Antenatal retinoic acid administration increases trophoblastic retinol-binding protein dependent retinol transport in the nitrofen model of congenital diaphragmatic hernia

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BACKGROUND: Low pulmonary retinol levels and disrupted retinoid signaling pathway (RSP) have been implicated in the pathogenesis of congenital diaphragmatic hernia (CDH) and associated pulmonary hypoplasia (PH). It has been demonstrated that nitrofen disturbs the main retinol-binding protein (RBP)-dependent trophoblastic retinol transport. Several studies have demonstrated that prenatal treatment with retinoic acid (RA) can reverse PH in the nitrofen-induced CDH model. We hypothesized that maternal administration of RA can increase trophoblastic RBP-dependent retinol transport in a nitrofen model of CDH.

METHODS: Pregnant rats were treated with nitrofen or vehicle on gestational day 9 (D9) and sacrificed on D21. RA was given i.p. on D18, D19, and D20. Retinol and RA levels were measured using high-performance liquid chromatography. Immunohistochemistry was performed to evaluate trophoblastic expression of RBP. Expression levels of the primary RSP genes were determined using quantitative real-time PCR and immunohistochemistry.

RESULTS: Markedly increased trophoblastic RBP immunoreactivity was observed in CDH+RA compared to CDH. Significantly increased serum and pulmonary retinol and RA levels were detected in CDH+RA compared to CDH. Pulmonary expression of RSP genes and proteins were increased in CDH+RA compared to CDH.

CONCLUSION: Increased trophoblastic RBP expression and retinol transport after antenatal administration of RA suggest that retinol-triggered RSP activation may attenuate CDH-associated PH by elevating serum and pulmonary retinol levels.

The mortality rate of infants born with congenital diaphragmatic hernia (CDH) remains high despite prenatal diagnosis and improved postnatal treatment strategies (1). The high morbidity and mortality is mainly attributed to pulmonary hypoplasia (PH) and associated persistent pulmonary hypertension (2). Much of the current understanding of the pathogenesis of PH in CDH originates from experimental animal studies. Maternal exposure to nitrofen (2,4-dichlorophenyl-*p*-nitrophenyl ether) in rodents during midgestation results in a high rate of CDH and associated PH in their fetuses, which is strikingly similar to the human condition (3).

It is well understood that retinoids—vitamin A and its derivatives—are essential for the morphogenesis of most developing organs and tissues, including the lungs (4). The placenta has a major role in retinol homeostasis in fetal life (5). As the fetus cannot synthesize retinol, it relies on circulating maternal retinol, which reaches the fetus by crossing the maternal–fetal barrier in the placenta (6). Although retinol-binding protein (RBP) is essential for retinol transport within the circulation (7), maternal RBP does not cross the placenta (8). Therefore, in order to enter the fetal circulation, maternal retinol bound to maternal RBP must be released at the maternal–fetal interface. Fetal RBP is produced by the trophoblast and then forms a complex with retinol to be subsequently released into the fetal circulation and delivered to the target organs (8). This expression is tightly controlled by the placental retinol level through the expression of trophoblastic retinoid acid receptors (RARα) (8).

In recent years, disruption of the retinoid signaling pathway (RSP) has been implicated in the pathogenesis of PH and CDH in rodents (9) and human newborns with CDH (10). We have previously shown that serum and pulmonary total retinol levels are significantly decreased in nitrofen-induced hypoplastic lungs during late lung morphogenesis, supporting the hypothesis that reduced retinol levels contribute to the pathogenesis of CDH (11). In human newborns with CDH, both retinol and its binding protein in the serum, RBP, have been reported to be decreased, whereas maternal levels were comparable between mothers of CDH patients and mothers of healthy children (10). Further experimental studies have demonstrated that the trophoblastic expression of RBP and RARα is decreased in rats with nitrofen-induced CDH, indicating that nitrofen disturbs the mobilization of retinol from the placenta into the fetal circulation (12).

Several studies have demonstrated that antenatal treatment with retinoic acid (RA), the active metabolite of retinol, during

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gestation increases the expression levels of several genes which are key regulators of the RSP, reverting PH in the nitrofen-induced CDH model (13). It has been shown that maternal administration of RA can lead to it directly crossing the placenta, elevating the fetal serum RA level (14). However, the precise mechanism by which RA acts in the nitrofen-induced CDH model still remains unclear. We designed this study to test the hypothesis that maternal administration of RA increases trophoblastic RBP-dependent retinol transport, which in turn results in elevated serum and pulmonary retinol levels in the nitrofen model of CDH.

RESULTS

Fetus Weights

Administration of nitrofen decreases total body weight and causes placental hypoplasia and PH in both the affected and contralateral lungs (**Table 1**; $P < 0.05$).

Prenatal administration of RA increased significantly both placenta and total body weight in Control+RA compared to controls (**Table 1**; $P < 0.05$). Both lungs as well as placenta and total body weight were significantly higher in the CDH+RA group compared with CDH group (**Table 1**, $P < 0.05$).

