern Memorial Hospital, Chicago, IL)

noninvasive source for biospecimen collection, would reflect the dose of iNO and relate to pulmonary outcome.

Study Design—Studies were performed on 125 infants who required mechanical ventilation at 7 to 14 days and received 24 days of iNO at 20–2 ppm. A control group of 19 infants did not receive iNO.

Results—In NO-treated infants there was a dose-dependent increase of both NO_x and cGMP per creatinine (maximal 3.1- and 2-fold, respectively, at 10–20 ppm iNO) compared with off iNO. NO_x and cGMP concentrations at both 2 ppm and off iNO were inversely related to severity of lung disease during the 1st month, and the NO_x levels were lower in infants who died or developed BPD at term. NO_x was higher in Caucasian compared with other infants at all iNO doses.

Conclusion—Urinary NO_x and cGMP are biomarkers of endogenous NO production and lung uptake of iNO, and some levels reflect the severity of lung disease. These results support a role of the NO–cGMP pathway in lung development.

Keywords

nitric oxide; cyclic GMP; bronchopulmonary dysplasia; premature; urine

Inhaled nitric oxide (iNO) is currently used in infants, children, and adults as treatment for pulmonary hypertension, exploiting the selective and potent vasodilation properties of iNO that are mediated through activation of soluble guanylate cyclase to produce the secondary messenger cyclic guanosine monophosphate (cGMP). NO also promotes formation of nitrosylated proteins that act independent of cGMP to affect protein structure, location, and function including transcriptional activity.¹

iNO has been used in clinical trials with premature infants for prevention of bronchopulmonary dysplasia (BPD) and later pulmonary morbidity. The hypothesis underlying this therapy is that iNO corrects a developmental deficiency of endogenous NO production in premature infants and promotes alveogenesis in the injured, immature lung. Benefit from iNO has been observed in some studies, but not in others, which may reflect the dosing regimen and/or racial/ethnic composition of the study group.² Other possible factors in efficacy may be individual variability related to delivery of iNO to the lung periphery, rate of metabolism of NO, or maturity/efficiency of cGMP formation.

Animal studies support a role for NO in lung development. Epithelial and neuronal NO synthase (NOS) are present in lung epithelium of fetal baboons, and both NOS activity and plasma nitrate increase in the third trimester.^{3,4} Infant baboons delivered prematurely are deficient in endogenous NO production, and NOS activity remains suppressed after premature birth.⁵ iNO treatment of these animals increased lung weight and volume, enhanced cell proliferation, and normalized elastin deposition.⁶ In infant rats, downregulation of vascular endothelial growth factor (VEGF) decreased NO production and delayed lung growth and alveolar development; these parameters were restored with iNO treatment.⁷ Using the bleomycin model of lung disease, iNO treatment improved alveolization following lung injury in infant rats.⁸ Prematurely delivered lambs have

diminished levels of endothelial NOS and soluble guanylate cyclase plus findings of reduced alveolar growth and increased respiratory tract resistance, which are preserved with prolonged iNO treatment.^{9–11}

Because NO is rapidly metabolized in the lung and circulation, measurement of NO metabolites (NOx), which include nitrate, nitrite, and nitrosylated compounds, has been used as a surrogate marker of NO production/content. Previously, we reported that iNO treatment at 2–20 ppm in premature infants with lung disease results in a dose-dependent increase in NOx concentrations in plasma and tracheal aspirate.¹² Increases in plasma NOx are also reported for infants with pulmonary hypertension who received iNO.^{13,14} While these findings document delivery and uptake of NO in lungs of iNO-treated infants, there is currently no information on whether levels of cGMP change with iNO as an indication of stimulated activity of soluble guanylate cyclase in lung cells.

In the current study, we hypothesized that levels of NOx and cGMP in urine, as a noninvasive source for biospecimen collection, would reflect the dose of iNO to premature infants and relate to pulmonary outcome near term. We describe an iNO dose-dependent relationship for both NOx and cGMP levels in urine and significant influences of race/ethnicity and severity of lung disease on urinary NOx.

