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ORIGINAL ARTICLE

Altered regulation of retinoic acid synthesis in nitrofen-induced hypoplastic lung

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Abstract Retinoids are a group of molecules derived from vitamin A, which play an important role in lung development. Within the cell, retinol can either be oxidized to retinal or esterified to retinyl esters by lecithin : retinol acyltransferase (LRAT) for storage. Retinal is then oxidized to an active metabolite of vitamin A, retinoic acid (RA) by retinal dehydrogenase (RALDH). RA is the active metabolite of vitamin A. Cyp26 (a1,b1, and c1), which is a member of the cytochrome P450 family, acts by reducing the activity of RA. Cyp26 type b1 is the predominant subtype expressed in the murine lung. Several studies have suggested that nitrofen may interfere with the retinoid pathway resulting in congenital diaphragmatic hernia (CDH) and pulmonary hypoplasia. Recently, it was reported that nitrofen may act by inhibiting RALDH2. The aim of this study was to examine the pulmonary expression of Cyp26b1, LRAT, and RALDH2, the key enzymes involved in the synthesis of RA, in order to understand the mechanisms underlying pulmonary hypoplasia in the nitrofen CDH model. Pregnant rats were exposed to either olive oil or 100 mg of nitrofen on day 9 of gestation (D9). Fetal lungs were harvested at D15, D17, D19, and D21. D17, D19, and D21 lungs were divided into three groups: control, nitrofen without CDH and nitrofen with CDH, whereas D15 lungs were divided into only two groups; control and nitrofen as the diaphragm is not fully formed yet at this stage. Real- time PCR was performed to evaluate the relative level of Cyp26b1, LRAT, and RALDH2 expression in the lung. Relative levels of Cyp26b1 mRNA were significantly decreased in the lungs of nitrofen with CDH (D17;0.19 ± 0.09, D19;0.70 ± 0.20, D21;0.40 ± 0.36) and nitrofen without CDH (D17;0.14 ± 0.06, D19;0.54 ± 0.42, D21;0.51 ± 0.56) compared to controls (D17;0.35 ± 0.16, D19; 1.15 ± 0.48, D21;1.28 ± 0.78) ($P < 0.05$). LRAT expression was also significantly decreased in nitrofen with CDH (D17; 19.3 ± 7.8, D19; 4.3 ± 1.1, D21; 3.3 ± 1.6) and nitrofen without CDH (D17; 21.2 ± 11.1, D19; 4.5 ± 3.6, D21; 4.1 ± 1.6) compared to controls (D17; 153.7 ± 29.8, D19; 26.8 ± 16.8 D21; 10.1 ± 3.8) ($P < 0.05$). There was no significant difference in the relative levels of Cyp26b1 and LRAT between nitrofen with CDH and nitrofen without CDH. There were no significant differences in RALDH2 expression among the groups at any stages. Down-regulation of Cryp26b1 and LRAT demonstrates that RA content is decreased in nitrofen induced hypoplastic lungs compared to controls. The finding that RALDH2 expression in the hypoplastic lung is not altered suggests that nitrofen may act by interfering with the retinoid metabolism during the early stage of the retinoid signaling pathway.

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Introduction

Pulmonary hypoplasia and persistent pulmonary hypertension are the primary causes of morbidity and mortality in infants with congenital diaphragmatic

hernia (CDH) [1]. Much of the current understanding of pathogenesis of CDH originates from experimental studies. Maternal exposure to nitrofen (2,4-dichlorophenyl-*p*-nitropheniyl ether) in rats during a specific time in gestation results in pulmonary hypoplasia and a high rate of CDH in the offspring, which is very similar to the human condition [2]. Although the nitrofen model of CDH has been widely used, the exact mechanism by which nitrofen acts is not clearly understood.