CDH in Fetus

The incidence rate of CDH was similar in both CDH and CDH+RA group (77/131, 58.7 vs. 73/127, 57.4%). In both CDH and CDH+RA group, the left side was the most commonly affected side (57/77, 74% and 54/73, 73.9%).

Maternal Serum Retinoid Levels

There were no differences in the serum total retinoid, retinol, RA, and retinyl-ester (RE) levels between control and nitrofen mothers (**Table 2**). Maternal administration of RA significantly increased maternal serum total retinoid, RA and RE levels both in control+RA and CDH+RA groups (**Table 2**; $P < 0.05$), and serum retinoid levels were similar in both the control+RA and CDH+RA groups (**Table 2**). Serum retinol levels were similar in all four groups (**Table 2**).

Fetal Serum and Pulmonary Retinoid Levels

As previously demonstrated, we simultaneously detected significantly increased total serum retinoid and decreased serum retinol levels in the CDH group compared with controls (**Table 3**; $P < 0.05$) (11). In controls, the primary retinoid present in the serum by proportion was retinol (97%), whereas in CDH, RE, at 92%, was proportionately the primary retinoid in the serum (**Table 3**).

Prenatal administration of RA significantly increased fetal serum RA as well as total retinoid, retinol, and RE levels in both control+RA and CDH+RA groups compared with controls and CDH (**Table 3**; $P < 0.05$). In contrast with the CDH group, retinol was the main retinoid (65.8%) in the serum by proportion in the CDH+RA group (**Table 3**).

Previously we demonstrated that both total pulmonary retinoid and pulmonary retinol levels were significantly decreased in the CDH group compared with controls (**Table 4**) (11). Significantly increased total pulmonary retinoid levels were measured in both control+RA and CDH+RA fetuses compared with controls and CDH (**Table 4**, $P < 0.05$). The pulmonary

Table 1. Fetus weights

Weight (g)	Control	Control+RA	CDH	CDH+RA
Placenta	0.576 ± 0.01	0.681 ± 0.02*	0.442 ± 0.01*,**	0.58 ± 0.07
Left lungs	0.054 ± 0.002	0.059 ± 0.003	0.025 ± 0.001*,**	0.039 ± 0.01
Right lungs	0.078 ± 0.004	0.085 ± 0.001	0.051 ± 0.002*,**	0.069 ± 0.002
Total body weight	5.1 ± 0.08	6.2 ± 0.1*	4.68 ± 0.1*,**	4.98 ± 0.07

In CDH group, pulmonary hypoplasia, hypoplastic placentas, and lower total body weight were compared with controls (*$P < 0.05$ vs. control). Both total body and lung weight were significantly higher in the CDH+RA group compared with CDH group (**$P < 0.05$ vs. nitrofen+RA).

Table 2. Maternal serum retinoid level

Maternal serum (μM/g)	Control (n = 6)	Control+RA (n = 6)	CDH (n = 6)	CDH+RA (n = 6)
Total retinoid	0.669 ± 0.001	1.049 ± 0.001*,**	0.702 ± 0.001	1.051 ± 0.001*,**
RA	0.01 ± 0.001	0.1 ± 0.001*,**	0.01 ± 0.001	0.1 ± 0.001*,**
Retinol	0.198 ± 0.002	0.213 ± 0.001	0.208 ± 0.001	0.225 ± 0.001
Retinyl ester	0.461 ± 0.001	0.736 ± 0.003*,**	0.484 ± 0.002	0.726 ± 0.002*,**

In every group, the maternal serum retinol levels were similar. Prenatal administration of RA increased total retinoid, RA, and retinyl ester level in control+RA and CDH+RA group compared with control and CDH (*$P < 0.05$ vs. control; **$P < 0.05$ vs. CDH).

Table 3. Fetal serum retinol levels

Fetal serum (μM/g)	Control (n = 8)	Control+RA (n = 8)	CDH (n = 8)	CDH+RA (n = 8)
Total retinoid	0.0897 ± 0.002 (100%)	0.196 ± 0.003* (100%)	0.198 ± 0.008* (100%)	0.296 ± 0.003*,** (100%)
RA	0.0001 ± 0.001 (0.1%)	0.001 ± 0.002*,** (0.5%)	0.0001 ± 0.001 (<0.01%)	0.001 ± 0.002*,** (0.3%)
Retinol	0.0872 ± 0.001 (97%)	0.181 ± 0.001*,** (92%)	0.0151 ± 0.003* (7.6%)	0.195 ± 0.004*,** (65.8%)
Retinyl ester	0.00245 ± 0.002 (2.7%)	0.014 ± 0.004*,** (7.1%)	0.1829 ± 0.005* (92%)	0.0978 ± 0.002*,** (33%)

Compared with controls, the total retinoid and retinyl-ester levels were significantly increased; and retinol level was significantly decreased in serum in the CDH group (*$P < 0.05$ vs. control). Prenatal administration of RA significantly increases serum level of total retinoid and retinol and significantly decreases retinyl ester level in the CDH+RA group (**$P < 0.05$ vs. CDH). Retinol was the main retinoid in the serum, both in control and CDH+RA groups, whereas in CDH group retinyl ester was the main retinoid in the serum.