Methods

Patient Populations

Studies of iNO effects were performed on a subpopulation of infants with urine samples from the multicenter Trial of Late Surfactant to Prevent BPD (TOLSURF, 89 infants from 19 clinical sites) and the TOLSURF Pilot (36 infants from 8 clinical sites). Both trials enrolled premature infants who required mechanical ventilation at 7 to 14 days postnatal age. All infants received iNO by the dose regimen of the nitric oxide chronic lung disease (NO CLD) Trial¹⁵ beginning at enrollment and were randomized to receive either Infasurf or sham therapy. Results of TOLSURF Pilot have been published¹⁶ and data collection and analysis of TOLSURF is ongoing. Premature infants enrolled in the Prematurity and Respiratory Outcome Program, who did not receive iNO, were a control cohort (19 infants from three TOLSURF sites); this study enrolled all infants of < 29 weeks' gestation and < 7 days of age without regard to respiratory status. Extensive clinical data were obtained in all three studies. Race/ethnicity was self-reported per National Institutes of Health (NIH) guidelines. Severity of early lung disease was measured using the Respiratory Severity Score (RSS), which is defined as fraction of inspired oxygen (F_{iO_2}) \times mean airway pressure in intubated infants; for nonintubated infants with nasal cannula, RSS was calculated as $F_{iO_2} \times$ liters per minute flow rate. Pulmonary outcome was assessed at both 36 and 40 weeks postmenstrual age (PMA) using a room air challenge for infants in supplemental oxygen < 0.30¹⁷; BPD (moderate/severe) was diagnosed for infants on assisted ventilation, > 0.30 oxygen, on nasal cannula at > 4 L/min in room air or any flow rate with oxygen, and failing the room air challenge. Each of the studies was approved by Institutional Review Boards (IRBs) of participating institutions and required informed consent by the parents.

Urine Collection and Assays

Infant urine samples were collected by placing cotton balls in the diaper for 4 to 8 hours and then extruding the urine using a syringe; samples were stored at -80°C . NO_x were assayed as previously described,¹² using vanadium reduction and a Sievers Nitric Oxide Analyzer (GE Water & Power, Boulder, CO). cGMP and creatinine were assayed using commercial ELISA kits (Cayman Chemical Co., Ann Arbor, MI). Results for NO_x (nmol) and cGMP (pmol) were normalized to creatinine (μg) to adjust for renal excretory function. Because urine is collected from infants over several hours, we tested the effect of incubating urine at 37°C for 3 hours before assay; there was no effect of incubation on NO_x, cGMP, or creatinine values. Samples with $> 20\%$ variability of duplicates were re-assayed.

Data Analysis

In our analyses we used only data obtained 24 hours after a change in dose of iNO. Data with normalized distribution are expressed as mean \pm standard deviation or standard error and were analyzed by Student *t*-test and ANOVA for multiple comparisons; skewed data are expressed as the median value and were analyzed by the Wilcoxon rank-sum test. Time-course and dose-response data were examined by linear regression. Analysis of quartile distribution of NO_x data and pulmonary outcome was made using the two-sample test for equality of proportions with continuity correction.

Results

Studies were performed on urine samples from 125 premature infants who received iNO in the TOLSURF and TOLSURF Pilot trials and 19 premature infants not receiving iNO (control). The two groups were similar with regard to gestational age (range 23–29 weeks) and gender distribution, however, control infants had higher birth weight and less severe lung disease, as reflected by RSS and incidence of BPD at 36 weeks PMA, consistent with different enrollment criteria in the two studies (Table 1).

Control Infants

Fig. 1 presents data for urinary NO_x, cGMP, and cGMP/NO_x for control infants over 6 to 65 days, corresponding to the time interval for data from iNO-treated infants. NO_x values (Fig. 1A) ranged from 0.8 to 5.9 nmol/ μg creatinine and increased slightly with postnatal age. cGMP values (Fig. 1B) ranged from 1.2 to 11.5 pmol/ μg creatinine with a significant overall decline with postnatal age. The cGMP:NO_x ratio (Fig. 1C), which may represent the overall efficiency of NO_x signaling through soluble guanylate cyclase, ranged from 0.4 to 8.6 pmol/nmol and values declined with increasing postnatal age ($p = 0.0001$).

