PEDIATRIC ORIGINAL

Decreased surfactant phosphatidylcholine synthesis in neonates with congenital diaphragmatic hernia during extracorporeal membrane oxygenation

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Abstract *Purpose:* Congenital diaphragmatic hernia (CDH) may result in severe respiratory insufficiency with a high morbidity. The role of a disturbed surfactant metabolism in the pathogenesis of CDH is unclear. We therefore studied endogenous surfactant metabolism in the most severe CDH patients who required extracorporeal membrane oxygenation (ECMO).
Methods: Eleven neonates with CDH who required ECMO and ten ventilated neonates without significant lung disease received a 24-h infusion of the stable isotope [U-^{13}C] glucose. The ^{13}C-incorporation into palmitic acid in surfactant phosphatidylcholine (PC) isolated from serial tracheal aspirates was measured.

Mean PC concentration in epithelial lining fluid (ELF) was measured during the first 4 days of the study.
Results: Fractional surfactant PC synthesis was decreased in CDH-ECMO patients compared to controls (2.4 ± 0.33 vs. $8.0 \pm 2.4\%$/day, $p = 0.04$). The control group had a higher maximal enrichment (0.18 ± 0.03 vs. 0.09 ± 0.02 APE, $p = 0.04$) and reached this maximal enrichment earlier (46.7 ± 3.0 vs. 69.4 ± 6.6 h, $p = 0.004$) compared to the CDH-ECMO group, which reflects higher and faster precursor incorporation in the control group. Surfactant PC concentration in ELF was similar in both groups.
Conclusion: These results show that CDH patients who require ECMO have a decreased surfactant PC synthesis, which may be part of the pathogenesis of severe pulmonary insufficiency and has a negative impact on weaning from ECMO.

Keywords Surfactant metabolism · Lung injury · Stable isotopes · Surfactant phosphatidylcholine · Congenital diaphragmatic hernia

Introduction

Congenital diaphragmatic hernia (CDH) may result in severe neonatal pulmonary insufficiency due to lung hypoplasia, pulmonary hypertension and the sequelae of ventilatory support [1]. The mortality is variable depending on the case and center selection and the ability to use a variety of treatment modalities [2–4].

Several animal models, such as the surgically created CDH lamb model and the nitrofen-induced CDH rat model, suggest decreased surfactant parameters in lung tissue, but not on the individual cellular level [5–10]. In vitro studies in isolated type II cells of the CDH lamb model show decreased incorporation of precursor in surfactant phosphatidylcholine (PC), suggesting decreased surfactant synthesis [8, 9].

The scarce data in humans are more controversial. No difference in the amniotic lecithin/sphingomyelin ratio between CDH and control fetuses was found [11], but amniotic surfactant protein A (SP-A) was reported to be lower in CDH fetuses who died or required ECMO [12]. Autopsy studies in CDH infants showed decreased SP-A levels in the lungs [13, 14]. Recently, Boucherat et al. [15] concluded that no surfactant deficiency exists in lungs of fetuses with CDH. Earlier, we showed that the concentration of surfactant PC in bronchoalveolar lavage from CDH patients was not different from control patients [16]. Within this context, Cogo et al. [17–19] studied surfactant metabolism with the use of stable isotopes in infants with CDH on mechanical ventilation, but not on ECMO, and compared them with ventilated control infants. In CDH infants without ECMO they found decreased amounts of PC and SP-A in the tracheal aspirates, a decreased surfactant disaturated PC (DSPC) pool size and a higher DSPC turnover rate, but no decreased synthesis of surfactant DSPC. In an earlier study, we did not find a change in surfactant PC pool size or in turnover rate in high-risk CDH patients who required ECMO when compared to infants with meconium aspiration on ECMO or to ventilated infants without ECMO [20].

Although different studies from the CDH study group did not reveal a benefit of routine use of exogenous surfactant, abnormalities in surfactant metabolism kinetics or secondary inactivation of surfactant due to the method of artificial ventilation cannot be excluded [21, 22]. We hypothesized that surfactant synthesis in CDH patients who require ECMO is decreased compared to control infants. Therefore, we studied the surfactant PC kinetics in vivo in CDH patients who required ECMO with the use of infused stable isotope tracers.