RA, retinol, and RE levels were significantly increased in the CDH+RA group compared with CDH (**Table 4**, $P < 0.05$).

Immunohistochemical Staining of Trophoblastic RARα and RBP
We have shown earlier that nitrofen markedly decreases trophoblastic RARα and RBP expression (**Figures 1** and **2**) (11). Prenatal administration of RA markedly increased trophoblastic RARα and RBP immunoreactivity in control+RA and CDH+RA placentas compared with control and CDH (**Figures 1** and **2**).

Total Pulmonary Protein Level of RBP
In addition to the finding of reduced trophoblastic RBP expression, we also detected decreased pulmonary RBP levels in the CDH group compared with controls (11). In left lungs, significantly increased total RBP protein levels were detected in the CDH+RA group compared with CDH (**Table 5**; $P < 0.05$).

Pulmonary RSP Gene Expression
The relative mRNA expression levels of cellular *retinol-binding protein-1* (*CRBP1*), *retinol dehydrogenase-1a3* (*RALDH1a3*), *retinoic acid receptor α* (*RARα*), *retinoic acid receptor β* (*RARβ*), *and retinoic X receptor α* (*RXRα*) genes were significantly upregulated in CDH lungs compared with controls (**Table 6**) (9).

The relative pulmonary mRNA expression levels of main components of RSP genes were significantly increased in control+RA compared with controls and in the CDH+RA group compared with CDH (**Table 6**; $P < 0.05$). Immunohistochemistry showed that the number of alveoli was decreased in CDH compared with controls (13). We observed that the number of alveoli was markedly increased in CDH+RA group compared with CDH. Markedly increased immunoreactivity of RSP was observed in control+RA and CDH+RA lungs compared with control and CDH (**Figure 3**).

Table 4. Fetal pulmonary retinol levels

Fetal pulmonary (µM/g)	Control (n = 8)	Control+RA (n = 8)	CDH (n = 8)	CDH+RA (n = 8)
Total retinoid	0.7772 ± 0.05	0.874 ± 0.03*	0.5133 ± 0.01*	0.978 ± 0.02*,**
RA	0.0001 ± 0.001	0.001 ± 0.003*,**	0.0001 ± 0.001	0.001 ± 0.003*,**
Retinol	0.0816 ± 0.01	0.121 ± 0.01*	0.0717 ± 0.01*	0.151 ± 0.01*,**
Retinyl ester	0.69 ± 0.03	0.75 ± 0.02*	0.44 ± 0.02*	0.79 ± 0.04*,**