iNO-Treated Infants

Infants in the TOLSURF studies were enrolled at a mean postnatal age of 9 days and received iNO at 20 ppm for 2 to 4 days followed by 7 days each at doses of 10, 5, and 2 ppm. Sixty infants received doses of Infasurf and 65 received sham instillation. There were no significant differences in levels of urinary NO_x or cGMP between treated and control groups; all analyses are accordingly presented for the entire cohort of TOLSURF infants. Urines were collected after enrollment but were not consistently obtained before beginning

iNO. Data were obtained for 1,078 samples from 125 infants with an average of nine samples/infant and including 311 samples off iNO at 18 to 58 days after enrollment. Because of the short time on 20 ppm iNO and the variability in timing of initial urine collections, reliable data for NO_x and cGMP at this dose were obtained for only 19 infants; in this subpopulation there were no significant differences between levels of NO_x, cGMP, and cGMP/NO_x between 20 and 10 ppm iNO. All data are, therefore, presented as mean values for 10–20, 5, 2, and 0 ppm dose iNO.

Fig. 2A shows the time course of NO_x and cGMP in one infant, illustrating the declining concentrations as the iNO dose decreases from 20 ppm and is stopped (0 ppm) 23 days after enrollment. Summary results are shown in Fig. 2B along with mean values for the control infants. Values for NO_x at all doses of iNO are statistically elevated compared with off iNO, and cGMP at 10 to 20 ppm is significantly elevated compared with other doses and off iNO. The ratio of cGMP to NO_x is lower at all iNO doses compared with both off iNO (average decrease of 38%, $p < 0.0001$) and untreated controls. NO_x and cGMP levels for untreated control infants are lower than the 0 ppm values, but cGMP/NO_x is similar. In Fig. 2C, mean NO_x and cGMP values are plotted against the dose of iNO and indicate a nearly linear increase with maximal increases of 3.1- and 2-fold, respectively.

We tested for an association between NO_x and cGMP levels and favorable pulmonary outcome near term, defined as survival without BPD at 36 and 40 weeks PMA, with results shown in Fig. 3. NO_x levels are modestly higher in infants surviving without BPD at all concentrations of iNO with statistical significance reached at 2 ppm for 36 weeks (Fig. 3A) and both 2 ppm and off iNO for 40 weeks outcome (Fig. 3C). In addition, an analysis of NO_x levels at 2 ppm by quartile distribution indicated significantly higher proportion of infants without BPD at 40 weeks in the highest quartile (20/26, NO_x range 6.2–15.8 nmol/ μ g creatinine) compared with the lowest quartile (11/26, NO_x range 0.9–3.2 nmol/ μ g creatinine, $p = 0.02$); these two groups of infants had similar gestational age (25.5 ± 1.1 vs. 25.0 ± 1.1 , non-significant). By contrast, there were no significant differences by pulmonary outcome for levels of cGMP (Fig. 3B, D).

We next tested for relationships between NO_x and cGMP and clinical factors known to influence pulmonary outcome: gender, gestational age, race, and severity of lung disease. Based on the results shown in Fig. 3, we focused these analyses on NO_x and cGMP results at 2 ppm iNO and off iNO. No gender differences were observed (data not shown). With increasing gestational age, there were trends for increasing NO_x levels and decreasing cGMP and cGMP/NO_x; a significant slope occurred only for cGMP at 0 ppm iNO (slope -0.66 pmol/ μ g/d, $p = 0.04$, $r = 0.19$). Fig. 4 shows dose-response plots for iNO and NO_x levels by race. NO_x was higher for Caucasian infants than both Hispanic and African American infants off iNO and at each dose of inhaled gas. However, the increment in NO_x level above baseline at each dose of iNO (e.g., NO_x level at 2 ppm minus the 0 ppm concentration) was similar for all three groups (not shown). There were no racial differences for levels of cGMP or cGMP/NO_x (data not shown). In this cohort, survival without BPD was similar for Caucasian, African American, and Hispanic infants at both 36 weeks PMA (33.3, 36.6, and 38.5%) and 40 weeks (62.5, 56.1, and 61.5%), respectively.

The mean RSS during the first 2 weeks after enrollment was used to assess severity of early lung disease. By regression analysis, both NO_x and cGMP levels at 2 ppm iNO decreased significantly with increasing RSS (Fig. 5). There was no change in cGMP/NO_x at 2 ppm iNO and negative slopes for NO_x and cGMP versus RSS off iNO were not statistically significant (not shown).