Retinoids, vitamin A and its derivatives, play an important role in lung development [3]. Within the cell, retinol can either be oxidized to retinal or esterified to retinyl esters by lecithin:retinol acyltransferase (LRAT) for storage [4]. Retinal is then oxidized to active metabolite of vitamin A, retinoic acid (RA) by retinal dehydrogenase (RALDH). RA exerts its biological effects through biding to nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Fig. 1). Cyp26 (a1,b1, and c1), a newly recognized member of the cytochrome P450 family, disposes of excess RA by oxidizing it. Cyp26a1 and Cyp26b1 have similar catalytic activity but the tissue specific expression patterns are different. Cyp26b1 is the predominant subtype expressed in developing murine lung [5, 6]. Although the concentration of RA is closely regulated, the mechanism underlying the regulation of RA synthesis is not well understood. It has been reported that the 'on' signal for RA is its localized synthesis from retinol via retinal using the RALDHs and the 'off' signal is considered to be the catabolism of RA by Cyp26 [7, 8]. On the other hand, Ross has suggested that LRAT and Cyp26 are the two key factors in retinoid homeostasis, both of which are regulated in response to RA [9].

Recent studies suggest that retinoid signaling pathway may be disturbed in nitrofen model of CDH. An in vitro assay has demonstrated that nitrofen inhibits RALDH2, the enzyme catalyzing the final step in RA production [10]. Antenatal administration of vitamin A reduced the incidence and severity of CDH in the nitrofen rat model [11]. Furthermore, using fetal lung rat explants we have recently demonstrated that the RA improves nitrofen-induced hypoplastic lung growth [12]. This suggests that retinoid pathway is disturbed in nitrofen-induced hypoplastic lungs as well as in CDH.

To understand the mechanisms underlying pulmonary hypoplasia in the nitrofen CDH model, we designed this study to examine pulmonary expression of RALDH2, Cyp26b1, and LRAT, the key enzymes involved in the regulation of RA synthesis.

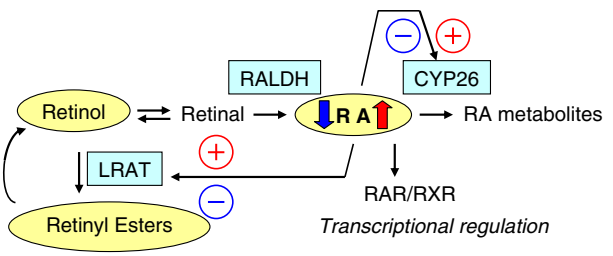

Fig. 1 Schematic view of the retinoid signaling pathway and regulation of RA by LRAT and CYP26. *RA* retinoic acid, *LRAT* lecithin:retinol acyltransferase, *RALDH* retinal dehydrogenase, *RAR* retinoic acid receptor, *RXR* retinoid X receptor

Materials and methods

Animal model

Adults Sprague–Dawley rats were mated overnight. Twelve hours later the presence of spermatozoids in the vaginal smear was verified and was considered as gestational day 0. Pregnant female rats were then randomly divided into two groups. Animals in the experimental group received 100 mg of nitrofen (Wako Chemicals, Osaka, Japan), dissolved in 1 ml of olive oil, via a stomach tube on day 9 of gestation (D9) (term = 22 days), whereas those in the control group received only vehicle. Fetuses were delivered by caesarean section on D15, D17, D19, and D21. Under a dissecting microscope (Leica S8 APO, Leica Microsystems, Wetzlar, Germany), the diaphragm was carefully inspected for the presence of a hernia. Fetuses with diaphragmatic defects were defined as the nitrofen with CDH group, whereas the fetuses exposed to nitrofen with an intact diaphragm represented the nitrofen without CDH group. The control group consisted of animals that only received olive oil. D17, D19, and D21 fetuses were divided into three groups: control, nitrofen without CDH and nitrofen with CDH, whereas D15 fetuses were divided into only two groups; control and nitrofen as the diaphragm is not fully formed yet at this stage. Lungs were dissected from each fetus and kept in RNA LATER (Ambion, Huntingdon, UK) and stored at –20°C until analysis. The department of Health and Children approved all the animal experiments (ref.B100/3621) under the Cruelty to Animals Act, 1876; as amended by European Communities Regulations 2002.

