Effects of parenteral cysteine and glutathione feeding in a baboon model of severe prematurity

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ABSTRACT

Background: The availability of cysteine for glutathione synthesis is low in premature infants with respiratory distress.

Objective: The effects of gestational age, oxygen delivery, and cysteine infusion or glutathione infusion, or both, on plasma total cysteine and other methionine metabolites were studied in a baboon model of severe premature birth with respiratory distress.

Design: Premature baboons were studied as part of the multi-investigator National Institutes of Health Collaborative Project on Bronchopulmonary Dysplasia. Premature baboons, 125 d (69% of term) or 140 d (78% of term) of gestational age, were maintained in neonatal intensive care units for ≤14 d. Parenteral feeding with or without supplemental cysteine and glutathione infusions was given. Plasma total cysteine, methionine, \( N \)-methylglycine, cystathionine, and the other methionine metabolites were monitored by capillary gas chromatography–mass spectrometry.

Results: Cord plasma total cysteine was the lowest in the 125-d-old premature baboons. Plasma total cysteine decreased in the first 3 d after delivery in the 125-d-old (but not in the 140-d-old) premature baboons even when cysteine was infused. Supplementation with glutathione from the first day of life raised plasma total cysteine markedly. Plasma cystathionine increased in all animals after birth but increased 4-fold in 125-d-old animals with glutathione infusion. At 6 and 10 d postdelivery, the arterial–alveolar oxygen gradient was significantly higher in the 125-d-old animals that received glutathione infusions.

Conclusions: Glutathione, but not supplemental cysteine, infusions prevented the postdelivery decline in plasma cysteine concentrations in premature baboons. Glutathione infusions resulted in marked elevations of plasma cystathionine concentration.


KEY WORDS Baboons, prematurity, glutathione therapy, cystathionine, cysteine, \( N \)-methylglycine

INTRODUCTION

Previously, we showed decreases in plasma total cysteine and liver and plasma glutathione (GSH) in mice under conditions of oxidant stress (1). Human newborns with respiratory distress also show a marked decrease in plasma total cysteine compared with fasted or fed control infants (2). GSH is the most important intracellular reductant and requires cysteine for its synthesis (3). Studies also showed that human premature infants have a GSH deficiency in circulating plasma, which may be more related to gestational age than to respiratory distress (4).

Methionine may be obtained from the diet or synthesized from homocysteine by either of 2 enzymes (5). One reaction, as shown in Figure 1, is catalyzed by methionine synthase (EC 2.1.1.13), which is present in all cells, and requires 5-methyltetrahydrofolate and vitamin B-12. The other reaction, catalyzed by betaine-homocysteine methyltransferase (EC 2.1.1.5), utilizes betaine to methylate homocysteine generating \( N,N \)-dimethylglycine and methionine and is confined mostly to the liver (6). Methionine is the precursor for \( S \)-adenosylmethionine (SAM), which is the source of methyl groups important in the synthesis of creatine, DNA, phospholipids, neurotransmitters, and many other important compounds (5, 7). Homocysteine is at a branch point and either can be remethylated, especially under conditions of starvation and methionine deficiency, or can be condensed with serine to form cystathionine (8). Cystathionine is further cleaved to cysteine and \( \alpha \)-ketobutyrate. Therefore, cysteine can be synthesized from methionine or supplied directly in the diet. Methionine and methyl group deficiencies favor both increased methylation of homocysteine to form methionine and decreased transsulfuration. SAM is a necessary activator of cystathionine \( \beta \)-synthase (EC 4.2.1.22), the enzyme that produces cystathionine from homocysteine. Therefore, if SAM decreases, the synthesis of cysteine should decrease (8). In conditions of excess methionine or excess SAM, a hepatic enzyme, glycine \( N \)-methyltransferase, methylates glycine to form \( N \)-methylglycine (sarcosine) (9).

Recent investigations of the regulation of \( L \)-methionine \( S \)-adenosyltransferase (EC 2.5.1.6) showed that the enzyme is inactivated when GSH is depleted (7, 10). This could result in

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decreased concentrations of SAM and N-methylglycine and decreased conversion of homocysteine to cystathionine and ultimately cysteine. Although such a response to oxidant stress and GSH depletion might conserve ATP, it might also have the negative effect of decreasing the synthesis of GSH. Newborn animals may be especially vulnerable to cysteine and GSH depletion because cystathionine γ-lyase (γ-cystathionase; EC 4.4.1.1) activity is not fully expressed until 1–2 wk after birth (11). We measured plasma concentrations of total cysteine and a complete panel of methionine metabolites in a premature baboon model during treatment of respiratory distress to determine whether gestational age, feeding practices, or oxygen delivery had effects on the concentrations of these amino acids.

MATERIALS AND METHODS

Animal care protocols

The Institutional Animal Care and Use Committee at the Southwest Foundation for Biomedical Research, San Antonio, TX, approved the study. All animals were cared for according to the National Research Council’s guidelines for the care and use of laboratory animals and were part of the multiinvestigator National Institutes of Health Collaborative Project on Bronchopulmonary Dysplasia (12). In this project, ≈100 premature baboons are delivered per year and are each studied by multiple investigators. There were no special treatments of the pregnant baboon mothers. The newborn baboons were delivered by hysterotomy at 125 ± 2 d (69% of term), 140 ± 2 d (78% of term), 160 ± 2 d, or 175 ± 2 d of gestation. Term gestation of the baboon is 184 ± 2 d. After delivery, all animals were resuscitated immediately with positive-pressure ventilation (12, 13). In addition, newborns at 125 d gestation received a dose of artificial surfactant shortly after delivery and at 6 h (Survanta; Ross Products, Columbus, OH) (14). At 125 d gestation, the animals were ventilated with as needed (PRN) oxygen necessary to keep the arterial oxygen tension (P_{aO2}) between 7.3 and 9.3 kPa (55 and 70 mm Hg). Newborns delivered at 140 d gestation were treated with either 100% oxygen (n = 20) or PRN oxygen (n = 18): these 2 groups were designated 140d 100% and 140d PRN, respectively. At both gestational ages, ventilation was adjusted to maintain the partial pressure of carbon dioxide in alveolar gas (P_{aCO2}) at 6.0–7.3 kPa (45–55 mm Hg). After delivery, the baboons were supported in a state-of-the-art neonatal intensive care unit for

![Pathways of methionine metabolism](image)
Previous investigations showed that bronchopulmonary dysplasia (BPD), with characteristic histopathology, develops in the 125-d-old animals after only PRN oxygen exposure (12, 13) and in the 140-d-old animals after 100% oxygen exposure.