Discussion

In this study, we demonstrate the utility of infant urine as a noninvasive source for measuring dose-dependent uptake of iNO and cGMP signaling in the lung. We found that urinary NO_x levels are influenced by racial/ethnic background and severity of lung disease. These results are consistent with a role for both endogenous and inhaled NO, acting through soluble guanylate cyclase, in promoting lung growth and alveolization after premature birth.

Measurement of plasma or urinary NO_x, in particular nitrate, has been explored in children and adults as a potential biomarker in a variety of clinical conditions.^{18–22} In normal adults, Kurioka et al²³ found urinary NO_x levels of 1.1 to 3.0 nmol/μg creatinine that were consistent day-to-day and independent of gender, age, and collection interval. For premature infants, mean urinary NO_x concentrations of 6.1²⁴ and 1.9²⁵ nmol/μg creatinine are reported. The range of NO_x concentrations in our control infants (~1–6 nmol/μg creatinine) is consistent with these previously reported values.

Short-term exposure to exogenous NO as a source of increased plasma nitrate is well documented in both adults^{26–28} and infants.^{12–14} To our knowledge, our results provide the first dose- and time-dependent data for urinary NO_x after prolonged iNO administration. Notably, the fold increases in urinary NO_x with iNO therapy are very similar to those for plasma in a similar premature infant population¹² and in term infants,¹⁴ indicating that urinary NO_x may be a reliable indicator of airway concentrations of iNO. We cannot rule out the possibility that anti-inflammatory effects of iNO, which have been observed in some lung injury studies,^{29,30} result in increased endogenous NO production that contributes to the increased plasma and urinary NO_x.

In numerous tissues NO stimulates soluble guanylate cyclase, increasing levels of cGMP that activate target proteins involved in vasodilation and other responses. The level of urinary cGMP in our control infants is comparable to that reported for newborn infants³¹ and several fold higher than reported in adults.^{32,33} One earlier report found increased plasma cGMP after iNO treatment of adults with right heart failure,²⁸ but there have been no descriptions of urinary cGMP for infants receiving iNO. We find that urinary cGMP levels increase with iNO administration, suggesting activation of soluble guanylate cyclase in lung epithelial and/or smooth muscle cells. Of interest, the cGMP/NO_x ratio is lower on iNO versus off gas, suggesting decreased efficiency of exogenous NO in signaling that may reflect in part the limited diffusion distance of NO from air spaces. In the lung, NO is a mediator of VEGF action on angiogenesis that accompanies formation of new alveoli,^{34,35} and studies in transgenic mice support the involvement of cGMP in lung growth, particularly during hyperoxic lung injury.³⁶ Our data support involvement of this pathway in infants.

iNO therapy is currently widely used with benefit in both children and adults as treatment for pulmonary hypertension secondary to a variety of causes. More relevant to the current findings, iNO has been used in premature infants with persistent, severe chronic lung disease, and suspected pulmonary hypertension.³⁷ iNO therapy for prevention of BPD has been beneficial in some clinical trials but not in others,² which may reflect in part better response for African American infants.^{15,38,39} Our findings that urinary NO_x levels are higher in Caucasian infants, and that there are no racial differences in cGMP levels, suggest that this trend in racial responsiveness is not due to differences in delivery or uptake of iNO or its signaling via cGMP.

We found that lower concentrations of urinary NO_x were associated with more severe lung disease in infants on low (2 ppm) or off iNO. This relationship was observed for NO_x and cGMP during the second postnatal month, with disease severity indicated by RSS, and at term for NO_x with occurrence of BPD. Not unexpectedly, the mean RSS during weeks 2 to 5 was higher for infants who died or had BPD at 40 weeks PMA (4.8 ± 1.9) compared with infants who survived without BPD (3.6 ± 1.4 , $p = 0.003$). Somewhat surprisingly, cGMP concentrations were not related to pulmonary outcome at term. This could reflect greater variability and less change in cGMP than NO_x values, precluding a significant association, or indicate an effect of NO independent of cGMP. In view of the animal data supporting the role of NO in alveogenesis,³⁻¹¹ we speculate that lower NO production on a genetic and/or developmental basis (in lung and possibly other tissues) contributes to delayed alveogenesis and chronic lung disease in premature infants. Alternatively, lower NO_x levels could result secondarily from more severe lung disease. Previously, improved pulmonary outcome was observed for iNO-treated infants with the lowest quartile of predose tracheal aspirate NO_x, perhaps reflecting a deficiency of endogenous NO that was corrected by iNO therapy.¹²

There are some limitations to this study. While the control data establish the increase in NO_x and cGMP observed in treated infants, the relatively small number of control infants is inadequate for analysis of NO_x levels and pulmonary outcome. Because of the interval required for urine collection and variability in initiation of iNO therapy relative to sample collection, we could not reliably define the change in NO_x and cGMP levels relative to pre-iNO in most infants. Due to study design of the trials, we did not collect plasma samples concurrent with urine samples and, thus, cannot provide paired comparisons of levels.