Patients and methods

Patients

The study was performed in the intensive care units of the Erasmus MC-Sophia in a 1.5-year period. We studied two groups of neonates (Table 1): neonates with left-sided CDH who required veno-arterial ECMO (CDH-ECMO) (n = 11) and a group of ventilated term neonates without

Table 1 Patient characteristics

	CDH-ECMO $n = 11$	Control $n = 10$
Gestational age (week)	38.2 ± 0.8	39.0 ± 0.4
Birth weight (g)	$3,077 \pm 150$	$3,189 \pm 237$
Apgar at 5 min	6.4 ± 0.8	7.0 ± 1.5
Male/female	7/4	5/5
Pneumothorax (n)[a]	5	1
Duration of ventilation (days)	28.3 ± 5.1	25.6 ± 9.9
Age at start study (h)	42.9 ± 4.5	137.6 ± 45.5[b]
Survivors (n)	5	8

[a] In all cases occurring before the start of ECMO, sometimes even directly after birth
[b] Significantly different ($p = 0.03$)

significant lung disease (control) (n = 10). The diagnoses of the ten ventilated control patients were varied (gastroschisis, cerebral infarction, small to moderate omphalocele (n = 2), anal atresia, thymic cyst, spina bifida, pentalogy of Cantrell and hypoxic ischemic encephalopathy (n = 2) without primary pulmonary anomaly or signs of abnormal lung development at the time of the study. The study was approved by the local medical ethics committee, and all patients were studied after receiving written informed consent from the parents.

Our institution uses a standardized protocol for treatment of CDH patients, consisting of: planned vaginal delivery in prenatal diagnosed cases, immediate endotracheal intubation at birth, followed by high frequency oscillation (Sensormedics) as the primary ventilator mode. We used low settings: frequency 8–10 Hz, MAP 12–14 mmHg, ΔP 30–40 mmHg and FiO_2 to maintain preductal saturations $\geq 95\%$, P_{CO_2} between 7.0 and 7.5 kPa, allowing permissive hypercapnia. Insertion of a double-lumen nasogastric tube with the application of continuous suctioning for decompression of the stomach, cardiac ultrasound to evaluate right–left shunting and structural defects, fluid restriction (40–60 ml/kg body weight on day 1), no muscle paralysis, sedation with midazolam [0.1 mg/(kg h)] and morphine [5–15 mcg/(kg h)], and support of systemic blood pressure by dopamine and eventually norepinephrine [23].

The entry criteria for ECMO were: gestational age of ≥ 34 weeks, birth weight of $\geq 2,000$ g, mechanical ventilation for <7 days, $AaDO_2$ of >600 torr (>80 kPa) for 8 h and OI >25 for 4 h. A minimal preductal PaO_2 of ≥ 75 torr (≥ 10 kPa) was an additional entry criterion for CDH patients to at least ensure a reasonable chance of weaning from ECMO later. During ECMO, ventilator settings were routinely reduced to a peak inspiratory pressure of 9–12 mmHg (12–16 cm H_2O), positive end-expiratory pressure of ~ 4 mmHg (5–6 cm H_2O), respiratory rate of 10–15 breaths/min, and FiO_2 of 0.25–0.3 using conventional ventilation (Babylog, Drager).

Isotope infusion and sample collection

All included patients received a 24-h continuous intravenous infusion of [U-^{13}C] glucose [0.17 mg/(kg min)], Campro Scientific, Veenendaal, The Netherlands, and Cambridge Isotope Laboratories, Inc., Andover, MA). The start of the isotope infusion was defined as the start of the study ($t = 0$). Before and during the label infusion, 1 ml of blood was drawn every 6 h for determination of ^{13}C-enrichment of plasma glucose. Samples were collected in an EDTA tube, immediately placed on ice and centrifuged at 2,500 rpm for 10 min. The plasma was stored at $-70°C$ until it was analyzed. Tracheal aspirates were obtained every 4–6 h during the time the infant was intubated, with a maximum of 2 weeks. The tracheal suctioning was performed during routine patient care and did not deviate from the normal clinical care, which consisted of tracheal suctioning every nursing shift, or more frequently if it seemed clinically necessary. Tracheal aspirates were immediately placed at $-20°C$, until further processing.