The 140-d-old premature baboon model has been studied extensively for >15 y. The PRN and 100% oxygen groups both develop an early requirement for supplemental oxygen, respiratory distress not attributable to other causes, and abnormal chest X-rays that showed reticular lung fields with peripheral air bronchograms and small lung volumes. Light microscopy on tissue samples from animals killed at 24 h showed the classic findings of hyaline membrane disease. If the 140-d-old animals are maintained in the neonatal intensive care unit, they improve during days 3–5; however, the 100% oxygen animals begin to deteriorate at ≈96 h postdelivery. If killed at 6 d, the PRN-treated 140-d-old group has resolved aletectomy and hyaline membrane disease on light microscopy. However, the hyperoxia-treated animals continue to have abnormalities. The difference between the 2 groups is even greater at 10 d. Thus, the 100% oxygen, but not the PRN-treated, 140-d-old premature baboon model reliably develops severe changes compatible with human hyaline membrane disease (12, 13).

The ultrapremature model, 125 d of gestation, was recently developed by the Southwest Foundation (14). These ultrapremature baboons required a dose of exogenous surfactant at birth and at 6 h of age; otherwise, there was 100% mortality. In this model, all animals have chest radiographs consistent with hyaline membrane disease and develop BPD-like chronic lung injury despite being treated with PRN oxygen of 25–40% of the fractional concentration of oxygen in inspired gas (FiO₂). Light microscopy has shown no significant progression of alveolarization, enlarged simplified terminal airspaces, diffuse focal intersaccular fibroplasia, and a lack of extensive airway epithelial hyperplasia–metaplasia (14).

All animals received an intravenous solution containing 5% dextrose and calcium, 150 mL·kg⁻¹·d⁻¹, from birth through 24 h of life. The dextrose solutions were adjusted as appropriate; most animals were able to tolerate a 10% dextrose solution at 150 mL·kg⁻¹·d⁻¹ by 144 h of age. The premature baboons were started on total parenteral nutrition (TPN) at 24 h after birth starting at ≈1 g·kg⁻¹·d⁻¹. The TPN was titrated to 2.5 g·kg⁻¹·d⁻¹ as tolerated over the next 24–48 h. All 140-d-old animals and the first group of 125-d-olds (n = 12) in our investigation received Trava-sol (Clintec Pharmaceuticals, San Juan, Puerto Rico) as the TPN solution. The amino acid solution was changed to TrophAmine (McGaw Pharmaceuticals, Irvine, CA) in late 1996 by the medical director at the Southwest Foundation for the 125-d-old model only. There are some differences in the relative quantities of amino acids in these 2 solutions and those pertinent to the investigation are as follows: methionine, 40 mg in Travasol compared with 34 mg in TrophAmine; serine, 50 compared with 38 mg; glycine, 103 compared with 36 mg; and taurine, 0 compared with 2.5 mg, respectively. Thus, those 125-d-old premature baboons infused with TrophAmine received 85% of the quantity of methionine, 76% of the quantity of serine, 35% of glycine, and a small amount of taurine compared with those receiving Travasol.

Preliminary studies in our 125-d-old group showed that plasma cysteine concentrations fell for 3 d after birth. Thus, the medical director also changed the feeding protocol for the 125-d-old animals only in late 1996 to include cysteine infusion [50 mg added to 100 mL of TPN (125 μmol·kg⁻¹·h⁻¹, or 0.6 mmol·kg⁻¹·d⁻¹)] starting at ≈24–48 h of life as tolerated. These animals were designated 125d Cys (n = 12) in our investigation and were all studied chronologically after the initial 125-d-old group, designated 125d. The 125d, 125d Cys, and 140-d-old animals were all shared with other members of the collaborative group. Therefore, group sizes, sampling time points, feeding, and other conditions of treatment were determined by the needs of the entire collaborative group, although feeding, resuscitation, and ventilation protocols were uniform. However, 6 additional 125-d-old baboons were treated with a GSH infusion as a unique project and in this report are designated 125d GSH. Ultrapure GSH (Sigma Chemical Co, St Louis) was dissolved in saline solution buffered with bicarbonate. The infusion was started at 24 μmol·kg⁻¹·h⁻¹ in the first hours of life and titrated up to 48 μmol·kg⁻¹·h⁻¹ by 24 h and continued throughout the rest of the experiment. The GSH was provided in the minimum volume possible. In addition, the TPN feeding program with TrophAmine and added cysteine was followed. The 125d GSH animals were treated in every other respect exactly like the 125d Cys group. Intravenous feeding of Intralipid (Kabi Pharmacia, Clayton, NC) was started on day 7 postdelivery in the 125-d-old animals. The 140-d-old animals did not receive lipids to maintain historical consistency in the model.

Fluid and acid base status was monitored as described previously (14). Intravenous sodium bicarbonate was given at 2 mmol/kg when the base deficit exceeded −8 mmol/kg. The number of doses and total dose of bicarbonate given were monitored. Baboon blood with heparin was transfused as necessary.

Pulmonary and hemodynamic monitoring was described previously (12). The serial arterial blood gas analysis and ventilator settings were recorded. The alveolar-arterial oxygen tension difference was calculated as follows: A–a gradient = [(PaO₂ – PH₂O) × FiO₂] – PaCO₂ – PₐO₂, where PaO₂ – PH₂O is assumed to equal 95 kPa (713 mm Hg) at sea level in San Antonio, TX. The oxygenation index (OI) was calculated as follows: OI = MAP × FiO₂ × 100/PₐO₂, where MAP is the mean airway pressure.