In summary, we report the first data for urinary NO_x and cGMP in premature infants receiving iNO. We found that levels were independent of late surfactant treatment and increased in dose-dependent and parallel manner, which support, but do not establish, that effects of NO on lung development in the human are mediated via cGMP signaling. NO_x concentrations are influenced by racial/ethnic background and by severity of initial lung disease, and infants who developed BPD or died had lower NO_x levels at 1 to 2 months while receiving low levels of iNO and after discontinuing the gas. Reduced endogenous NO production may contribute to persistent lung disease that occurs in a subpopulation of premature infants with initial respiratory failure. Additional studies in infants not receiving iNO are underway to better define this relationship.

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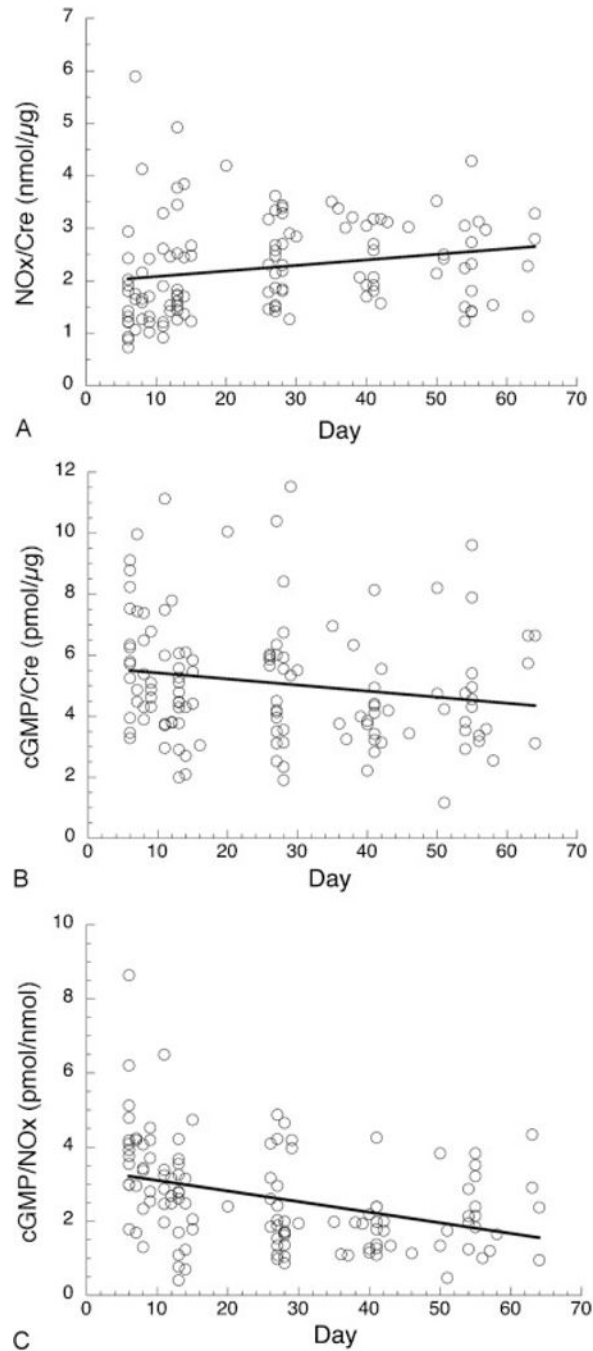


Fig. 1.