Analytical procedure

The plasma and tracheal aspirates were processed as described before [19, 24]. Briefly, plasma was delipidated, and glucose was isolated and derivatized to an aldonitril pentacetate derivative [25]. Organic extraction was used to isolate surfactant lipids in the tracheal aspirates [26], and surfactant PC was recovered by thin layer chromatography [27]. Isotopic enrichments were measured by mass spectrometry, as described before [24]. The ^{13}C-enrichments were expressed as atom percent excess (APE), which represents the increase in the percentage of ^{13}C atoms in total carbon dioxide from the combusted compounds above baseline enrichment (before infusion). Enrichments were corrected for the contribution of unlabeled carbon atoms added during derivatization.

Determination of composition and concentration

Fatty acid composition of surfactant PC and the amount of surfactant PC were determined by gas-chromatography (Hewlett-Packard, 5890 series II, Amstelveen, The Netherlands) [16]. The concentration of surfactant PC in the epithelial lining fluid (ELF) was calculated by correcting for dilution of the ELF during endotracheal suction: dilution factor = [urea]$_{serum}$/[urea]$_{supernatant}$ [28].

Calculations

Calculations were performed as described before [24]. Time of first appearance (T_{app}) is defined as the time delay between the start of the isotope infusion and the first appearance of the label in surfactant PC. Time of maximal enrichment (T_{max}) is the time where maximum enrichment is reached (E_{max}). Half-life of surfactant PC ($T_{1/2}$) was calculated from the downslope of the enrichment versus time curve. Fractional synthesis time (FSR) of palmitic acid in surfactant PC represents the percentage of the total PC-palmitate pool synthesized de novo from plasma glucose per day.

Data analyses

Data are presented as mean $\pm$ standard error of the mean (SEM). The non-parametric Mann-Whitney U test was applied to compare groups. Spearman correlation was used to evaluate correlations between surfactant kinetic parameters, surfactant PC concentration and patient characteristics. Significance was accepted at a value of $p < 0.05$ (two-sided).

Results

Seven CDH-ECMO patients were diagnosed prenatally and were born in our hospital; four were postnatal referrals. ECMO was started at 15.1 ± 2.7 h after birth and had a duration of 226 ± 34 h. The time on ECMO before the start of the study was 27.8 ± 4.2 h. No baby received treatment with exogenous surfactant. All patients were successfully decannulated. The non-survivors in the CDH-ECMO group died at 28.2 ± 5.5 days of life because of therapy-resistant pulmonary hypertension. Unfortunately, lung body weight ratios are not available as no non-survivor autopsies were authorized. Two patients with hypoxic ischemic encephalopathy in the control group died because of severe cerebral damage on day 6, respectively, day 15 of life. Clinical characteristics are described in Table 1.

Ventilatory parameters

Nine CDH patients were ventilated with high frequency oscillation (HFO) before ECMO; two CDH patients were conventionally ventilated. Ventilation and oxygenation characteristics of the CDH patients before the start of ECMO were as follows: mean airway pressure (MAP) was 13.5 ± 1.4 mmHg (18.3 ± 1.9 cm H_2O), oxygenation index [OI = (MAP $\times$ FiO$_2$)/PaO$_2$I] was 40.6 ± 8.1, and the alveolar-arterial oxygen gradient (AaDO$_2$ = PaO$_2$ $-$ [(713 $\times$ FiO$_2$) $-$ (PaCO$_2$/0.8)]) was 593.0 ± 10.2 torr (79 kPa). The ten control patients were conventionally ventilated for 26 ± 10 days following surgery or because of apneic attacks. These patients had mild ventilatory settings: FiO$_2$: 0.26 ± 0.03, MAP: 5.9 ± 0.6 mmHg