RNA extraction and real time RT-PCR

Total RNA was extracted from each lung using TRIZOL reagent (Life Technologies, Paisley, UK) accord-

Table 1 Sequences of primers for polymerase chain reaction

Name of primer	Forward primer	Reverse primer	Product size (bp)
RALDH2	ATGGGTGAGTTTGGCTTACG	AAGGAGGCCTGGTGATAGGT	134
Cyp26b1	AGAGCTGCAAGCTGCCTATC	CGCCCCAGTAAGTGTGTCTT	145
LRAT	CAGGCTGAGAAGTTTCAGGA	GATGCCAGGCCTGTGTAGAT	110
β-actin	TTGCTGACAGGATGCAGAAG	TAGAGCCACCAATCCACACA	108

ing to recommended protocol. DNA contamination was removed by DNase treatment. Real-time reverse transcription polymerase chain reaction (RT-PCR) was carried out to quantify the amounts of mRNA expression of LRAT in each lung. The sequence of the primers and the size of yielded products are shown in Table 1. Relative levels of gene expression were measured by real-time PCR (iCycler iQ Multicolor Real Time PCR Detection System, Bio-Rad Laboratories, CA, USA) using QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. After reverse transcription at 50°C for 30 min, 45 cycles of amplification were carried out (denaturation at 95°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s). Serial dilutions of one sample RNA was prepared to create a standard curve for relative quantification of mRNA in the samples. Experiments were carried out in triplicate for each data point. The relative changes in levels of specific genes were expressed as a present of the control values that were set as 100%, after normalization by the level of β-actin expression in each sample.

Statistical analysis

All numerical data are presented as mean ± standard deviation. Differences between the three groups were tested by one-way analysis of variance (ANOVA). Differences between two groups were tested by unpaired *t*-test. Statistical significance was defined as <0.05.

Results

Relative levels of Cyp26b1 mRNA were significantly decreased in the D17, D19, and D21 lungs in the nitrofen with CDH and nitrofen without CDH groups compared to controls ($P < 0.05$) (Fig. 2 and Table 2). There were no significant differences between nitrofen with and without CDH at all stages. The Cyp26b1 expression in the D15 lungs in the nitrofen group was lower than control but did not reach significance. The mRNA expression of LRAT was significantly down-regulated in the lungs in the nitrofen with and without CDH groups compared to controls at all stages (Fig. 3 and Table 2). There was no significant difference in the LRAT expression between nitrofen with CDH and nitrofen without CDH. We did not find any significant difference in RALDH2 mRNA expression between nitrofen with CDH (D15; 67.62 ± 16.12, D17; 28.01 ± 13.97, D19; 16.55 ± 4.84, D21; 13.41 ± 2.65) and con-

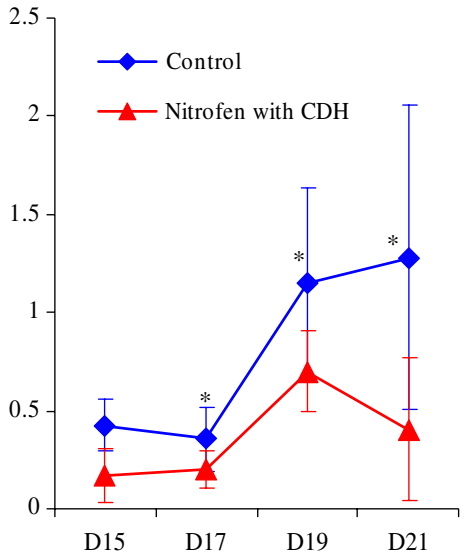

Fig. 2 Relative mRNA expression of Cyp26b1 *$P < 0.05$ (*filled triangular* at D15 represents nitrofen group)