Time course for urinary nitric oxide metabolites (NOx) and cyclic guanosine monophosphate (cGMP) in control infants. (A) Regression analysis of NOx/creatinine. There is a slight increase between 6 and 65 days postnatal age (slope = 0.01 nmol/μg/d, $p = 0.03$, $r = 0.20$); mean \pm standard deviation (SD), 2.25 ± 0.92 nmol/μg. (B) Regression analysis of cGMP/creatinine. Values decrease between 6 and 65 days postnatal age (slope = -0.02 pmol/μg/d, $p = 0.05$, $r = -0.18$); mean \pm SD, 5.1 ± 2.0 pmol/μg. (C) Regression analysis of cGMP/NOx. Values decrease with time (slope = -0.03 nmol/μg/d, $p < 0.0001$, $r =$

-0.37); mean \pm SD, 2.62 ± 1.37 pmol/nmol. Creatinine levels (not shown) did not change significantly with time (mean \pm SD, 17.6 ± 7.1 mg/dL). $N = 120$ samples from 19 infants.

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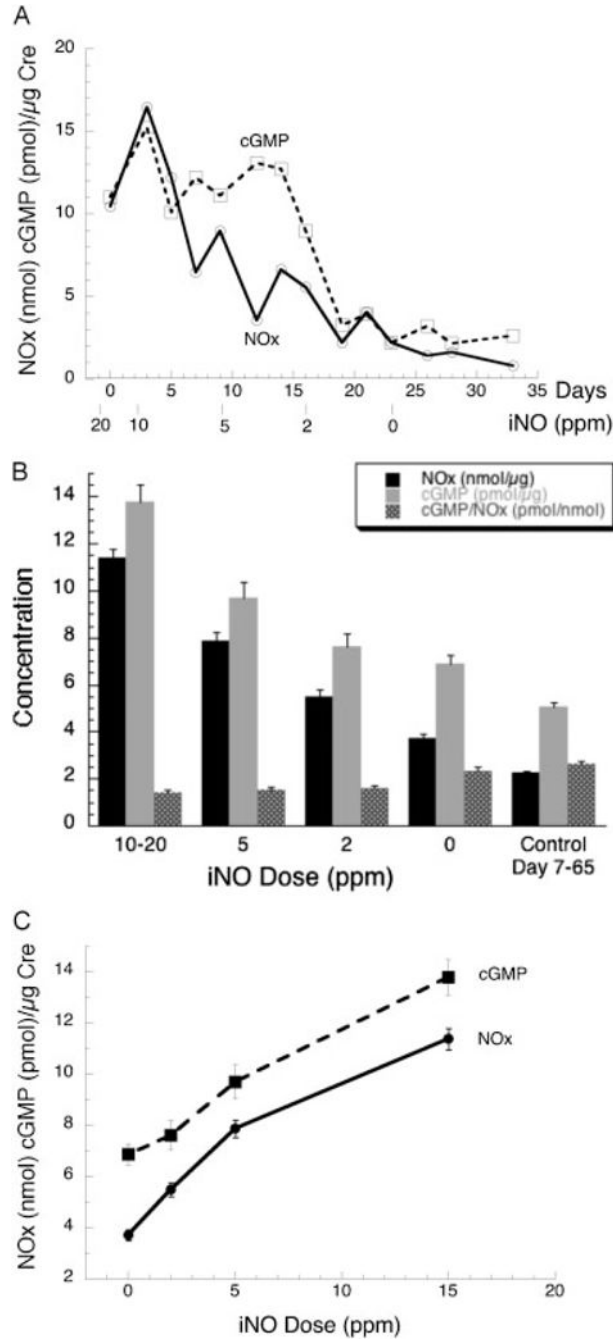


Fig. 2. Time course for urinary nitric oxide metabolites (NOx) and cyclic guanosine monophosphate (cGMP) in inhaled nitric oxide (iNO)-treated infants. (A) Example of the time course for one infant by days in the study and iNO dose: 24 weeks' gestation, 640 g birth-weight infant who was enrolled on postnatal day 7. (B) Summary of levels by iNO dose. Data are mean ± standard error (SE) for 2 to 5 values for 102 to 120 iNO-treated infants at each dose and mean ± SE for 120 samples from 19 control infants over the same age range. For iNO-treated infants, all values for NOx are significantly different ($p < 0.01$)

from each other, the cGMP level at 10 to 20 ppm is greater than at other doses ($p < 0.001$), and cGMP/NOx off iNO is greater than at all iNO doses ($p < 0.001$). Creatinine levels (not shown) increased with postnatal age (mean \pm SD, 12.6 ± 7.5 , slope = 0.13 mg/dL/d, $r = 0.22$, $p < 0.001$). (C) Regression plots of NOx and cGMP values by iNO dose. Parallel slopes are observed with maximal increase for NOx and cGMP of 3.1-fold and 2-fold, respectively; on linear regression, p for slope is 0.02 for both parameters. Data from part (B).