The methods used to compare pathologic changes by light microscopy in the 125d Cys and 125d GSH animals were described previously (12). The inflation pattern, presence of inflammatory cells, and degree of fibrosis were noted. In addition, the ductus arteriosus was studied grossly at necropsy in the 125d GSH and 125d Cys animals. The patency was described as wide open, open, or with varying degrees of closure.

Assay of amino acids

Blood was collected sequentially at birth, day 1 (≈24 h), day 3 (72 h), day 6, and day 10 and sometimes at day 14 when ventilation was continued for that duration. Some samples from days 2 and 9 were available also. At birth, blood was squeezed from the umbilical cord and heparin was added for amino acid analysis (20:1, blood–heparin ratio; 1 U heparin/L) and this is referred to as the cord plasma or day 0 sample. Plasma was separated by centrifugation at 2000 × g and 4°C for 20 min and frozen in liquid nitrogen. Frozen samples were shipped on dry ice by overnight courier to the University of Colorado and kept frozen until analyzed. Plasma total cysteine, total homocysteine, cystathionine, methionine, N-methylglycine, N,N-dimethylglycine, serine, glycine, and tryptophan were analyzed as described previously (15–18). A stable isotope–labeled internal standard was added for each amino acid quantitated and the sample was reduced to release cysteine and homocysteine from all sulphydryl-binding.
sites so that “total” cysteine and homocysteine were measured. The amino acids were quantitated by capillary gas chromatography–mass spectrometry using single ion monitoring.

Glutathione analysis

Glutathione (reduced + oxidized glutathione) samples were prepared by aspirating 0.5 mL whole blood with heparin into a syringe containing 0.5 mL of 50 mmol serine borate/L, 50 mmol KPO₄/L (pH 7.4), and 17.5 mmol EDTA/L. The sample was centrifuged for 10 min at 600 × g and 4°C then processed through a Sep-Pak C₁₈ column (Waters Associates, Milford, MA) and acid precipitated with trichloroacetic acid to a final concentration of 15%. The supernate was removed and frozen in liquid nitrogen. All sequential samples on days 1, 3, 6, and 10 were processed in a similar manner and held in liquid nitrogen until further biochemical analysis. Samples were obtained only from the 125d Cys and 140-d-old animals and on cord blood from 160-d-old and 175-d-old animals. GSH was analyzed by using a modification of the method ofPastore et al (19). Briefly, the sample was diluted with 15% trichloroacetic acid and then reduced with NaBH₄ and dithiothreitol. After 1 min, the samples were derivatized with N-ethylmorpholine and bromobutanol. After acidification, they were extracted with methylene chloride and the aqueous layer was subjected to HPLC analysis with fluorescence detection. The excitation wavelength was 390 nm and the emission wavelength was 478 nm. A new standard curve was used. For comparisons of 2 groups, Student’s unpaired t test was used. For comparisons of ≥3, a one-way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference test for multiple comparisons was used. The means ± SDs are shown in the text and tables. P < 0.05 was considered statistically significant.

RESULTS

Metabolites in cord blood

Plasma metabolites were quantitated in cord blood samples obtained at the time of delivery from baboons of 4 gestational ages (125, 140, 160, and 175 d) and are shown in Table 1. Plasma total cysteine and total homocysteine were lower and methionine was higher than the values found in 3 plasma samples from adult female baboons. Plasma total cysteine was lower in 125-d-olds than in baboons of greater gestational ages (160 and 175 d). In contrast, plasma cystathionine and methionine were significantly higher at the youngest gestational age. N-Methylglycine was not significantly different at the various gestational ages, in contrast with the related metabolite N,N-dimethylglycine, which was lower at the 2 younger gestational ages. Plasma GSH may have been higher at the older gestational ages but, because of the limited number of samples available, this difference was not significant.

Changes in metabolite concentrations after delivery

Mean total cysteine fell after delivery for 72 h in both the 125d (unsupplemented) and 125d Cys animals despite the infusion of cysteine in the latter group. By 72 h, the total cysteine concentration was only 70% of the value in the cord plasma in the 125d animals, as shown in Figure 2 (103 ± 9 compared with 73 ± 18 μmol/L). For the 125d Cys animals, the value for total cysteine was only 63% of the cord plasma value at 48 h and was similar to that in the 125d group at 72 h. Cysteine was added to the TPN as tolerated between 24–48 h and was usually at full strength at ∼72 h in the 125d Cys group. The resulting plasma total cysteine value increased by 6 d to be significantly greater than that during the previous 3 d (ages 24 and 72 h) in these animals. However, total cysteine value at day 6 was higher in the 125d-old group that did not also receive cysteine. Although the absolute value was lower in the unsupplemented group (121 ± 50 compared with 167 ± 50 μmol/L), the difference was not significant. The total cysteine concentration was stable after 6 d in the 125d Cys group until 14 d, when it was