Table 2 Surfactant kinetic parameters

	CDH-ECMO $n = 11$	Control $n = 10$	p Value
FSR (%/day)	2.4 ± 0.3	8.0 ± 2.4	0.04
T_{app} (h)	18.0 ± 2.6	11.7 ± 1.1	0.11
T_{max} (h)	69.8 ± 6.6	46.7 ± 3.0	0.004
E_{max} (APE)	0.09 ± 0.02	0.18 ± 0.03	0.04
Half life (h)	69.0 ± 10.3	63.4 ± 10.7	0.79
E_{gluc} (APE)	2.0 ± 0.1	2.2 ± 0.3	0.75
PC (mg/ml ELF)	6.6 ± 1.9	12.8 ± 2.6	0.78

Phosphatidylcholine (PC) concentration in epithelial lining fluid (ELF) during the first 4 days of the study. Data are expressed as mean ± standard error of mean (SEM)

FSR Fractional surfactant synthesis, T_{app} time of first appearance of the label, T_{max} time of maximal enrichment, E_{max} maximal enrichment expressed as atom percent excess (APE), E_{gluc} mean enrichment of plasma glucose in steady state

The non-parametric Mann–Whitney U test was applied to compare groups

(8.0 ± 0.8 cm H_2O), ventilatory rate: 31 ± 4/min, positive inspiratory pressure (PIP): 14.1 ± 1.3 mmHg (19.2 ± 1.8 cm H_2O) and positive end-expiratory pressure (PEEP): 3.2 ± 0.1 mmHg (4.4 ± 0.2 cm H_2O).

Surfactant kinetic parameters (Table 2)

The ^{13}C-enrichment of plasma glucose was in steady state (E_{gluc}) in all infants between $t = 6$ and 24 h, and was similar in both groups (Table 2). Despite a larger blood volume in ECMO patients due to the extracorporeal circuit, this was expected as the plateau ^{13}C enrichment of glucose during a continuous infusion is determined by the rate of appearance of glucose in the plasma and not by the volume of distribution. Figure 1 shows the ^{13}C-glucose incorporation of surfactant PC
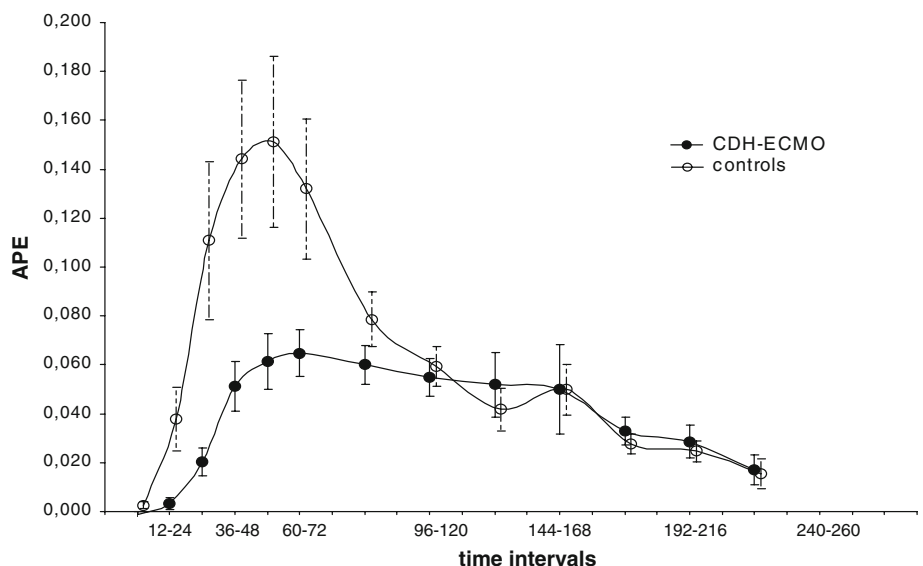
palmitate in sequential tracheal aspirates in the CDH-ECMO patients and control patients. The incorporation of ^{13}C from the precursor glucose into surfactant PC palmitate started somewhat later (T_{app}) in the CDH-ECMO patients, but the difference with controls was not significant. The CDH-ECMO group had a significantly lower maximal enrichment (E_{max}), which also was reached later (T_{max}) compared to the control group. These indices reflect reduced precursor incorporation into surfactant PC in the CDH-ECMO group (Table 2). FSR was more than three times lower in CDH-ECMO patients than in control patients, but the half-life of label disappearance from surfactant PC was not different between groups (Table 2). No correlation between surfactant kinetic parameters and patient characteristics were found, except for a negative correlation between surfactant half-life and gestational age in the CDH-ECMO group ($p = 0.03$).