Table 2 Relative levels of Cyp26b1 and LRAT in the lung

	D17		D19		D21	
	Cyp26b1	LRAT	Cyp26b1	LRAT	Cyp26b1	LRAT
Control	0.35 ± 0.16	153.7 ± 29.8	1.15 ± 0.48	26.8 ± 16.8	1.28 ± 0.78	10.1 ± 3.8
Nitrofen without CDH	0.14 ± 0.06*	21.2 ± 11.1*	0.54 ± 0.42*	4.5 ± 3.6*	0.51 ± 0.56*	4.1 ± 1.6*
Nitrofen with CDH	0.19 ± 0.09*	19.3 ± 7.8*	0.70 ± 0.20*	4.3 ± 1.1*	0.40 ± 0.36*	3.3 ± 1.6*

*$P < 0.05$ versus control

trols (D15; 62.07 ± 17.46 D17; 26.18 ± 10.44; D19; 20.82 ± 5.57 D21; 15.60 ± 5.25) (Fig. 4).

Discussion

The nitrofen model of CDH has been widely used and has vastly contributed to our understanding of this congenital malformation. This model has shown that the pulmonary hypoplasia occurs in two developmental insults. The first insult occurs before diaphragm formation and affects both lungs, followed by the second insult, which affects ipsilateral lung due to compression by herniated viscera [13]. This concept suggests that pulmonary hypoplasia in CDH could be a disease of impaired lung development associated with, but not always caused by, a structural defect of the diaphragm [14].

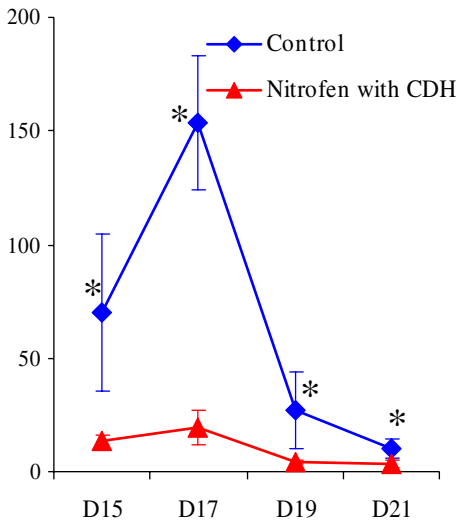

Fig. 3 Relative mRNA expression of LRAT $*P < 0.05$ (*filled triangular* at D15 represents nitrofen group)

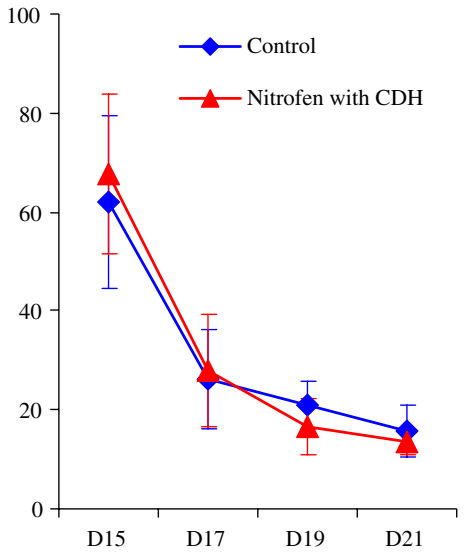

Fig. 4 Relative mRNA expression of RALDH2 (*filled triangular* at D15 represents nitrofen group)

The first evidence linking retinoids with CDH was published in 1941. The authors found that there was a 25% incidence of CDH in the litters born to dams with vitamin A deficient diets [15]. A small clinical study revealed that the infants with CDH had lower plasma retinol levels compared to healthy infants [16]. A study using genetically engineered mice has shown a pronounced suppression of retinoid response element by nitrofen [17]. RARα/RARβ double knock out mice have demonstrated a significant proportion of lung agenesis/hypoplasia and/or diaphragmatic defect [18]. When vitamin A is administered to pregnant rats along with nitrofen, the incidence and severity of CDH is reduced and lung maturation is restored in their offspring [11, 19, 20]. Furthermore, we have recently demonstrated that RA improves growth in the nitrofen-induced hypoplastic lungs [12]. The above studies strongly suggest that the retinoid pathway is disturbed in CDH and associated pulmonary hypoplasia.

