Concentration of nitric oxide products in bronchoalveolar fluid obtained from infants who develop chronic lung disease of prematurity

Julian R Vyas, Andrew E Currie, David E G Shuker, David J Field, Sailesh Kotecha

Abstract

**Aims**—To determine if nitric oxide (NO) products (nitrate and nitrite) are increased in bronchoalveolar lavage (BAL) fluid obtained from infants who develop chronic lung disease of prematurity (CLD).

**Methods**—One hundred and thirty six serial bronchoalveolar lavages were performed on 37 ventilated infants (12 with CLD, 18 with respiratory distress syndrome (RDS), and seven control infants) who did not receive inhaled NO.

**Results**—During the first week of life nitrate concentration was between 25–31 µmol/l in all three groups. Thereafter, the concentration of BAL fluid nitrate decreased to 14 µmol/l and 5.5 µmol/l, respectively in the RDS and control groups by 14 days of age. In contrast, nitrate in the CLD infants remained constant until 28 days of age (31.3 µmol/l at day 14; p<0.05). In all BAL fluid samples the mean concentration of nitrite was <1.2 µmol/l throughout the first 28 days with no significant differences noted among the three groups.

**Conclusion**—The similar concentration of BAL fluid nitrate in all groups during the first week of life suggest that NO may be important in the adaptation of the pulmonary circulation after birth. However, persistence of nitrate in the BAL fluid of infants with CLD during the second week may reflect pulmonary maladaptation, or, more likely, persisting pulmonary inflammation.


Keywords: chronic lung disease of prematurity; nitric oxide; nitrate; bronchopulmonary dysplasia

Chronic lung disease of prematurity (CLD) is a common sequelae of respiratory distress syndrome (RDS) in preterm infants. Although the risk factors for CLD, which include oxygen toxicity, barotrauma, immaturity, infection, and patent ductus arteriosus, have been identified, the exact pathophysiology of CLD remains unknown. However, persisting pulmonary inflammation has been increasingly reported in association with the development of CLD. Many of these pro-inflammatory cytokines, including tumour necrosis factor α, interleukin-1, and interleukin-6, can increase the expression of inducible nitric oxide synthase (iNOS) in a variety of cell types.

In 1987, Palmer et al. and Ignarro et al. reported that endothelial derived relaxing factor was nitric oxide (NO). It has since been shown that NO is a ubiquitous molecule with many diverse physiological functions, including relaxation of smooth muscle in blood vessels, neurotransmission, effects on immune function and platelet function. NO production may be increased in diseases, such as asthma, pneumonia, and sepsis. NO may therefore have an important role in the pathophysiology of respiratory disease. The role of NO in the pathogenesis of CLD is currently unknown.

NO is formed by the enzyme nitric oxide synthase from L-arginine and it has a short half life of < 1 second in blood. The oxidation reactions of NO are complex and involve nitrogen in many different oxidation states. NO combines extremely rapidly with superoxide radicals to produce peroxynitrite. This reaction occurs preferentially to that of superoxide inactivation by superoxide dismutase. Peroxynitrite is unstable at physiological pH and readily decomposes to form nitrite and nitrate. Both these products may also be formed from nitrogen dioxide, low concentrations of which may be present if endogenous NO is produced in the inflammatory environment of infants who subsequently develop CLD.

As pulmonary inflammation is associated with the development of CLD, and because pro-inflammatory cytokines increase the expression of iNOS, we thought that infants who developed CLD would have increased production of endogenous NO. To assess the production of endogenous NO, we determined the concentration of nitrate and nitrite in bronchoalveolar lavage (BAL) fluid obtained from infants who did and did not develop CLD, but who had not received inhaled NO.

**Methods**

Newborn infants requiring mechanical ventilation were recruited to the study. Three groups of infants were studied: (a) those with CLD, comprising infants who were initially ventilated for RDS and who were oxygen dependent and had chest radiographic changes at 28 days of age; those with RDS, comprising infants who were ventilated for RDS, but who were nursed in air, and had a normal chest x-ray picture by 28 days of age; and controls who required ventilation for non-respiratory reasons and required an FIO2 of less than 0.28.