Both total retinoid and retinol levels were significantly decreased in lungs in CDH compared with controls (*$P < 0.05$ vs. control). Prenatal administration of RA increases both total retinoid and retinol levels in left lungs in control+RA and CDH+RA compared with control (*$P < 0.05$ vs. control) and CDH (**$P < 0.05$ vs. CDH).
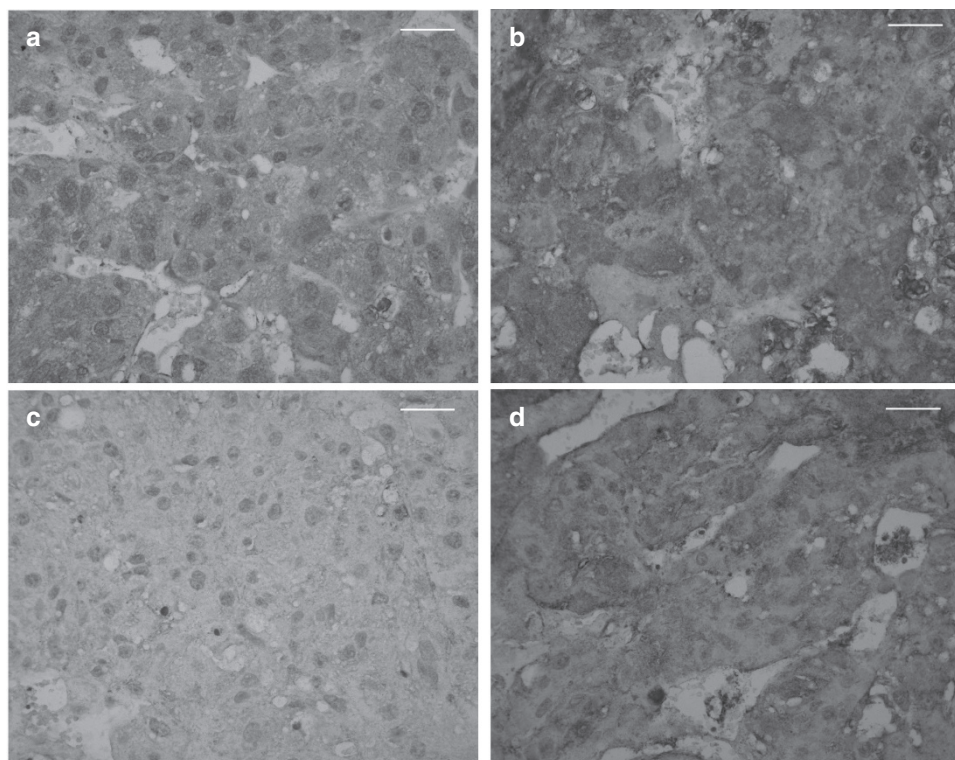
Figure 1. Trophoblastic RARα expression. Trophoblastic RARα expression on ×20 magnification. (Bar = 1 nm. (**a**) control, (**b**) control+RA, (**c**) CDH, (**d**) CDH+RA). (**c**) Nitrofen markedly decreases trophoblastic RARα immunoreactivity. Prenatal administration of RA markedly increases trophoblastic RARα immunoreactivity in (**b**) control+RA and (**d**) CDH+RA group compared with (**a**) control and (**c**) CDH.
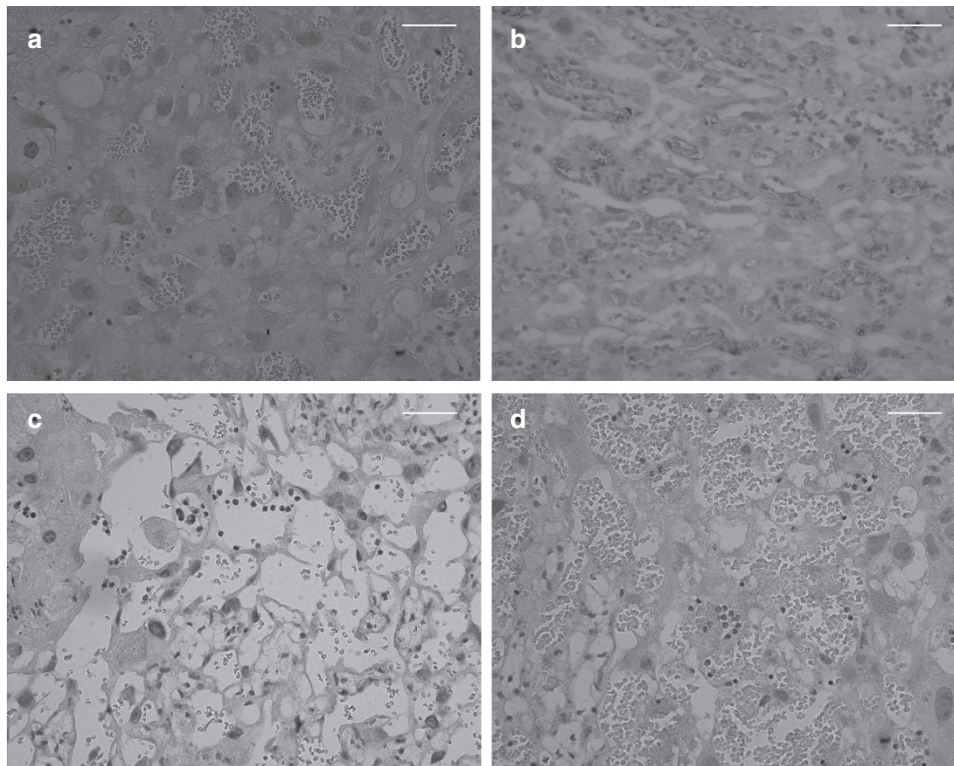
Figure 2. Trophoblastic RBP expression. Trophoblastic RBP expression on ×20 magnification. (Bar =1 nm. (**a**) control, (**b**) control+RA, (**c**) CDH, (**d**) CDH+RA). (**c**) Nitrofen markedly decreases trophoblastic RBP immunoreactivity. Prenatal administration of RA markedly increases trophoblastic RBP immunoreactivity in (**d**) CDH+RA group compared with (**a**) control and (**c**) CDH.

Table 5. Pulmonary RBP levels

	Control	Control+RA	CDH	CDH+RA
Pulmonary RBP level (ng/ml)	409 ± 0.01	421 ± 0.02*,**	260 ± 0.003*	379 ± 0.012**

In addition to the reduced trophoblastic RBP expression, the pulmonary RBP level was significantly lower in CDH compared with controls (*$P < 0.05$ vs. control). The pulmonary RBP level was significantly increased in CDH+RA compared with CDH (**$P < 0.05$ vs. CDH).