The most biologically active metabolite of vitamin A, RA plays a pivotal role in lung development. Although the concentration of RA is closely regulated, the mechanism underlying the regulation of RA synthesis is not well understood. It has been reported that the 'on' signal for RA involves the RALDH enzymes and the 'off' signal is considered to be the catabolism of RA by Cyp26 [7, 8]. On the other hand, a study by Ross has suggested that LRAT and Cyp26 are the two key factors in retinoid homeostasis, both of which are regulated in response to RA [9]. The result of this study showed that LRAT level was significantly reduced in the lung of vitamin A deficient rats and similarly, Cyp26 expression in the lung is down-regulated by vitamin A deficiency and up-regulated by excess RA. However, recent studies suggest that RALDHs are minimally affected by the presence or absence of RA, whereas the Cyps are dramatically affected by both their substrate, RA, and their catabolic products leading to a situation involving positive feedback and resultant degradation of the RA signal [8].

In the normal rat, significant storage of retinol in the lung occurs in the latter one-third of prenatal life. These stores are rapidly depleted during late pregnancy and the early weeks of postnatal life as the lungs grow and develop. A sufficient and continuous availability of retinol (either from the blood or from local storage sites) is pivotal, especially for a time-dependent

regulation of the lung development and related formation of the active metabolite RA [21, 22]. Therefore, the disturbance of the retinoid signaling pathway during this critical period may cause irreversible damage on lung development. The safety margins for RA-mediated regulation of primordial diaphragm and heart development are also relatively low, suggesting those tissues are more susceptible to perturbations of the retinoid pathway compared to other tissues [10]. This could explain the diaphragmatic defect and cardiac anomalies observed in the nitrofen rat model.

Although the exact mechanism by which nitrofen acts on this pathway is not fully understood, some researchers have tried to answer these questions. It has been reported that nitrofen and its metabolites may competitively bind with RARs during early embryonic exposure and thus lead to abnormal development of lungs and diaphragm [23]. On the other hand, an in vitro assay has demonstrated that nitrofen inhibits RALDH2, the enzyme catalyzing the final step in RA production [10].

Our data show that both LRAT and Cyp26 were down-regulated while RALDH2 remains unaltered, which is identical to the response seen in the vitamin A deficient condition [8, 9]. This suggests that nitrofen disturbs retinoid signaling at a very early stage of this pathway, such as during retinol transportation from the serum to the cell, rather than at a later stage by blocking RALDH or competing retinoid receptors, as mentioned above. These results support our previous study where we reported significantly decreased levels of retinol in the nitrofen-induced hypoplastic lungs compared to controls [24]. Decreased retinol causes insufficient RA, and thus, LRAT expression is down-regulated causing a shift of retinol from storage to utilization, and at the same time Cyp26, the enzyme responsible for degradation of RA, is also down-regulated.

These data confirm that the retinoid signaling pathway is disturbed in lung hypoplasia in the nitrofen induced CDH. Further studies investigating the therapeutic potential of retinoids in lung maturation are required.