### Table 1

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>125 d (n = 41)</th>
<th>140 d (n = 53)</th>
<th>160 d (n = 10)</th>
<th>175 d (n = 10)</th>
<th>Adult (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/L</td>
<td>µmol/L</td>
<td>µmol/L</td>
<td>µmol/L</td>
<td>µmol/L</td>
</tr>
<tr>
<td>Cysteine</td>
<td>106 ± 11±2,3</td>
<td>112 ± 16</td>
<td>125 ± 13</td>
<td>125 ± 28</td>
<td>260 ± 33</td>
</tr>
<tr>
<td>Cystathionine</td>
<td>1.6 ± 0.82,4</td>
<td>1.1 ± 0.3</td>
<td>1.0 ± 0.6</td>
<td>0.9 ± 0.3</td>
<td>0.6 ± 0.08</td>
</tr>
<tr>
<td>Methionine</td>
<td>49 ± 147</td>
<td>40 ± 10</td>
<td>42 ± 9</td>
<td>41 ± 14</td>
<td>26 ± 6</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>1.3 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>1.5 ± 0.4</td>
<td>1.5 ± 0.5</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>N-Methylglycine</td>
<td>2.6 ± 1.3</td>
<td>2.4 ± 1.1</td>
<td>2.8 ± 0.6</td>
<td>2.8 ± 0.8</td>
<td>2.4 ± 1.2</td>
</tr>
<tr>
<td>N,N-Dimethylglycine</td>
<td>4.8 ± 1.2,3</td>
<td>5.3 ± 1.5</td>
<td>7.6 ± 1.8</td>
<td>6.9 ± 0.7</td>
<td>4.4 ± 1.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>360 ± 66</td>
<td>354 ± 85</td>
<td>364 ± 105</td>
<td>382 ± 110</td>
<td>350 ± 20</td>
</tr>
<tr>
<td>Serine</td>
<td>139 ± 245</td>
<td>128 ± 23</td>
<td>117 ± 25</td>
<td>118 ± 17</td>
<td>109 ± 14</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>63 ± 24</td>
<td>69 ± 33</td>
<td>65 ± 32</td>
<td>65 ± 31</td>
<td>61 ± 1</td>
</tr>
<tr>
<td>Glutathione</td>
<td>4.2 ± 1.3</td>
<td>4.4 ± 2.0</td>
<td>5.7 ± 3.3</td>
<td>6.9 ± 2.8</td>
<td>NA</td>
</tr>
</tbody>
</table>

1 ± SD.
2 Significantly different from 160 d, P < 0.05.
3 Significantly different from 140 d, P < 0.05.
FIGURE 2. Mean (±SE) metabolite concentrations of cord blood on days 0, 1, 3, 6, and 9 and 10 combined for the 4 groups of baboons: 125d, 125 d gestation (●; n = 12); 125d Cys, 125 d gestation with added cysteine (○; n = 11); 125d GSH, 125 d gestation with added glutathione (▲; n = 6); and 140d PRN, 140 d gestation with oxygen given as needed (△; n = 18). The n values shown are the maximum possible; some values are missing from some time points. Significant differences (P < 0.05) are as follows.

Total cysteine: 125d GSH > all other groups on days 0, 3, 6, and 9 and 10 combined; 140d PRN > 125d and 125d Cys on day 1; and 125d GSH > 125d and 125d Cys on day 1.

Cystathionine: 125d GSH > all other groups on days 3, 6, and 9 and 10 combined; 125d Cys > 140d PRN on day 1; and 125d GSH > 125d and 140d PRN on day 1.

Methionine: 140d PRN > 125d Cys on day 3; 125d > 125d Cys and 125d GSH on day 6; 140d PRN > 125d Cys on day 6; 140d PRN > 125d Cys on days 9 and 10 combined.

Total homocysteine: 140d PRN > 125d and 125d Cys on day 3; 125d > 125d Cys and 125d GSH on day 6; 140d PRN > 125d Cys on day 6; 140d PRN > 125d Cys on days 9 and 10 combined.

N,N-Dimethylglycine: 140d PRN > 125d Cys and 125d GSH on day 3; 140d PRN > 125d Cys and 125d GSH on day 9.

N-Methylglycine: 140d PRN > 125d on day 1; 125d GSH > 125d on day 3.

Glycine: 125d and 140d PRN > 125d Cys on day 6.

Serine: 140d PRN > 125d Cys on day 3; 125d and 140d PRN > 125d Cys on day 6; 140d PRN > 125d Cys on days 9 and 10 combined.
224 μmol/L, which was significantly higher than that during the first 9 d and very similar to adult total cysteine concentrations. Later time points were not available in the 125d group.

In contrast with cysteine infusion, supplementation with GSH was very effective in raising plasma total cysteine. The 125d GSH animals had significantly higher plasma cysteine concentrations (156 ± 32 μmol/L) than did the other 125-d-old animals by 24 h and had higher concentrations than did the 140-d-old group by day 3. The plasma total cysteine concentration increased significantly more by day 3 (283 ± 132 μmol/L) and plateaued during the remainder of the infusion time at a concentration similar to that in adults.

In contrast with the 125-d-old animals, the 140-d-old animals (140d PRN and 140d 100% combined) maintained plasma total cysteine concentrations over the first 3 d of life similar to those of cord blood. These values increased significantly by day 10. On day 1, the 140d PRN group had significantly higher plasma total cysteine values than did the 2 groups of 125-d-old animals that did not receive GSH (127 ± 31 compared with 111 ± 30 μmol/L).

The plasma methionine concentration also decreased sharply in the 24 h after birth and stayed at a relatively low plateau in the 125d Cys animals for 10 d. In contrast with the cysteine-, GSH- or cysteine- and GSH-supplemented animals, the 125d and 140d PRN groups had a rise in plasma methionine on day 3 (40 ± 21 and 61 ± 25 μmol/L, respectively) and maintained a high concentration, which in the 125d group was significantly greater than that in the supplemented animals at day 6 (P < 0.05) and that in the 140d PRN animals at 9 and 10 d of life.

N-Methylglycine concentrations showed a pattern similar to that of methionine, with a small decline in the first 24 h and then an increase in the unsupplemented groups so that the 125d (3.6 ± 1.8 μmol/L) and 140d PRN (3.0 ± 1.3 μmol/L) values were significantly higher than those in the 125d Cys group (1.5 ± 0.7 μmol/L; P < 0.05). The 125d GSH group had a marked increase in N-methylglycine to 9.7 ± 14.4 μmol/L (more than 2.5-fold on day 3) followed by a decline that resulted in intermediate values, as shown in Figure 2. In contrast with N-methylglycine, the postdelivery plasma N,N-dimethylglycine concentration showed a rapid rise in the 140d PRN animals (7.9 ± 3.3 μmol/L) on day 6, and was also significantly higher than that in the supplemented groups, 125d Cys and 125d GSH (3.7 ± 1.4 and 3.4 ± 1.4, respectively). The values were fairly stable in the 2 groups receiving additional intravenous cysteine or GSH.