DISCUSSION

The nitrofen-induced CDH model is one of the most widely used animal models to study the pathogenesis of PH in CDH (9,13,15). However, the exact molecular mechanism underlying nitrofen-induced PH in this model is still not fully understood. It is known that retinoids, especially retinol, play a key role during fetal lung morphogenesis (4). It has been shown that retinol deficiency alone can induce PH (16). Because *de novo* synthesis of retinol does not occur, the developing mammalian embryo requires retinol from the maternal circulation for normal organ development (16). Retinol normally crosses the placenta via the trophoblast. It has been reported that the placenta buffers retinoid delivery by releasing retinol to the fetus when maternal intake is deficient and by storing it to protect the embryo from a potential toxic excess of maternal retinoids (17). Retinol is bound in a complex with the transport protein RBP (7). Quadro *et al.* (8) investigated fetal retinoid delivery in mice and found that maternal RBP does not cross the placenta. Hence, they concluded that RBP of embryonic origin plays a key role in distributing retinol to the developing tissues (16). It has been reported that the liver is the main source of RBP in adults, whereas fetal RBP levels are mainly dependent on trophoblastic RBP synthesis (16). It has been found in RBP-knockout mice that, when RBP-dependent retinol transfer is not working, the retinol is transferred through the placenta alternatively with RE RBP-independent retinol transfer (7).

At a cellular level, target cells take up the retinol–RBP complex or alternatively RE, and retinol is bound to CRBP (18). The CRBP–retinol complex can either be esterified to RE for storage or act as the substrate in the metabolic pathway which leads to the production of the biologically active metabolite, RA by retinol dehydrogenase (RALDH) (9). RA exerts its biological effects through binding to nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (18). RA signaling is tightly regulated by a negative feedback mechanism: elevated RA suppresses the production of CRBP which in turn downregulates the activation of RARs and RXRs (18).

Several authors have shown that the RSP is disrupted in both human and animal models of CDH, and decreased serum and pulmonary retinol levels have been associated with the development of PH in CDH (9–11). The importance of the RSP during fetal development and especially in lung morphogenesis is further supported by a tissue culture study showing that RA administration attenuates the development of PH in the nitrofen-induced CDH model (13). Beurskens *et al.* (10) have found decreased serum RBP and retinol levels in human newborns with CDH compared with controls, whereas mothers of both have comparable levels of RBP and retinol. These authors

Table 6. Pulmonary RSP genes mRNA expression levels

	CRBP1	RALDH1a3	RARα	RARβ	RXRα
Control (n = 8)	0.78 ± 0.09	0.03 ± 0.01	0.54 ± 0.04	0.08 ± 0.01	0.14 ± 0.01
Control+RA (n = 8)	1.52 ± 0.07*,**	0.04 ± 0.01*,**	0.94 ± 0.04*,**	0.13 ± 0.01*,**	0.38 ± 0.01*,**
CDH (n = 8)	1.02 ± 0.40*	0.07 ± 0.01*	0.68 ± 0.03*	0.09 ± 0.01*	0.36 ± 0.03*
CDH+RA (n = 8)	1.71 ± 0.10*,**	0.10 ± 0.01*,**	1.22 ± 0.08*,**	0.19 ± 0.01*,**	0.48 ± 0.03*,**