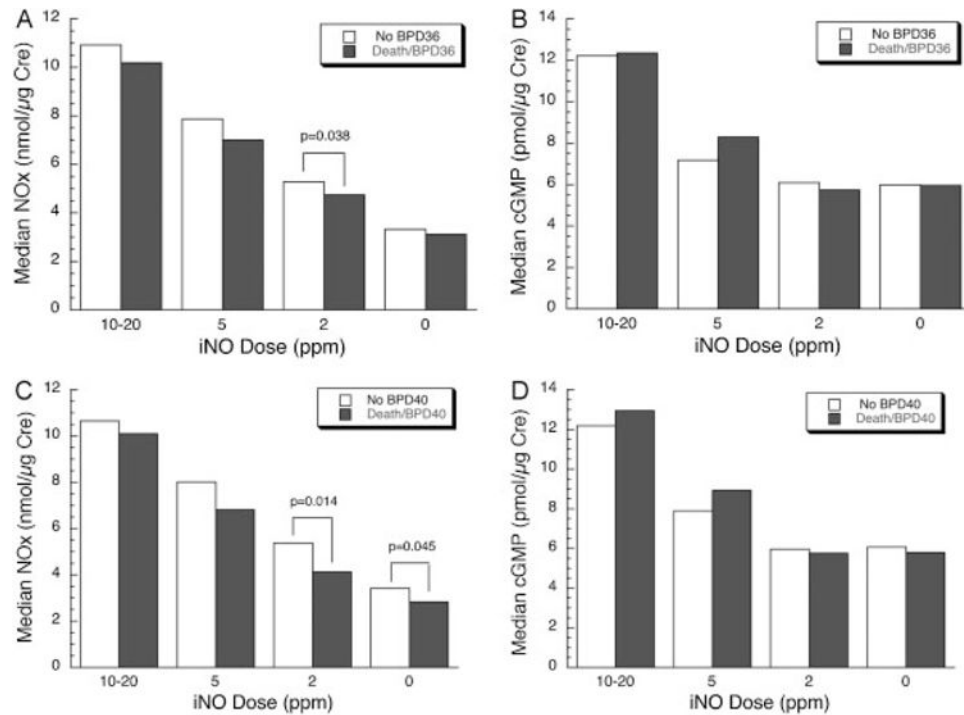


Fig. 3. Nitric oxide metabolites (NOx) and cyclic guanosine monophosphate (cGMP) levels related to pulmonary outcome. (A) Urinary NOx at each inhaled nitric oxide (iNO) dose and diagnosis of moderate/severe bronchopulmonary dysplasia (BPD) at 36 weeks postmenstrual age (PMA). The median NOx level was significantly greater on 2 ppm iNO for infants surviving without BPD. (B) Urinary cGMP and BPD at 36 weeks PMA. There was no significant difference at any iNO dose. (C) Urinary NOx and BPD at 40 weeks PMA. The median NOx level was significantly higher on 2 ppm iNO and off iNO for infants surviving without BPD. (D) Urinary cGMP and BPD at 40 weeks PMA. There was no significant difference at any iNO dose. Data are median values; $n = 42$ to 44 and 65 to 72 for no BPD and death/BPD at 36 weeks, respectively, and 65 to 72 and 41 to 47 for no BPD and death/BPD at 40 weeks, respectively.