Plasma cystathionine concentrations increased in all of the animals by 24 h of life and stayed at a high plateau. The 125d GSH animals had a profound 4-fold increase in plasma cystathionine by day 3 (8.9 ± 4.2 μmol/L), which was maintained thereafter. The total homocysteine concentration was not significantly changed over the first 24 h of life but increased in all 4 groups of animals by day 3. By day 6, a pattern similar to that seen for methionine and N,N-dimethylglycine emerged in that the supplemented animals (125d Cys and 125d GSH) had significantly (P < 0.05) lower total homocysteine concentrations than did those that were unsupplemented. Glycine concentrations began to increase within the first 24 h after birth and eventually showed a pattern similar to that for methionine, N-methylglycine, N,N-dimethylglycine, and homocysteine. The supplemented groups were receiving only ~35% of the amount of glycine as the 125- or 140-d-old unsupplemented groups. Thus, the 125d Cys group had lower concentrations at days 6, 9, and 10 than did the unsupplemented animals and the 125d GSH group had intermediate values. There was a slight, nonsignificant decrease in plasma serine in the first 24 h of life and then an increase in the unsupplemented animals so that by day 6, again, the 125d and 140d PRN groups had higher values than did those receiving cysteine.

**Effects of oxygen and gestational age on metabolites after delivery**

The 140d animals were either treated with PRN oxygen or maintained on 100% oxygen because 100% oxygen produces a more severe model of chronic lung disease (12). However, the plasma cysteine and related metabolites measured in this investigation showed no differences in the animals treated with 100% oxygen compared with PRN oxygen (data not shown).

Plasma cysteine concentration did not decline after birth in the 140d animals (both PRN and 100% oxygen analyzed together) and, in fact, the 24-h value was significantly higher than that in cord blood. By age 10 d, the value was significantly higher than any of the previous concentrations. In contrast, the plasma methionine concentration did decline in the first 24 h to its lowest point, and then it increased to its highest concentration at 3 d of life. Except for the lack of a decline in plasma cysteine, the pattern of metabolites over time was very similar for the 140-d-old animals and the 125-d-old animals that had not received cysteine or GSH supplements (Figure 2).

Plasma GSH concentrations were stable over the first 10 d of life in the 140-d-old animals. However, the plasma GSH concentration on day 6 was significantly lower than that of cord plasma in the 125d group (1.6 compared with 4.2 μmol/L; P < 0.05). There was no significant difference in plasma GSH concentrations between the 125d newborns and those either in the 140-d-old animals. No samples were available for the 125d Cys or GSH groups.

**Oxygenation in premature baboons**

The mean arterial-alveolar (A-a) gradient and the OI values for the groups of premature baboons over the first 10 d of life are shown in Table 2. The 3 groups of 125-d-old animals were compared by ANOVA with the 140d PRN group because they all received PRN oxygen supplementation. The 140d 100% group was compared only with the 140d PRN group. On the first day of life the 140d PRN animals had a higher A-a gradient and OI than did any of the 125-d-old groups. The 125d animals had a lower A-a gradient than did the 125d GSH animals. The 140d 100% animals had a higher A-a gradient, but not OI, than did the 140d PRN animals. As expected, the A-a gradient was higher throughout the entire 10 d in the 140d 100% animals. The OI was significantly lower in the 140d PRN group on days 9 and 10. On day 6, the 140d PRN group had the lowest A-a value.

The A-a gradient was significantly higher in the 125d GSH group than in the other 3 groups after 3 d, and by 9 and 10 d the 125d GSH animals had a 3–4-fold higher A-a gradient than did the other groups. The OI was also 2–3 times higher in the 125d GSH group than in the 125d Cys and 140d PRN groups. The A-a gradient and OI were not different in the 125d Cys compared with the 125d group.

The 125d and 125d Cys groups were balanced well in terms of sex. Sex did not affect the A-a gradient, OI, or total cysteine concentrations on days 3 and 6 in the 125d group. The A-a gradient and OI value were lower in females than in males in the 125d Cys group on day 3 (P < 0.05), but there were no significant differences on day 6. The 125d GSH group was unbalanced, with 4 males to 1 female on day 3 and 4 males to 2 females on day 6.
TABLE 2
Arterial-alveolar (A-a) oxygen gradient and oxygen index (OI) values in premature baboons over the first 10 d of life