The surfactant PC concentration in ELF during the first 4 days of the study was equal in both groups (Table 2). No correlations were found between the surfactant PC concentration and the clinical or surfactant PC kinetic parameters.

Surfactant PC concentration in ELF during the first 4 days of the study was not different between the survivors and non-survivors of the CDH-ECMO group (9.5 ± 3.6 vs. 4.5 ± 1.8 mg/ml ELF, $p = 0.27$). There were also no differences in surfactant kinetic parameters between the survivors and non-survivors of the CDH-ECMO group. Non-survivors had a lower gestational age than survivors of the CDH-ECMO group (37.1 ± 0.6 vs. 39.6 ± 0.5 weeks, $p = 0.02$).

In the current study, we also measured fractional surfactant PC synthesis in two CDH patients who did not require ECMO. The kinetic parameters of these two CDH patients not requiring ECMO were: T_{app} 13 and 26 h,

Fig. 1 ^{13}C-enrichment of palmitic acid in surfactant phosphatidylcholine in sequential tracheal aspirates. Neonates with congenital diaphragmatic hernia on extracorporeal membrane oxygenation (CDH-ECMO) (*closed circles*) and control patients (*open circles*) received a 24-h [U-^{13}C] glucose infusion as precursor for phosphatidylcholine-palmitate in surfactant. Data are expressed as mean and standard error of the mean (SEM) of time intervals of 12 and 24 h (with the 24 h interval starting at 72 h)

T_{max} 39 and 43 h, E_{max} 0.17 and 0.23 APE, FSR 7 and 14%/day, PC concentration in ELF 3.7 and 7.6 mg/ml, half-life 56.8 h in one patient only.

Seven CDH-ECMO patients and two control patients of the current study had also received an endotracheal tracer to calculate the total lung surfactant pool size [20]. By multiplying the FSR by the pool size in these patients, we were able to calculate the 'net absolute synthesis rate.' The mean net synthesis rate of seven CDH-ECMO patients was 2.7 ± 0.8 mg/(kg d), which is comparable with that of premature infants [2.7 ± 0.8 mg/(kg d)] [29]. In the two control patients the net synthesis rate was much higher at 11.2 and 11.5 mg/(kg d).

Discussion

We report a significantly reduced surfactant PC synthesis in infants with CDH who require ECMO compared to ventilated control infants. The surfactant PC synthesis was studied in vivo with the use of stable isotopes by measuring incorporation rates of precursor glucose into surfactant PC (FSR 2.4 ± 0.33 vs. 8.0 ± 2.4%/day, $p = 0.04$). These results are in line with studies in animal models that also found decreased PC synthesis in isolated type II cells or lungs from CDH fetuses of the rat and lamb model [8, 9, 30].

In an earlier report in CDH infants who did not require ECMO, we found no decreased fractional synthesis rates of surfactant DSPC in comparison to ventilated control infants [18, 19]. A plausible explanation for the difference in surfactant synthesis is that the CDH infants who require ECMO have more severe pulmonary hypoplasia and/or immaturity than CDH patients who do not require ECMO. Indeed, pulmonary insufficiency in the CDH infants who did not require ECMO in our two previous studies was clearly less severe with a lower OI (7.8–10 vs. 40.6), other ventilator parameters and a lower mortality. In agreement with our previous studies, the kinetic parameters of the two CDH patients not requiring ECMO were very similar to these in the ventilated control group.

The decrease in surfactant synthesis in this study on CDH-ECMO patients is felt to be secondary to progressing injury to the lungs. Bohlin et al. [31] measured surfactant synthesis in term infants using ^{13}C-acetate as precursor. They found a decreased FSR in term infants with severe respiratory failure, similar to the FSR of preterm infants with respiratory distress syndrome, suggesting that a lower FSR might be reflective of severely injured and dysfunctional lungs. The FSR we found in our CDH patients is also similar to the FSR in preterm infants with RDS (~ 2.7%/day) after ^{13}C-glucose infusion as precursor [24].