Infants who were diagnosed as having persistent pulmonary hypertension of the new-
Table 1  Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>CLD</th>
<th>RDS</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>12</td>
<td>18</td>
<td>7</td>
</tr>
<tr>
<td>Female:male</td>
<td>5:7</td>
<td>8:10</td>
<td>1:6</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>780 (630–1070)</td>
<td>1390 (970–3480)</td>
<td>2230 (2180–2970)</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>26 (23–28)</td>
<td>29.5 (27–39)</td>
<td>35 (31–39)</td>
</tr>
<tr>
<td>Antenatal dexamethasone</td>
<td>11 (92%)</td>
<td>11 (61%)</td>
<td>1 (14%)</td>
</tr>
<tr>
<td>Surfactant administration</td>
<td>12 (100%)</td>
<td>18 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Day 1 maximum peak inspiratory pressure (cm H2O)</td>
<td>18 (14–36)</td>
<td>19 (13–22)</td>
<td>16 (15–20)</td>
</tr>
<tr>
<td>Day 1 maximum mean airway pressure (cm H2O)</td>
<td>8.5 (5–18)</td>
<td>9 (5–13)</td>
<td>6 (4–8)</td>
</tr>
<tr>
<td>Day 1 maximum FIO2</td>
<td>0.46 (0.24–1.0)</td>
<td>0.35 (0.25–0.76)</td>
<td>0.25 (0.21–0.31)</td>
</tr>
<tr>
<td>Day 1 maximum oxygenation index</td>
<td>6.4 (2–21.3)</td>
<td>7.9 (1.2–14.2)</td>
<td>2.4 (2.2–3.6)</td>
</tr>
</tbody>
</table>

Median values (range) are shown. CLD = chronic lung disease of prematurity; RDS = respiratory distress syndrome.

Born by echocardiography or who had received inhaled nitric oxide treatment were excluded from the study. Infants of mothers who had infection or prolonged rupture of the membranes (of greater than 48 hours) were also excluded as were infants with infection at any time verified by positive blood or endotracheal secretions cultured.

Informed consent was obtained from the parents and the study was approved by the local research ethics committee.

Bronchoalveolar lavage was performed twice weekly at the time of routine endotracheal tube suction until extubation or 28 days of age, as described before. The infant was placed supine with the head to the left. A 5 FG suction catheter was passed until resistance was felt. One ml/kg of physiological saline was then instilled and aspirated immediately. This process was repeated and the 2 aliquots were pooled. The fluid was centrifuged within 10 minutes of collection at 500 × g for 10 minutes and the supernatant fluid stored at −70°C for subsequent analysis of nitrite and nitrate.

ASSAY METHODS

Nitrite assay

The samples were thawed on ice to prevent degradation of nitrite and were centrifuged in 12 kDa molecular weight cutoff ultrafiltration microcentrifuge tubes (Whatman, Maidstone) at 12000 rpm for 35 minutes to remove proteins which may interfere with the assay. Nitrite concentration was determined using a commercial kit (Nitrate/Nitrite Fluorometric Assay Kit, Cayman Chemicals, Ann Arbor, USA) based on a fluorometric method described by Misko. Nitrite reacted with 2,3-diaminonaphthalene (DAN) to produce a fluorescent compound (excitation 365 nm and emission 450 nm). The limit of detection for this assay was 0.2 μM nitrite.

Nitrate assay

Nitrate was measured according to the manufacturer’s instructions (Nitrate/Nitrite Fluorometric Assay Kit, Cayman Chemicals, Ann Arbor, USA). Briefly, nitrate was reduced to nitrite by the Aspergillus species nitrate reductase (E.C.1.6.6.2) in the presence of essential cofactors NADPH and flavine adenine dinucleotide (FAD). Nitrite was subsequently assayed as described above. The limit of detection was also 0.2 μM.

The median (range) is shown for gestation and birthweight of the infants studied. Because of skewed data, the means (SEM) of the log transformed data for nitrite and nitrate concentrations were calculated for which the anti-logs are illustrated. Multiple groups were compared using the non-parametric Kruskal-Wallis test and comparisons between groups were made using the Mann-Whitney U test. A p value of <0.05 was considered significant.