<table>
<thead>
<tr>
<th>A-a and OI by day</th>
<th>125d (n = 12)</th>
<th>125d Cys (n = 12)</th>
<th>125d GSH (n = 6)</th>
<th>140d PRN (n = 18)</th>
<th>140d 100% (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-a [kPa (mm Hg)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20.7 ± 11.0</td>
<td>25.5 ± 12.1</td>
<td>37.5 ± 10.2</td>
<td>46.4 ± 13.6</td>
<td>64.8 ± 10.5</td>
</tr>
<tr>
<td></td>
<td>(156 ± 83)</td>
<td>(192 ± 91)</td>
<td>(282 ± 77)</td>
<td>(349 ± 102)</td>
<td>(487 ± 79)</td>
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<tr>
<td>OI [cm H₂O/kPa (cm H₂O/mm Hg)]</td>
<td>44.4 ± 23.3</td>
<td>46.6 ± 26.3</td>
<td>57.1 ± 16.5</td>
<td>106.8 ± 39.1</td>
<td>87.2 ± 57.9</td>
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<td>(5.9 ± 3.1)</td>
<td>(6.2 ± 3.5)</td>
<td>(7.6 ± 2.2)</td>
<td>(14.2 ± 5.2)</td>
<td>(11.6 ± 7.7)</td>
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<td>A-a [kPa (mm Hg)]</td>
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<td>3</td>
<td>37.5 ± 10.8</td>
<td>26.7 ± 15.8</td>
<td>50.0 ± 17.2</td>
<td>31.9 ± 23.1</td>
<td>62.2 ± 14.5</td>
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<td>(282 ± 81)</td>
<td>(201 ± 119)</td>
<td>(376 ± 129)</td>
<td>(240 ± 174)</td>
<td>(468 ± 109)</td>
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<td>OI [cm H₂O/kPa (cm H₂O/mm Hg)]</td>
<td>69.2 ± 25.6</td>
<td>44.4 ± 15.0</td>
<td>110.8 ± 88.0</td>
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<td>58.6 ± 53.4</td>
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<td>(5.9 ± 2.0)</td>
<td>(14.4 ± 11.7)</td>
<td>(6.9 ± 4.8)</td>
<td>(7.8 ± 7.1)</td>
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<td>A-a [kPa (mm Hg)]</td>
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<td>6</td>
<td>31.8 ± 17.3</td>
<td>17.6 ± 12.4</td>
<td>37.6 ± 12.5</td>
<td>14.1 ± 14.0</td>
<td>47.1 ± 16.2</td>
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<td>(239 ± 130)</td>
<td>(132 ± 93)</td>
<td>(283 ± 94)</td>
<td>(106 ± 110)</td>
<td>(354 ± 122)</td>
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<tr>
<td>OI [cm H₂O/kPa (cm H₂O/mm Hg)]</td>
<td>51.9 ± 32.3</td>
<td>35.3 ± 23.3</td>
<td>60.2 ± 21.0</td>
<td>33.8 ± 31.6</td>
<td>28.6 ± 41.4</td>
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<td>(6.9 ± 4.3)</td>
<td>(4.7 ± 3.1)</td>
<td>(8.0 ± 2.8)</td>
<td>(4.5 ± 4.2)</td>
<td>(3.8 ± 5.5)</td>
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<tr>
<td>A-a [kPa (mm Hg)]</td>
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<tr>
<td>9, 10</td>
<td>10.6 ± 6.5</td>
<td>37.6 ± 13.6</td>
<td>8.8 ± 8.1</td>
<td>64.6 ± 11.7</td>
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<tr>
<td></td>
<td>(80 ± 49)</td>
<td>(283 ± 102)</td>
<td>(66 ± 61)</td>
<td>(486 ± 88)</td>
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</tr>
<tr>
<td>OI [cm H₂O/kPa (cm H₂O/mm Hg)]</td>
<td>30.1 ± 12.0</td>
<td>53.4 ± 17.3</td>
<td>21.0 ± 12.0</td>
<td>51.1 ± 40.6</td>
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<tr>
<td></td>
<td>(4.0 ± 1.6)</td>
<td>(7.1 ± 2.3)</td>
<td>(2.8 ± 1.6)</td>
<td>(6.8 ± 5.4)</td>
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</table>

1, 2 ± SD; the n shown is the maximum possible (some values are missing from some time points). 125d, 125d gestation; 125d Cys, 125d with supplemental cysteine; 125d GSH, 125d with supplemental glutathione; 140d PRN, 140d with oxygen given as needed; 140d 100%, 140d with 100% oxygen given; NA, not applicable.

1 Significantly less than 140d PRN, P < 0.05 (ANOVA).
2 Significantly less than 125d GSH, P < 0.05.
3 Significantly different from 140d PRN, P < 0.05 (t test).
4 Significantly less than 125d, P < 0.05 (ANOVA).

day 6, although there were no significant differences in the A-a, OI, or metabolites on those days between the sexes.

Feeding schedule

The TPN was titrated to tolerance over the first 3 d of life, starting after the 24 h sample in most of the groups. However, most of the metabolite values were not significantly different in the 125d GSH animals who received one-half strength TPN by 24 h compared with those who did not, or in 3 of the 13 140d PRN animals who also were receiving one-half strength TPN by 24 h. The only difference in metabolites for all groups combined was that N-methylglycine was significantly higher in those not receiving TPN (2.1 ± 0.5 compared with 1.2 ± 0.5 μmol/L; P < 0.05) at 24 h. At 48 h, roughly half of the 125d Cys group had been switched to full-strength TPN and the total plasma cysteine was slightly higher in these animals than in those still receiving half-strength TPN (123 ± 63 compared with 73 ± 17 μmol/L; P = 0.082). In contrast, the methionine concentration was lower (17.3 ± 6.0 compared with 27.9 ± 10.5 μmol/L) in those fed full-strength compared with one-half strength TPN, respectively (P = 0.036). Samples were not collected in the other groups at 48 h. By day 3, most of the animals in the 125d Cys and GSH groups, as well as those in the 140d PRN group, were receiving full-strength TPN. However, in the 125d group there were 3 animals still receiving half-strength TPN compared with 5 receiving full-strength TPN on day 3. The mean total cysteine concentration was significantly lower in the half-strength group (55 ± 8 compared with 81 ± 15 μmol/L; P < 0.05). Total homocysteine also was lower in the half-strength group (1.5 ± 0.5 compared with 2.9 ± 0.5 μmol/L; P < 0.05) and N-methylglycine was nonsignificantly lower (1.0 ± 0.5 compared with 2.4 ± 1.2 μmol/L; P = 0.075) in the same groups, respectively. Methionine also was nonsignificantly lower in the half-strength group (22.9 ± 12.8 compared with 49.1 ± 21.8 μmol/L; P = 0.112). The lower mean plasma cysteine in the 125d group, which was still receiving half-strength TPN, was not associated with a worsening of respiratory status, however, because the A-a gradient was actually higher in those receiving full-strength TPN (43.5 ± 2.7 kPa [327 ± 20.7 mm Hg]) compared with 29.7 ± 2.5 kPa (223 ± 19 mm Hg); P < 0.05.