RSP genes were significantly upregulated in CDH compared with controls (*$P < 0.05$ vs. control). The relative mRNA expression levels of RSP genes were significantly increased in control+RA compared with control (*$P < 0.05$ vs. control) and in CDH+RA compared with CDH (**$P < 0.05$ vs. CDH).
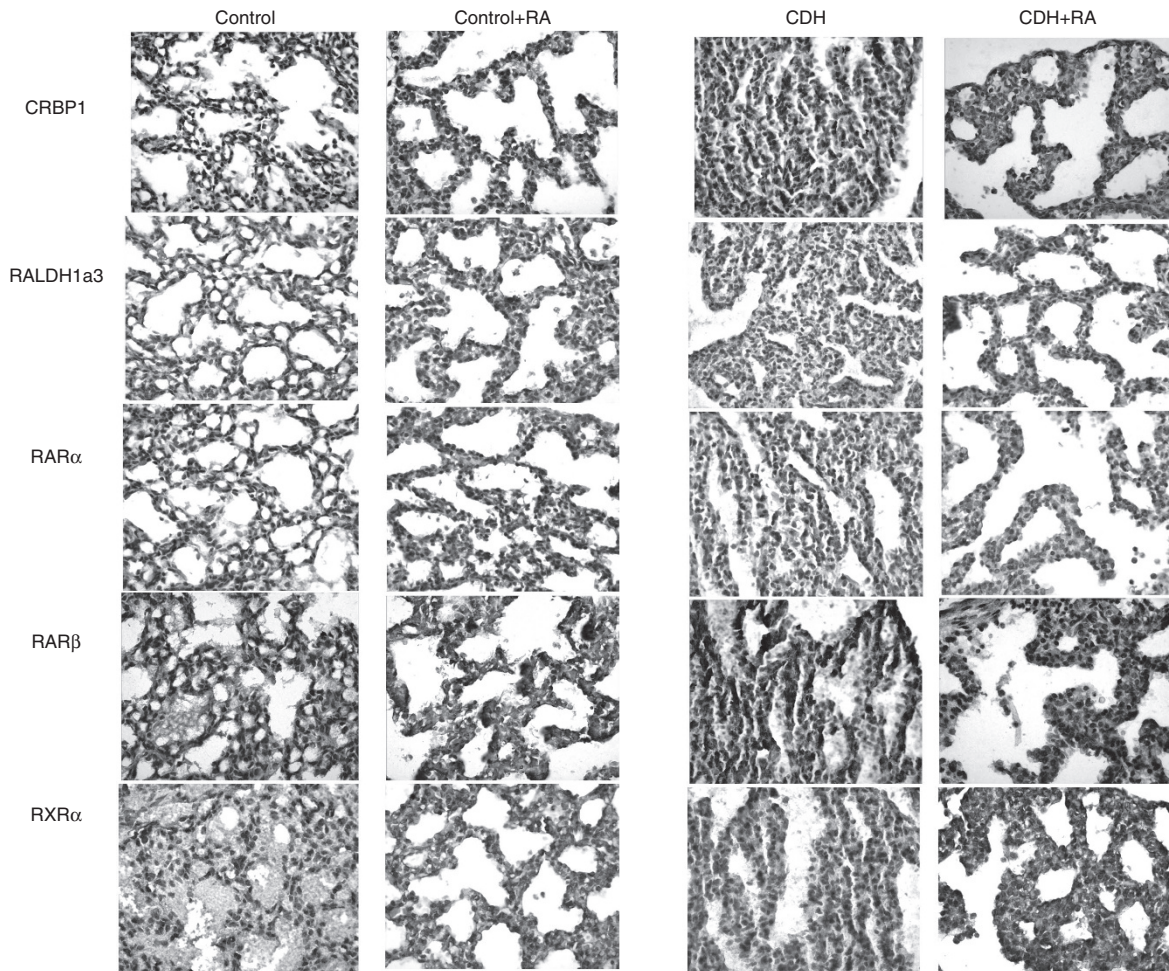
Figure 3. Immunohistochemistry of retinoid signaling pathway expression in lungs. Expression of the main component of RSP in fetal lungs on ×20 magnification. The number of alveoli was decreased in CDH compared with controls. Prenatal treatment with RA promoted pulmonary alveogenesis. Immunoreactivity of RSP was markedly increased in CDH+RA lungs compared with CDH.

concluded that the maternal–fetal transport may be disrupted, thus leading to CDH and associated PH.

Recently, we showed that nitrofen does not disturb the maternal–placental retinol transfer, but it disturbs somehow the RBP-dependent retinol mobilization from placenta to the fetus (19). That results in a high placental retinol level, which, through negative feedback, can decrease trophoblastic RARα and then RBP expression resulting in decreased pulmonary RBP and retinol level (19). In the circulation, retinol primarily exists in retinol-RBP formation, although at lower concentrations other forms of retinol circulate in the bloodstream such as RE incorporated in lipoprotein particles and RA (6). It has been demonstrated that nitrofen does not disturb the alternative, RBP-independent RE maternal–fetal retinol transport. This RE pathway is the main source of retinol during lung morphogenesis in the nitrofen CDH model (11). Tzimas *et al.* (14) have investigated the serum RA distribution in both maternal serum and in the embryo. They gave different doses of s.c. RA to the mother rats and measured the dams' RA levels. They found that embryonic/maternal concentration ratio of RA was similar after different doses of RA and that embryonic RA distribution was dependent on the maternal RA concentration. Several studies have demonstrated that

maternal i.p. administration of RA during gestation increases the expression levels of several genes which are key regulators of the RSP, reverting PH in the nitrofen-induced CDH model (13).

In this study, we found that nitrofen did not interfere with the concentration gradient RA maternal–fetal transport. Antenatal administration of RA, the active metabolite of retinol in the control+RA group, can increase trophoblastic RARα and RBP expression resulting in increased serum and pulmonary retinol levels. The increased trophoblastic RARα and RBP expression together with increased serum and pulmonary retinol levels in the CDH+RA group suggest that nitrofen disturbs the placental–fetal retinol mobilization through trophoblastic RARα expression. Furthermore, this may also suggest that the activation of RARα can reverse the nitrofen effect on decreased RBP-dependent placental–fetal retinol mobilization. Interestingly, we found that the highest retinol level was in the CDH+RA group. That may suggest that nitrofen can potentially interfere (negative feedback) with the regulation of RBP-dependent retinol mobilization resulting in high retinol mobilization and levels.

