ABNORMAL levels of pulmonary eicosanoids have been reported in infants with persistent pulmon-
ary hypertension (PPH) and congenital diaphragmatic hernia (CDH). We hypothesized that a
dysbalance of vasoconstrictive and vasodilatory
eicosanoids is involved in PPH in CDH patients.
The levels of several eicosanoids in lung homo-
genates and in bronchoalveolar lavage fluid of
controls and rats with CDH were measured after
caesarean section or spontaneous birth. In con-
trols the concentration of the stable metabolite
of prostacyclin (6-keto-PGF\textsubscript{1\alpha}), thromboxane A\textsubscript{2}
(TxB\textsubscript{2}), prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), and leukotriene
B\textsubscript{4} (LTB\textsubscript{4}) decreased after spontaneous birth. CDH
pups showed respiratory insufficiency directly
after birth. Their lungs had higher levels of 6-
keto-PGF\textsubscript{1\alpha}, reflecting the pulmonary vasodilator
prostacyclin (PGL\textsubscript{2}), than those of controls. We con-
clude that in CDH abnormal lung eicosanoid
levels are present perinatally. The elevated levels
of 6-keto-PGF\textsubscript{1\alpha} in CDH may reflect a compensa-
tion mechanism for increased vascular resistance.

Key words: Diaphragmatic hernia, Leukotrienes, Lung,
Newborn animals, Prostaglandins, Pulmonary hyper-
tension, Thromboxanes

Introduction

Eicosanoids are arachidonic acid metabolites
which are produced in different tissues in
human and animal species.\textsuperscript{1} They have been
studied extensively in relation to the perinatal
pulmonary circulation, and have been impli-
cated in several physiologic and pathologic
conditions such as persistent pulmonary hyper-
tension (PPH).\textsuperscript{2-7}

Prostacyclin (PGL\textsubscript{2}), prostaglandin E\textsubscript{2} (PGE\textsubscript{2}),
and thromboxane A\textsubscript{2} (TxA\textsubscript{2}) are all generated
via the cyclooxygenase pathway; the latter has a
pulmonary vasoconstricting activity, whereas
the other two are pulmonary vasodilators.\textsuperscript{6}
Increased circulating levels of PGE\textsubscript{2} may con-
tribute to the pathogenesis of patent ductus arteriosus.\textsuperscript{8} Leukotrienes, which are formed by
the 5-lipoxygenase pathway, may have a key
function in maintaining the elevated pulmonary
vascular resistance in the fetus,\textsuperscript{6,9} although this
could not be confirmed in other studies.\textsuperscript{7,10}

Children with congenital diaphragmatic hernia (CDH) have abnormal morphological develop-
ment of lungs and intrapulmonary blood vessels.\textsuperscript{11,12} The high neonatal mortality and
morbidity is ascribed to the extent of lung hypoplasia and PPH\textsuperscript{13} Increased levels of leuko-
trienes, and of metabolites of PGL\textsubscript{2} and TxA\textsubscript{2}
have been reported in plasma and in broncho-
alveolar lavage (BAL) fluid of both PPH patients
without CDH and children with CDH\textsuperscript{14-21}

We hypothesized that the pulmonary vascular abnormalities in CDH cause abnormal transition of
the pulmonary circulation at birth, associated with a dysbalance of vasoconstrictive and vasodilatory
eicosanoids. Therefore, we studied the content of different eicosanoids—metabolites
from the cyclooxygenase and one from the
lipoxygenase pathway—in lung homogenates and
in BAL fluid of perinatal rats with CDH\textsuperscript{22}
The pulmonary vascular abnormalities in these
rat pups strongly resemble those of children
with CDH\textsuperscript{23}

Materials and Methods

Animal model

Female Sprague-Dawley rats (Harlan Olac, UK) were mated during 1 h (day 0 of gestation). Nine of 18 pregnant rats received 100 mg of
2,4-dichloro-phenyl-p-nitrophenylether (Nitro-
fen: Rohm Haas Company, Philadelphia, PA) in
1 ml of olive oil orogastrically under light ether
anaesthesia on day 10 of gestation;\textsuperscript{22} the remaining
nine rats provided control pups. Nitrofen
induces a large left-sided diaphragmatic defect