Effects of GSH infusion on acid-base status

The mean arterial pH was 7.23 on day 1 and was significantly lower than the values on days 3 and 6. There were no significant differences between the 5 treatment groups for days 1 and 3. However, the mean pH in the 125d GSH group was 7.25 on day 6, significantly less than the value in the 125d group, 7.35, and the 140d PRN group, 7.33. There were no significant differences at day 10 or 14 for any of the groups. The total dose of sodium bicarbonate, the number of doses, and the doses given in the first 48 h of life were similar in all treatment groups. Therefore, it did not appear that the infusion of GSH or cysteine caused worsening of acidosis in these premature baboons.

Relations between variables

Pearson correlation coefficients were calculated for the metabolites and some of the other variables. Significant coefficients are shown in Table 3 for the entire group of animals at all time points. The A-a gradient was weakly positively correlated with the cystathionine concentration and N-methylglycine concentration in baboons at all time points analyzed. The A-a gradient was inversely correlated with total cysteine concentrations (r = −0.40, P = 0.005) in the 125d Cys group, but directly correlated in the
TABLE 3
Pearson correlation coefficients between variables in all treated premature baboons

<table>
<thead>
<tr>
<th></th>
<th>A-a</th>
<th>Cysteine</th>
<th>Cystathionine</th>
<th>N,N-Dimethylglycine</th>
<th>Glycine</th>
<th>Homocysteine</th>
<th>Methionine</th>
<th>N-Methylglycine</th>
<th>Serine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystathionine</td>
<td>0.18^2</td>
<td>0.67^2</td>
<td>-0.14^2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N,N-Dimethylglycine</td>
<td>NS</td>
<td>-0.21^2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>NS</td>
<td>0.23^2</td>
<td></td>
<td>0.34^2</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homocysteine</td>
<td>NS</td>
<td>0.21^2</td>
<td></td>
<td>0.16^2</td>
<td>0.20^2</td>
<td>0.70^2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td>0.12^3</td>
<td>0.62^2</td>
<td>0.55^2</td>
<td></td>
<td></td>
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<tr>
<td>N-Methylglycine</td>
<td>0.16^6</td>
<td>NS</td>
<td>0.21^2</td>
<td>NS</td>
<td>0.59^2</td>
<td>0.34^2</td>
<td>0.60^2</td>
<td>0.66^2</td>
<td>0.46^2</td>
</tr>
<tr>
<td>Serine</td>
<td>NS</td>
<td>0.16^2</td>
<td>0.21^2</td>
<td>0.21^2</td>
<td>0.84^2</td>
<td>0.71^2</td>
<td>0.66^2</td>
<td>0.46^2</td>
<td></td>
</tr>
</tbody>
</table>

1 A-a, Alveolar-arterial oxygen tension difference.
2 \( P < 0.000 \), [SPSS (SPSS Inc, Chicago) calculates only to 3 decimal places].
3 \( P = 0.001 \).
4 \( P = 0.002 \).
5 \( P = 0.01 \).
6 \( P = 0.03 \).
7 \( P = 0.009 \).
8 \( P = 0.004 \).

125d GSH and the two 140-d-old groups, with correlation coefficients of 0.35 \( (P = 0.04) \), 0.32 \( (P = 0.013) \), and 0.26 \( (P = 0.04) \) for the 3 groups, respectively. Methionine was positively correlated with A-a in the 125d GSH group \( (r = 0.34, P = 0.045) \) but negatively correlated in the 140d PRN group \( (r = -0.30, P = 0.02) \).

Cysteine and cystathionine were strongly positively correlated in the whole group and were also positively correlated on day 6 for each of the treatment groups. Cysteine was negatively correlated with N,N-dimethylglycine only for the group as a whole for all times points and for day 6, but not in the subgroups of animals. Total homocysteine and total cysteine were positively correlated all times points and for day 6, but not in the subgroups of animals.

Homocysteine and total cysteine were positively correlated in the whole group and in all of the subgroups analyzed. Correlation coefficients ranged as high as \( r = 0.93 \) \( (P < 0.001) \), which was found in the 125d GSH group. For the whole group, N-methylglycine also correlated with cystathionine, glycine, homocysteine, and serine. In contrast, the related metabolite N,N-dimethylglycine did not correlate with N-methylglycine. N,N-Dimethylglycine indirectly correlated with cystathionine and had weak correlations with homocysteine and methionine when the group was analyzed as a whole, but not in any subgroup.

Effects of GSH infusion on the status of ductus arteriosus and lung histology

The status of the ductus arteriosus was examined at necropsy in all 125d GSH and in 5 125d Cys animals who were treated in approximately the same period. All 6 of the 125d GSH and the five 125d Cys animals had wide-open or open patent ductus arteriosi on gross examination. According to light microscopy, there were no differences in the histopathologic changes in the lungs at necropsy of five 125d Cys compared with the six 125d GSH animals. Both groups had the expected changes of BPD, including areas of atelectasis and variable inflation, focal or diffuse interstitial thickening, and the presence of alveolar macrophages.

DISCUSSION

We showed that there may be decreased cysteine availability or increased cysteine utilization in very premature baboons similar to the abnormalities we found previously in both preterm and term human infants in respiratory distress (2). Plasma cysteine fell promptly after birth to its lowest point on the third day of life in the extremely premature 125-d-old animals, confirming the results of other investigations (20, 21). Past investigations reported conflicting results as to whether parenteral cysteine-HCl raises cysteine concentrations in premature infants (20, 22, 23), although most neonatal feeding recommendations include cysteine supplements for parenteral feeding of premature humans (24, 25). However, adding cysteine to the TPN solutions starting from 24–48 h after delivery did not significantly increase the plasma cysteine in this study at 3 d. In contrast, GSH infusion from the first day of life caused a marked rise in plasma total cysteine up to concentrations similar to those in adult baboons, which were also higher than results reported in adult humans (26, 27). The increase in total cysteine seen in the GSH-supplemented animals could be due to catabolism of the GSH by γ-glutamyl transferase or to decreased utilization of cysteine in the synthesis of GSH (26).