We observed, concurrently with elevated retinol levels, that prenatal administration of RA in the control+RA group can increase pulmonary RSP gene expression, lung weight, and maturation. Because the serum and pulmonary RA concentrations are relatively minute, even a 10-fold increase in RA level would not significantly increase the serum and pulmonary retinol levels and total body and lung weight. Previously, it has been shown in tissue culture that exogenous RA can rescue PH in nitrofen-induced hypoplastic lungs (13). We found that the level of RA to reach the fetal lungs after prenatal administration of RA would be thousands of times lower than the level that would reverse PH in the tissue culture. We believe that reversing PH in the live animal model may be attributable to the increased retinol level and not to the increased RA level. Our results show that increased retinol levels in CDH+RA group can increase total body weight and pulmonary weight. Therefore, we suggest that enhanced retinol levels accompanied by the increased gene expression of the main components of RSP may cause retinol-triggered RSP activation leading to reversal of PH in the nitrofen model of CDH.

In our series, the incidence rate of CDH was similar in both CDH and CDH+RA groups. Because we administrated RA only on D18, D19 and D20, after when the diaphragmatic defect developed in CDH, the increased retinol level could only affect late lung morphogenesis without impacting the incidence of CDH. The concept of a study examining whether early administration of RA alters the incidence of CDH is an intriguing one.

It is well known that RA is highly teratogenic. Further investigation needs to be performed to determine the lowest RA level which can reverse PH in this model or what other nontoxic drugs can increase trophoblasstic RARα expression. In this study, we have used only all-*trans* RA in one standard concentration, and we have not examined the levels of other RA metabolites such as 13-*cis*-RA or etertinate.

METHODS

Animals, Drugs, and Experimental Design

Eighty-eight adult Sprague-Dawley rats were mated, and females were checked daily for plugging. The presence of spermatozoids in vaginal smear was considered as a proof of pregnancy; the day of observation determined gestational day 0 (D0). Pregnant female rats were then randomly divided into four groups (control, control with RA, nitrofen, and nitrofen with RA). At D9 (term, 22 d), animals in the experimental group received 100 mg of nitrofen (WAKO Chemicals, Osaka, Japan) dissolved in 1 ml of olive oil intragastrically under short anesthesia, whereas those in the control group received only vehicle. Five milligrams per kilo of all-*trans* RA (Sigma-Aldrich, Steinheim, Germany) were given i.p. under short anesthesia on D18, D19, and D20 in nitrofen with RA group. Fetuses were harvested by cesarean section on D21 and divided into four groups: control, control with RA (control+RA group), nitrofen with CDH (CDH group), and nitrofen with RA (CDH+RA group). The animals that received nitrofen, and in whom CDH was not present, were not investigated. Only the animals that had left side CDH were included in this study, and only left lungs were studied. In the control group 8 out of 107, in the control+RA group 8 out of 104, in the CDH group 8 out of 72, and in the CDH+RA group 8 out of 88 fetuses were randomly chosen for investigation.

The Department of Health and Children approved the protocol of these animal experiments (Ref: B100/4378) under the Cruelty to Animals Act, 1876; as amended by European Communities Regulations 2002 and 2005, and all animals were treated according to the current guidelines of animal care.

Tissue Collection

After sedation with isoflurane, term fetuses were harvested free from the dams. Placentas and lungs were dissected from each fetus. Maternal and fetal blood was taken by intracardiac puncture for retinol determination. Freshly prepared serum samples for high-performance liquid chromatography (HPLC) were stored in aliquots at −80 °C after clotting for 2 h and centrifugation for 10 min at 1,000g. Fetal lung samples for quantitative real-time PCR (qRT-PCR) were kept in a TRIzol reagent (Invitrogen, Carlsbad, CA) and stored at −20 °C until further analysis. Fetal lung samples for determination of RBP levels by enzyme linked immunoassay were snap-frozen in liquid nitrogen and then stored at −80 °C. Placenta and lung samples for immunohistochemistry were fixed in 4% formalin and embedded in paraffin.

Immunohistochemistry

The paraffin-embedded fetal lungs and placentas were sectioned at a thickness of 5 μm, deparaffinized with xylene, and then rehydrated through ethanol and distilled water. Tissue sections were immersed in target retrieval solution (DAKO Ltd, Cambridgeshire, UK) heated for 10 min at 121 °C followed by incubation in 0.03% H_2O_2 for 30 min to block endogenous peroxidase activity. Sections were incubated overnight at 4 °C with a 1:100 dilution of rabbit polyclonal primary antibody against RXRα (sc553; Santa Cruz Biotechnology, Dallas, TX), 1:50 dilution of goat polyclonal primary antibody against RARα