References

1. Gosche JR, Islam S, Boulanger SC (2005) Congentital diaphragmatic hernia: searching for answers. Am J Surg 190:324–332
2. Kluth D, Kangah R, Reich P, et al (1990) Nitrofen induced diaphragmatic hernia in rats: an animal model. J pediatr Surg 25(8):850–854
3. Mactier H, Weaver LT (2005) Vitamin A and preterm infants: what we know, what we don't know, and what we need to know. Arch Dis Child Fetal Neonatal Ed 90:F103–F108
4. Paik J, Vogel S, Quadro L, et al (2004) Vitamin A: overlapping delivery pathways to tissues from the circulation. J Nutr 134(1):276S–280S
5. Blomhoff R, Blomhoff HK (2006) Overview of retinoid metabolism and function. J Neurobiol 66(7):606–630
6. Abu-Abed S, MacLean G, Fraulob V, et al (2001) Differential expression of the retinoic acid-metabolizing enzymes CYP26A1 and CYP26B1 during murine organogenesis. Mech Dev 110(1–2):173–177
7. Duester G (1996) Involvement of alcohol dehydrogenase, short-chain dehydrogenase/reductase, aldehyde dehydrogenase, and cytochrome P450 in the control of retinoid signaling by activation of retinoic acid synthesis. Biochemistry 35(38):12221–12227
8. Reijntjes S, Blentic A, Gale E, et al (2005) The control of morphogen signalling: regulation of the synthesis and catabolism of retinoic acid in the developing embryo. Dev Biol 285(1):224–237
9. Ross AC (2003) Retinoid production and catabolism: role of diet in regulating retinol esterification and retinoic Acid oxidation. J Nutr 133(1):291S–296S
10. Mey J, Babiuk RP, Clugston R, et al (2003) Retinal dehydrogenase-2 is inhibited by compounds that induce congenital diaphragmatic hernias in rodents. Am J Pathol 162(2):673–679
11. Thebaud B, Tibboel D, Rambaud C, et al (1999) Vitamin A decreases the incidence and severity of nitrofen-induced congenital diaphragmatic hernia in rats. Am J Physiol 277:L423–L429
12. Montedonico S, Nakazawa N, Puri P (2006) Retinoic acid rescues lung hypoplasia in nitrofen-induced hypoplastic foetal rat lung explants. Pediatr Surg Int 22(1):2–8
13. Keijzer R, Liu J, Deimling J (2000) Dual-hit hypothesis explains pulmonary hypoplasia in the nitrofen model of congenital diaphragmatic hernia. Am J Pathol 156(4):1299–1306
14. Gallot D, Marceau G, Coste K, et al (2005) Congenital diaphragmatic hernia: a retinoid-signaling pathway disruption during lung development? Birth Defects Res A Clin Mol Teratol 73(8):523–531
15. Andersen DH (1941) Incidence of congenital diaphragmatic hernia in the young of rats bred on a diet deficient in vitamin. Am J Dis Child 62:888–889
16. Major D, Cadenas M, Leclerc S (1998) Retinol status of newbnorn infants with congenital diaphragmatic hernia. Pediatr Surg Int 13:547–549
17. Chen MH, MacGowan A, Ward S, et al (2003) The activation of the retinoic acid response element is inhibited in an animal model of congenital diaphragmatic hernia. Biol Neonate 83(3):157–161
18. Mendelsohn C, Lohnes D, Decimo D, et al (1994) Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development 120(10):2749–7271
19. Thebaud B, Barlier-Mur AM, Chailley-Heu B, et al (2001) Restoring effects of vitamin A on surfactant synthesis in nitrofen-induced congenital diaphragmatic hernia in rats. Am J Respir Crit Care Med 164:1083–1089
20. Babiuk RP, Thebaud B, Greer JJ, et al (2004) Reductions in the incidence of nitrofen-induced diaphragmatic hernia by vitamin A and retinoic acid. Am J Physiol Lung Cell Mol Physiol 286(5):L970–L973

21. Biesalski HK, Nohr D (2003) Importance of vitamin-A for lung function and development. Mol Aspects Med 2:431–440
22. Shenai JP, Chytil F (1990) Vitamin A storage in lung during perinatal development in the rats. Biol Neonate 57(2):126–132
23. Chinoy MR, Chi X, Cilley RE (2001) Down-regulation of regulatory proteins for differentiation and proliferation in murine fetal hypoplastic lungs: altered mesenchymal–epithelial interactions. Pediatr Pulmonol 32(2):129–141
24. Nakazawa N, Montedonico S, Takayasu H, et al Disturbance of retinol transportation causes nitrofen-induced hypoplastic lung. J Pediatr Surg (In Press)