Results

Patient characteristics are shown in table 1. One hundred and thirty six lavages were performed on 37 infants receiving mechanical respiratory support. Twelve infants (5 girls, 7 boys) developed CLD, 18 had acute RDS (8 girls, 10 boys) and seven infants (1 girl, 6 boys) were ventilated for non-respiratory reasons (4 for abdominal surgery, 1 for tracheo-oesophageal fistula with oesophageal atresia, 1 for mild asphyxia at birth and 1 for apnoea). Antenatal dexamethasone had been given to 11 of 12 mothers in the CLD group, 11 out of 18 in the RDS group, and one of seven in the control group. The birthweight and gestational age of the CLD group was significantly lower than that of the RDS or control groups (birthweight: CLD vs RDS p<0.05, CLD vs controls p<0.05; gestational age: CLD vs RDS p<0.05, CLD vs controls p<0.05).

Surfactant (Survanta, Abbott Laboratories) was given to all the infants in both the CLD and RDS groups but none was given to infants in the control group. The first bronchoalveolar lavage was performed before the first dose of surfactant was administered. There were no differences between the CLD and RDS groups for maximum peak inspiratory pressure, maximum FIO2, mean airway pressure or oxygenation index on day 1 (table 1). As expected, the control group had a significantly lower maximum FIO2 and mean airway pressure (p<0.05) than the other two groups.

Nitrate concentration in BAL fluid for all three groups during the first week of life was between 25 and 30 μmol/l with little differences seen between the groups (fig 1). By 14 days of age, the concentration of nitrate in lavage fluid had decreased in the RDS and control groups to 14.2 μmol/l and 5.5 μmol/l, respectively. In contrast, it remained at around the same level.
in the CLD group until 28 days of age (31.3 μmol/l at day 14, p<0.05 compared with the RDS and control groups. The mean concentration of nitrite in BAL fluid obtained from all three groups throughout the study period was less than 1.2 μmol/l with no differences noted between the groups (fig 2).

Discussion

The results have shown that nitrate concentration in BAL fluid from all three groups was very similar during the first week of life. During the second week of life, nitrate concentration remained high in the infants who progressed to CLD. In contrast, it decreased in the RDS and control groups. Nitrite concentration in BAL fluid was less discriminatory between the three groups, being less than 1.2 μmol/l throughout the study period.

The similar concentration of nitrate in all three groups during the first week of life suggests that there is a common cause for its production, independent of gestational age. In animal models NO is thought to have a role in transition of the fetal circulation to the adult circulation. Abman et al have shown that inhibition of NO synthase by the L-arginine analogue nitro-L-arginine methyl ester (L-NAME) worsened hyperoxic acute pulmonary injury, suggesting that NO may protect the lungs from such injury. However, the mechanism of increased nitrate and nitrite production in BAL fluid with hyperoxia is unclear as iNOS expression was not increased. It may be that there is a balance of beneficial against adverse effects of NO depending on other prevailing factors.

In the inflammatory environment of the lung of infants who develop CLD, endogenously produced NO, as shown by our study, is likely to react with reactive oxygen species to form pro-oxidant products such as peroxynitrite. Peroxynitrite is an unstable product which rapidly nitrates tyrosine residues to form nitrotyrosine. Nitrotyrosine has been used as a marker of the presence of peroxynitrite in many inflammatory disorders, including rheumatoid arthritis, adult RDS, and atherosclerosis. Further preliminary studies by our group have shown that infants who develop CLD (and who are not treated with inhaled NO) have increased nitrotyrosine in proteins obtained from BAL fluid (data not shown). This finding has been confirmed by others. More importantly, the formation of nitrotyrosine may interfere with phosphorylation of tyrosine residues in critical cell signalling pathways which result in cell proliferation and differentiation.

We were unable to show any differences between the groups for BAL fluid nitrite. This is likely to have been due to either the instability of nitrite which is readily converted to nitrate in the oxygen enriched environment of the infant receiving oxygen treatment or due to oxidation to nitrate in storage even at −70°C. Other commonly encountered problems with studies such as ours include regular extubation of study infants as their clinical condition improves, the lack of a satisfactory marker for dilution of epithelial lining fluid, and difficulty of obtaining “normal” data for preterm infants.

In conclusion, our data show that, during the first week of life, nitrate in BAL fluid from ventilated infants was similar in all three groups studied. This suggests that NO may have a role in the transition of the fetal circulation to the postnatal circulation. Pulmonary NO production seems to persist in infants who develop...
CLD, possibly reflecting maladaptation of the pulmonary circulation in these infants, or ongoing pulmonary inflammation.

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