Table 7. Primers for quantitative real-time PCR

Gene		Sequence
β-Actin	Forward	5′-TTGCTGACAGGATGCAGAAG-3′
	Reverse	5′-TAGAGCCACCAATCCACACA-3′
CRBP1	Forward	5′-AGTGCATGACCACAGTGAGC-3′
	Reverse	5′-ACCCTCTGCTCTCATCTCCA-3′
RALDH1A3	Forward	5′-TCGAGAGTGGGAAGAAGGAA-3′
	Reverse	5′-AGAAGACGGTGGGTTTGATG-3′
RARα	Forward	5′-CCTGCCTCGAATCTACAAGC-3′
	Reverse	5′-GATACTGAGTCGGAAGAAGC-3′
RARβ	Forward	5′-CCAGGTATACCCCAGAGCAA-3′
	Reverse	5′-GTCAGTCAGAGGACCGAAGC-3′
RXRα	Forward	5′-GGTACTTCGTGGGGTCTTCA-3′
	Reverse	5′-TGGGGGTACTCCAAACAGAGG-3′

(ab28767, Abcam, Cambridge, UK), 1:50 dilution of goat polyclonal primary antibody against RALDH1A3 (sc26713; Santa Cruz Biotechnology), 1:100 dilution of mouse monoclonal primary antibody against CRBP1 (ab24090; Abcam), 1:100 dilution of primary antibody against RARβ (ab53161; Abcam), and 1:100 dilution of mouse monoclonal primary antibody against RBP (sc48384; Santa Cruz Biotechnology). After intensive washing, sections were incubated with corresponding horseradish peroxidase-labeled secondary antibodies and then processed using a DAKO EnVision kit (DAKO), developed with a diaminobenzidine–H_2O_2 substrate complex, and counterstained with hematoxylin.

HPLC analysis

Preparation of samples for measurement of total retinol concentration (including retinol, RE, retinoid acid, and other metabolites of Vitamin A) was performed by modification of a previously described protocol (9). Total retinol concentrations of fetal lungs and fetal and maternal serum were analyzed by HPLC provided with SPD-10A Shimadzu UV-Vis detector on a 3.9 × 150 mm, 5-µm reverse phase Resolve C18 column (Waters, Milford, MA). The elution phase was acetonitrile/methanol/ dimethylsulfoxide (90:10:1), and the flow rate was 1.0 ml/min. Chromatograms were extracted at a wave lengths of 325 nm. The concentration of each sample was extrapolated from a calibration curve obtained with pure retinol samples (Sigma-Aldrich) between 0.1 and 10 µg/ml in concentration.

Preparation of samples for simultaneous determination of retinol, RE, and RA was performed by modification of a previously described protocol (20). Retinol, RE, and RA concentrations of serum and lungs were analyzed by HPLC as described above.

RNA Isolation and qRT-PCR

Total RNA of fetal lungs was isolated using a TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Quantification of total RNA was performed spectrophotometrically (NanoDrop ND-1000 UV-Vis Spectrophotometer). Total RNA (1 µg) was reverse-transcribed using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, West Sussex, UK) according to the manufacturer's instruction. Following reverse transcription at 44 °C for 60 min, qRT-PCR was performed using a LightCycler 480 SYBR Green I Master (Roche Diagnostics) according to the manufacturer's protocol. Gene-specific primer pairs are listed in **Table 7** (9). After an initial denaturation step of 5 min at 95 °C, 45 cycles of amplification for each primer pair were carried out. Each cycle included a denaturation step (10 s at 95 °C), an annealing step (15 s at 60 °C), and an elongation step (10 s at 72 °C). Final elongation temperature was 65 °C for 1 min. Relative levels of gene expression were measured using a LightCycler 480 (Roche Diagnostics) according to the manufacturer's instructions. The relative changes in the expression levels of *CRBP1*, *RALDH1A3*, *RARα*, *RARβ*, and *RXRα* genes were normalized against the level of β-actin gene expression in each sample. Experiments were carried out at least in duplicate for each data point.

Protein Extraction and Enzyme-Linked Immunoabsorbent Assay

The total protein of left lung derived from fetuses was isolated using TRIzol Reagent (Invitrogen), according to the manufacturer's protocol. The obtained protein pellet was dissolved in 1% SDS, and total protein was dissolved in diethyl pyrocarbonate-treated water. Total protein concentration was determined spectrophotometrically (NanoDrop ND-1000 UV–vis spectrophotometer) by Bradford assay using a quantification kit and bovine serum albumin (2 mg/ml) as a standard. After extraction, proteins were used for measuring the total RBP (Immunology Consultants Laboratory, Portland, OR) levels by rat ELISA kit according to the manufacturer's protocol. The results were measured at 450 nm with Synerg Mx microplate reader (BioTek, Vinooski, VT) immediately after adding the stop solution. Experiments were carried out at least in duplicate for each data point.

Statistical Analysis

All numerical data are presented as mean ± SEM. Differences between groups at D21 were tested using an unpaired *t*-test or *U*-test, depending on the distribution of data. Statistical significance was accepted at *P* values < 0.05.

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