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with severe lung hypoplasia in up to 80% of the offspring using this regimen. Food and water were supplied ad libitum during the whole period of pregnancy. Nine pregnant dams were anaesthetized by inhalation of diethylether and a caesarean section was performed on day 22 (nitrofen-exposed litters \( n = 5 \); control litters \( n = 4 \)). While they were kept in the membranes to prevent any breathing, the fetuses died after cervical intersection with a needle, and were weighed. Only rat pups that could be processed within the first 30 min of anaesthesia were included. In the remaining litters (nitrofen-exposed \( n = 4 \), and controls \( n = 5 \)) spontaneous birth on day 22–23 was awaited; within 5–10 min after birth they were killed as described above, and weighed. The presence of a diaphragmatic defect in all nitrofen-exposed rat pups was revealed by autopsy. To obtain a homogeneous group only nitrofen-exposed rat pups with left-sided or bilateral diaphragmatic defects with concomitant severe lung hypoplasia were included, and nitrofen-exposed pups with small right-sided defects or without CDH were excluded. Thus four different groups were studied: CDH rat pups after caesarean section or born spontaneously, and control pups after caesarean section or born spontaneously. Either BAL procedure or dissection of the lungs for preparation of homogenates was then performed.

Lung homogenates

The lungs were removed, stripped of non-pulmonary tissue, separated, weighed, frozen in liquid N\(_2\), and stored at \(-70^\circ\text{C}\) until further processed. They were homogenized in 1 ml in Krebs-solution, and centrifuged at 2500 \(g\). The content of eicosanoids and protein was measured in the supernatant. Ten samples were obtained in CDH pups after caesarean section and four in spontaneously born pups. In controls the numbers were \( n = 11 \) and \( n = 23 \), respectively.

BAL procedure

After opening of the abdominal cavity and assessment of the diaphragmatic defect in the nitrofen-exposed pups, the thorax was opened, and a tracheotomy was performed. A polyethylene catheter (Portex, UK; outer diameter 0.61 mm or 1.0 mm, inner diameter 0.28 or 0.5 mm, for CDH pups and controls respectively) was inserted into the trachea and ligated. A 1 ml-syringe with NaCl 0.9% heated to 37°C was connected to the catheter, and the lungs were washed as previously described. In CDH pups the lungs were washed with seven to 10 times 0.05 to 0.1 ml. Lungs from control pups were washed four times with 0.25 to 0.45 ml, until 1 ml of fluid had been recovered. Samples that were visibly contaminated with blood were excluded. Ten samples were obtained in each CDH group, and 13 samples in each control group. The BAL fluid was directly frozen in liquid N\(_2\) and stored at \(-70^\circ\text{C}\) until assay.

Measurement of eicosanoids and total protein

The following eicosanoids were measured by radioimmunoassay: 6-keto-PGF\(_{1\alpha}\) (the stable metabolite of prostacyclin), PGE\(_2\), TxB\(_2\) (the stable metabolite of TxA\(_2\)), all three generated by the cyclooxygenase pathway, and leukotriene B\(_4\) (LTB\(_4\)), a lipoxygenase-derived metabolite of arachidonic acid. All assays were performed as described in detail previously. Total protein was measured by ELISA at 595 nm using a commercially available protein reagent and protein standard (Instruchemie B.V., Hilversum, the Netherlands).

Data analysis

All eicosanoid levels are expressed as pg/\(\mu\)g protein (mean ± SEM), unless stated otherwise. Differences between groups were tested by Student’s \(t\)-test or by the non-parametric Mann–Whitney test if appropriate. Statistical significance was assumed at 5% level.

Results

All spontaneously born control pups had a regular respiration rate and were pink within minutes after birth. Respiratory insufficiency with gasping and cyanosis was observed in rat pups with CDH, but not in controls, directly after birth.

The lung weights in spontaneously born control pups were significantly lower than those in controls delivered by caesarean section (Table 1; \( P < 0.001 \)). This was not the case in the CDH pups: the lung weights were similar in both groups (Table 1). Control lungs were significantly heavier than lungs in CDH (\( P < 0.001 \)).