There are continuing controversies over cysteine supplementation in severely premature human infants because, despite some evidence that cysteine concentrations increase, there are not a lot of data showing that the clinical condition or the nitrogen retention of the infants is improved (23) and there is a risk of worsening metabolic acidosis. We did not see differences in pH or in the amount of base given in relation to cysteine infusion, confirming the results of another recent investigation of premature humans (28). The cause of the decrease in total cysteine after birth is not known and could be due to either decreased synthesis or increased utilization or excretion of cysteine. Cysteine is a precursor of taurine, which may be an essential amino acid for newborns (25). However, the 125d Cys group received taurine in their amino acid solution, in contrast with the 125d group, and there was no apparent sparing effect on total cysteine concentrations between the 2 groups. It is possible that the ultrapremature baboons required more cysteine for GSH synthesis and thus utilized more of their pool of cysteine. Preterm infants have intact GSH synthesis and it is likely that the availability of cysteine is limiting (29). The finding that GSH infusion markedly increased plasma total cysteine would be compatible with that explanation.
The decrease in total cysteine accompanied by the marked increase in cystathionine is consistent with the results of past investigations that suggest that cystathionine γ-lyase is not fully active until several weeks after birth (11). To our knowledge, the marked elevation in cystathionine with GSH infusion was not reported before. Tracer studies with GSH infusion in healthy adults showed previously that transsulfuration was significantly decreased whereas the remethylation of homocysteine to methionine was increased (27). If transsulfuration had been decreased in the 125d GSH animals, then there must have been impaired clearance of cystathionine to explain the 4-fold increase in cystathionine that was seen in the present investigation. Surgical stress was shown previously to decrease cystathionine γ-lyase activity in adult rat liver (30), and similar unknown mechanisms might have been operating in the instrumented, mechanically ventilated baboons in this study.

Supplementation with cysteine and GSH resulted in lower N,N-dimethylglycine, N-methylglycine, homocysteine, and methionine concentrations at 6 d. The higher N,N-dimethylglycine and N-methylglycine concentrations in unsupplemented animals suggests that betaine-homocysteine methyltransferase was activated, compatible with the results of previously reported investigations (31, 32). However, in this study plasma methionine was actually maintained at a higher concentration in the absence of supplemental cysteine. Because GSH is required to activate L-methionine S-adenosyltransferase in the liver (7), it is possible that the unsupplemented group may have had decreased formation of SAM and, thus, decreased utilization of methionine. A problem with that explanation, however, is that the N-methylglycine concentrations were higher in the unsupplemented animals and correlated very strongly with the methionine concentrations. N-Methyglycine is synthesized from N,N-dimethylglycine but is also synthesized by glycine N-methyltransferase utilizing SAM as a donor to remove excess methyl groups (9). If SAM had been reduced, then it would have been expected that the N-methylglycine concentrations would decrease. The GSH-supplemented animals showed an interesting discrepancy between the concentrations of N,N-dimethylglycine and N-methylglycine in that the former remained at a constant concentration whereas the N-methylglycine increased 4-fold by day 3 postdelivery. This could be explained by augmentation of liver L-methionine S-adenosyltransferase activity by GSH as reported by Pajares et al (33) with resulting buildup of SAM and removal of excess methyl groups in the form of N-methylglycine. A buildup of SAM could also have activated cystathionine β-synthase, which could have shunted homocysteine away from transmethylation and toward cystathionine and cysteine. This could be a partial explanation for the high total cysteine seen with the group receiving the GSH infusion. The significantly lower total homocysteine with GSH supplementation would also be compatible with this explanation. Limitations of the current study are that only plasma rather than liver or lung tissue homogenates were available because of constraints of the in vivo model.

Even though total cysteine was increased by them, the GSH infusions in the 125d GSH group may have been harmful because the A-a gradient was significantly higher on days 6 and 10 than in the 125d Cys group. It is unlikely that changes in animal husbandry or neonatal intensive care practices would explain the differences between the 125d Cys and 125d GSH animals because these animals were studied concurrently under similar conditions.

In addition, there was no difference in the status of the ductus arteriosus in these 2 groups of animals, because both groups had open ducti on necropsy. Therefore, the basis for poorer gas exchange in animals treated with GSH infusions is not apparent. We speculate that increased plasma GSH increased the bioavailability of the endogenous vasodilator nitric oxide (34). Independent of any systemic effects, an increased action of nitric oxide could have caused increased perfusion of poorly ventilated areas of the lung, resulting in increased ventilation-perfusion mismatching and worsening gas exchange. An alternative or additional explanation for poorer gas exchange in animals given GSH infusions could be related to increases in left-to-right shunting through the open ductus arteriosus, resulting in worsening of pulmonary mechanics. Available data indicate that nitric oxide plays a role in maintaining ductile patency (R Clyman, unpublished observation, 1999). Therefore, a GSH effect on the ductus could also be mediated through prolongation of the biological half-life of nitric oxide. Such an effect could also occur through altered metabolism of prostaglandins. Specifically, prostaglandin E2 may help maintain ductile patency and prostaglandin E2 production and release is increased by reduced glutathione (35, 36). Regardless of the precise mechanism, poorer gas exchange in GSH-infused animals could have resulted from nonselective pulmonary vasodilatation, an increased left-to-right shunt through a local action on the ductus, or both. Although the appearance of lungs from treated and untreated groups was not different under light microscopy, the possibility that differences in vascular reactivity in the lung or ductus arteriosus occurred cannot be excluded. We cannot recommend the current GSH infusion as being beneficial in severely premature infants, and further investigations should be accompanied by successful surgical or pharmacologic closure of the patent ductus.

We thank the BPD Resource Center for their technical support of the baboon BPD model, Infrasonics Inc (San Diego) for the loan of pressure-limited infant ventilators to the BPD Resource Center, Ron Clyman for generously supplying data regarding the status of the ductus arteriosus, and Bev Raab and Linda Farb for their technical support in performing the metabolite assays.

REFERENCES