Our results of a lower FSR in CDH-ECMO cannot be simply explained by the smaller size of the lungs. Firstly, if pool size were just decreased in relation to smaller lungs, FSR would not be influenced when synthesis per lung unit would remain the same. Secondly, we did not find a decreased PC pool size in our previous study on CDH-ECMO patients in comparison to infants with meconium aspiration on ECMO or to non-ECMO patients [20]. Thirdly, no difference in net absolute synthesis between CDH infants without ECMO and ventilated controls was found by the use of dual tracers [18]. The net absolute synthesis rate calculated in seven CDH-ECMO patients is comparable with that of premature infants [2.7 ± 0.8 mg/(kg d)] [29]. In the two control patients the net synthesis rate was much higher.

An alternative explanation for the decreased surfactant synthesis in CDH-ECMO patients is the severe pulmonary hypertension leading to ECMO requirement. It could be speculated that reduced blood flow through the lungs due to severe pulmonary hypertension provides less substrate for surfactant synthesis. In addition, during VA-ECMO the lung blood flow is also significantly lower since the circuit blood flow drained from the right atrium is shunting the lungs and directly diverted to the ascending aorta. Earlier we found an increased surfactant synthesis in premature infants who had a clinically relevant persistent ductus arteriosus, a situation that leads to an increased lung blood flow [29]. Furthermore, ECMO is known to result in increased vascular permeability and cytokine release, which could influence surfactant kinetics [32].

From animal studies it is known that physical stretch of the alveoli stimulates surfactant synthesis and secretion [33]. Physical stretch during ECMO treatment is reduced because low ventilatory settings (PIP 9–12 mmHg) are used as part of the concept of "lung rest" and could possibly decrease surfactant synthesis. However, we found no difference in surfactant synthesis in preterm infants ventilated with high frequency oscillation compared to conventional ventilation, which argues against the influence of stretch in vivo on surfactant synthesis [34].

In the current study we did not find a significant difference in the concentration of PC in ELF during the first 4 days between the CDH patients on ECMO and controls (Table 2). The PC concentration in ELF of the CDH-ECMO patients in this study (6.6 mg/ml) is comparable with other data of CDH patients on ECMO (~ 6.5 mg/ml) and with the results of CDH patients not requiring ECMO (~ 4 mg/ml) [16, 19, 20]. In preterm infants, using the same method, a lower surfactant PC concentration was found (2.4 mg/ml) [35]. Earlier, we showed that surfactant PC pool size in CDH patients requiring ECMO was not decreased when compared with the pool sizes of neonates with meconium aspiration syndrome requiring ECMO and neonates who did not require ECMO [20]. However, Cogo et al. found lower concentrations of DSPC in ELF and SP-A in tracheal aspirates of CDH patients who were not on ECMO compared to controls.

Surfactant PC concentration in ELF during the study was not significantly lower in the non-survivors of our

CDH-ECMO group, though in the literature a correlation between surfactant composition and survival have been mentioned [12, 36].

In conclusion, the surfactant PC synthesis in neonates with CDH on ECMO is decreased compared to ventilated control patients. Our study does not completely solve the problem of which mechanism is responsible for this decreased synthesis. In fact, it would have been better if we also included another CDH no ECMO group to compare, which would be more convincing to show the relation of the severity of the CDH on the surfactant metabolism. As in previous studies, we did not find a decreased DSPC synthesis in milder CDH patients who did not require ECMO, and we therefore speculate that the decreased PC synthesis in CDH-ECMO patients is related to the severity of the pulmonary insufficiency, either caused by immaturity or by ventilator-induced injury, and/or by the low pulmonary blood flow during severe pulmonary hypertension and especially during ECMO. This is also supported by the study of Boucherat et al. [15] who found no deficient surfactant storage in human fetuses with CDH. From the present study it has become clear that CDH patients who require ECMO are quite a different group of patients than those with CDH without ECMO. The ECMO patients have a structural and functional pulmonary immaturity that sets them apart [37].

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