Results in lung homogenates

First, data from the left and the right lungs in all groups were analysed separately to determine whether there were consistent differences in
eicosanoid levels between the ipsilateral and contralateral lungs in CDH (data not shown). This was not the case, however, and data from both lungs were therefore pooled.

In CDH pups protein per mg wet lung weight was higher than in controls: 28.8 ± 0.8 µg and 27.5 ± 1.1 µg after caesarean section and spontaneous delivery in CDH, respectively, and 13.5 ± 0.1 µg and 18.5 ± 0.6 µg in controls, respectively (P < 0.001). In controls the protein content per mg lung weight was significantly lower in pups who were delivered by caesarean section than in spontaneously born pups (P < 0.001), but this was not true for the total protein content in both lungs (2020 ± 9 µg after caesarean section and 2060 ± 24 µg after spontaneous birth). In all control pups the total amount of protein was higher than in CDH pups, whose lungs contained 1760 ± 12 µg and 1710 ± 8 µg protein in the respective groups (P < 0.001).

The eicosanoid concentrations per µg protein measured in the lung homogenates are shown in Fig. 1. In controls the concentrations of all eicosanoids per µg protein (Fig. 1A–D) and the total amount of eicosanoids (Table 1) were significantly lower in spontaneously born pups, compared with those in the caesarean section group. In CDH pups the eicosanoid levels were not affected by the delivery mode; this was also the case for the eicosanoid content per mg lung weight (data not shown).

The levels of 6-keto-PGFₑα per µg protein (Fig. 1A; P < 0.001) and the total amount of 6-keto-PGFₑα (Table 1; P < 0.001) were significantly higher in CDH than in controls. In addition, the ratio of 6-keto-PGFₑα to TxB₂ was calculated for each group; in the caesarean section group it was 0.38 ± 0.03 and 0.16 ± 0.01 for CDH and controls, respectively (P < 0.001), and for spontaneously born rat pups 0.39 ± 0.02 and 0.16 ± 0.01, respectively (P < 0.001).

Controls born by caesarean section had higher total TxB₂ than CDH pups (Table 1; P = 0.006) and a tendency towards higher TxB₂ per µg protein (Fig. 1B; P = 0.08). No such differences for TxB₂ were observed in the spontaneously born rat pups. PGE₂ per µg protein was significantly higher in control pups delivered by caesarean section than in CDH pups (Fig. 1C; P = 0.003). The total amounts of PGE₂ were higher in controls than in CDH pups, irrespective of the delivery mode (Table 1). The concentration of LTB₄ per µg protein (Fig. 1D) and the total amount of LTB₄ (Table 1) were significantly higher in controls than in CDH pups after caesarean section (P < 0.001), whereas both groups showed similar LTB₄ levels after spontaneous delivery.

**Eicosanoids in BAL fluid**

In BAL fluid a wide range of eicosanoid concentrations was observed. In controls the concentrations per ml BAL fluid of 6-keto-PGF₁α, TxB₂, and LTB₄ were higher after spontaneous birth than after caesarean section (Table 2; P = 0.01, 0.06, and < 0.001, respectively). However, after correction for dilution, with total protein as marker, only LTB₄ was significantly higher after spontaneous birth (Table 2; P = 0.02). CDH pups showed higher uncorrected concentration levels of TxB₂ and LTB₄ in spontaneously born rats compared with pups delivered by caesarean section (Table 2; P = 0.04 and 0.05, respectively). The same volumes in CDH pups were so small that the protein concentration could only be measured in eight samples (n = 4 per group).
The ratio of 6-keto-PGF\(_{1\alpha}\) and TxB\(_2\) in BAL fluid was significantly higher in CDH pups than in controls who were delivered by caesarean section (7.93 ± 2.95 and 2.22 ± 0.5, respectively; \(P = 0.02\)). A similar tendency was observed for the spontaneously born rat pups (3.63 ± 1.13 for CDH and 1.48 ± 0.32 for controls; \(P = 0.06\)).
Discussion

In the present study higher levels of 6-keto-PGF$_{1\alpha}$, the stable metabolite of the pulmonary vasodilator PGI$_2$, were found in the lungs of CDH pups than in those of controls, irrespective of the mode of delivery. Lungs of CDH pups had similar or lower levels of TxB$_2$, PGE$_2$, and LTB$_4$ than control pups. All eicosanoids studied were higher in the lungs of control pups delivered by caesarean section than in those born spontaneously; this was not the case in CDH pups.