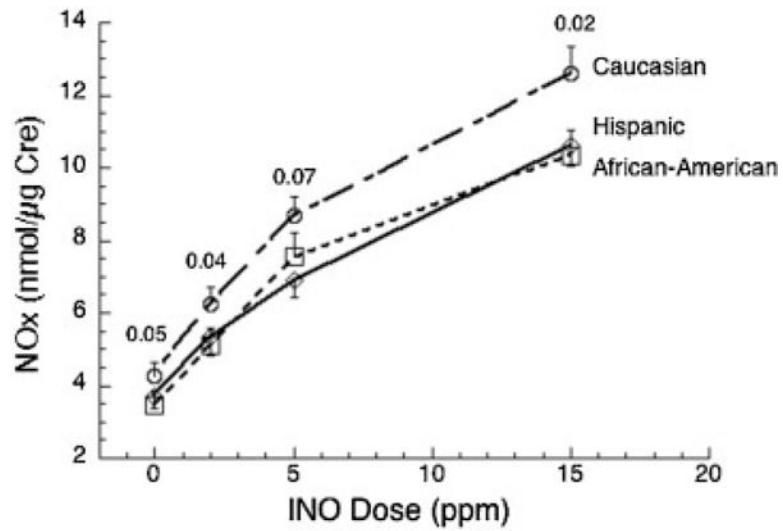


Fig. 4. Nitric oxide metabolites (NOx) levels by maternal race/ethnicity. Concentrations are higher for Caucasian infants compared with African American and Hispanic infants at each inhaled nitric oxide (iNO) dose. There were no differences between groups for gestational age, birth weight, or respiratory severity score (RSS). Data are mean \pm SE with number of infants as shown in Table 1; *p* values are indicated for Caucasian infants versus all other infants.

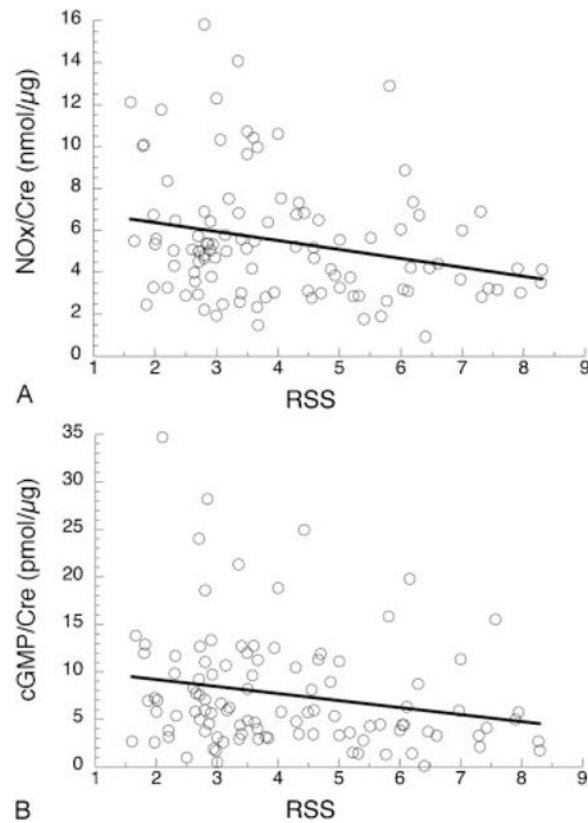


Fig. 5. Respiratory severity score (RSS) and levels of nitric oxide metabolites (NOx) and cyclic guanosine monophosphate (cGMP). (A) NOx concentrations on inhaled nitric oxide (iNO) dose of 2 ppm. By regression analysis, NOx decreases with increasing mean RSS; slope = -0.43 , $p = 0.01$, $r = 0.25$ for 104 infants. (B) cGMP concentrations on iNO dose of 2 ppm. cGMP levels decrease with increasing RSS; slope = -0.70 , $p = 0.05$, $r = 0.20$ for 113 infants. RSS is mean of 3 to 5 daily values during the dosing period (7–14 days).

Table 1

Demographics of study groups

	iNO treated	Control	<i>p</i>
<i>n</i>	125	19	
Gestational age (wk)	25.3 ± 1.1	25.7 ± 1.2	NS
Birth weight (g)	733 ± 168	833 ± 207	0.04
Gender (male/female)	71/54	12/7	NS
Race (caus/aa/his/other)	48/41/26/9	4/8/7/0	NS
Multiples	24 (19.2%)	2 (10.6%)	NS
Initial RSS	4.1 ± 1.7	2.0 ± 1.8	<0.001
Survive without BPD at 36 wk	46 (36.8%)	12 (63.2%)	0.04
Survive without BPD at 40 wk	79 (63.2%)	14 (73.7%)	NS

Abbreviations: aa, African American; BPD, bronchopulmonary dysplasia; caus, Caucasian; his, Hispanic; iNO, inhaled nitric oxide; RSS, respiratory severity score.