The lower lung weights in spontaneously born controls compared with those delivered by caesarean section probably indicate that lung fluid was absorbed to a large extent during the first adequate breaths. The gasping, irregular breathing movement in the spontaneously born CDH pups have been insufficient to overcome the pressure that is needed to initiate lung expansion and to provide adequate lung aeration and absorption of lung fluid, thus explaining the similar lung weights in both CDH groups.

We studied the eicosanoid concentration both in lung homogenates and in BAL fluid to determine whether the concentration in BAL fluid adequately reflects the situation in the lung tissue. We found widely varying eicosanoid concentrations in BAL fluid of the neonatal rat pups. After correction for protein, only the concentration of 6-keto-PGF$_{1\alpha}$ in control pups showed comparable results between BAL fluid and lung homogenates. The concentration of TxB$_2$ was generally 10 times higher in lung homogenates than in BAL fluid, which suggests that thromboxane is mainly present in the pulmonary vasculature and not into the airspaces. The same may be true for the concentration of LTB$_4$ during intrauterine life. Our data support earlier observations that the eicosanoid content in the pulmonary vasculature is more adequately reflected in tissue homogenates than in BAL fluid. However, the ratio of 6-keto-PGF$_{1\alpha}$ and TxB$_2$ was significantly higher in lung homogenates and in BAL fluid of CDH pups than in that of controls, suggesting that this parameter in BAL fluid reflects the values in lung tissue. A high ratio of 6-keto-PGF$_{1\alpha}$ and TxB$_2$ was also found in BAL fluid of two CDH patients with evidence of PPH (unpublished data).

During normal transition from intrauterine to extraterine life, the pulmonary vascular resistance rapidly declines within the first 30 s, and declines more slowly over the next 10–20 min. The first phase occurs irrespective of prostaglandin synthase blockade by indomethacin, but several studies in lambs and goats indicate that a transient prostacyclin production in the lungs, which is stimulated by tissue stress during establishment of gaseous ventilation and rhythmic ventilation, is important to sustain further pulmonary vasodilatation within the first hours after birth.

The lower levels of all eicosanoids in lung tissue of normal controls compared with CDH pups following spontaneous birth in this study seem to contradict the earlier findings in newborn lambs and goats. Perhaps the described loss of prostacyclin from the lungs shortly before birth continued immediately after birth, and the rat pups died before the prostacyclin concentration began to increase. However, the CDH pups could not survive much longer without artificial ventilation and supplemental oxygen.

Persistent pulmonary hypertension is a serious problem in neonatology which largely contributes to the neonatal mortality and morbidity in isolated cases of PPH, and in children with CDH. Improvement of oxygenation parameters in some children with PPH has been reported after intravenous or inhaled administration of prostacyclin, although other patients seem irresponsible to vasodilator therapy like prostacyclin or inhaled nitric oxide.

Increased levels of 6-keto-PGF$_{1\alpha}$, TxB$_2$, PGE$_2$, and leukotrienes have been reported in plasma and in bronchoalveolar lavage fluid of PPH patients and in plasma of CDH patients with PPH. A decrease in all eicosanoid levels was observed during clinical improvement, especially in patients who were being treated with extracorporeal membrane oxygenation. It has been suggested that LTC$_4$, LTD$_4$, and TxB$_2$ have a pulmonary vasoconstricting activity, whereas 6-keto-PGF$_{1\alpha}$ opposes the hypoxic vasoconstriction.

In a fetal lamb model of chronic intrauterine pulmonary hypertension increased pulmonary levels of 6-keto-PGF$_{1\alpha}$ and TxB$_2$ were detected shortly before, and 2 h after birth. Our study did not reveal significant differences in eicosanoid content in the lungs of the CDH rats during transition from intrauterine to extraterine life; this may be due to the short period of survival after birth and the lack of adequate respiratory movements.

We found increased levels of 6-keto-PGF$_{1\alpha}$ per µg protein, and decreased levels of PGE$_2$ and LTB$_4$ in lung tissue of CDH pups in the caesarean section group. Surprisingly, the concentration of TxB$_2$ per µg protein was similar in
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