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Disturbance of retinol transportation causes nitrofen-induced hypoplastic lung

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Abstract

Purpose: Retinoids play a key role in lung development. Recent studies suggest that retinoid signalling pathway may be disrupted in the nitrofen model of congenital diaphragmatic hernia (CDH), but the exact mechanism is not clearly understood. We hypothesized that nitrofen interferes with cellular uptake of retinol during lung morphogenesis and therefore designed this study to examine total retinol levels in lung, liver, and serum, and the gene expression of main components of the retinoid pathway in the nitrofen model of CDH.

Methods: Pregnant rats were exposed to vehicle or 100 mg of nitrofen on day 9 of gestation. Term fetuses were divided in control and nitrofen with CDH and without CDH groups. Retinol levels in serum, lungs, and liver were measured using high-performance liquid chromatography. Reverse transcriptase–polymerase chain reaction was performed to evaluate the relative amount of cellular retinol-biding protein I, retinal dehydrogenase 1a2 and 1a3 (Aldh1a2 and Aldh1a3), retinoic acid receptors α and β (RARα, RARβ), and retinoid X receptor α (RXRα) expression in the lung.

Results: Total retinol levels in the lungs were significantly lower in both nitrofen with CDH (1.78 ± 0.37 μg/g) and nitrofen without CDH (1.61 ± 0.24 μg/g) groups compared with controls (2.43 ± 0.31 μg/g) ($P < .001$), whereas serum retinol levels were significantly higher in nitrofen with and without CDH groups (0.77 ± 0.13 and 0.75 ± 0.11 μg/g, respectively) compared with controls (0.58 ± 0.12 μg/g) ($P < .001$). There was no significant difference in liver retinol levels between the 3 groups. Relative expression of cellular retinol-biding protein I, Aldh1a3, RARα, RARβ, and RXRα were significantly up-regulated in the lungs of the nitrofen with CDH group (0.70 ± 0.15, 3.94 ± 0.91, 2.15 ± 0.47, 3.49 ± 1.00, 1.88 ± 0.42, respectively) and the nitrofen without CDH group (0.61 ± 0.14, 3.72 ± 0.31, 1.66 ± 0.20, 3.28 ± 1.02, 1.38 ± 0.24, respectively) compared with controls (0.43 ± 0.11, 2.71 ± 0.47, 0.79 ± 0.42, 1.85 ± 0.69, 0.57 ± 0.22, respectively) ($P < .05$).

Conclusion: Our data clearly show that lung retinol storage is decreased in the nitrofen model of CDH. The associated increase in gene expressions of most downstream components of the retinoid signalling

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pathway may be a feedback reaction to the deficiency of lung retinol. These results suggest that nitrofen acts by interfering with the cellular uptake of retinol during lung morphogenesis resulting in pulmonary hypoplasia in this model.

Despite significant advances in neonatal resuscitation and intensive care, newborn infants with congenital diaphragmatic hernia (CDH) continue to have high mortality and morbidity. Pulmonary hypoplasia in CDH, characterized by immaturity and small size, produces respiratory failure that is considered to be the principal contributor to the high mortality [1]. Much of the current understanding of pathogenesis of CDH originates from experimental studies. A teratogenic model of CDH in rodents has been widely used. Maternal exposure to nitrofen (2,4-dichlorophenyl-*p*-nitropheniyl ether) in both mouse and rat models during a specific time in gestation results in a high rate of CDH and associated pulmonary hypoplasia to their embryos, which is strikingly similar to the human condition [2]. Although the nitrofen model of CDH has been widely used, the exact pathogenesis of pulmonary hypoplasia, particularly in the nitrofen-induced animals without CDH, is not clearly understood.

Retinoids, vitamin A and its derivatives, are essential for growth, development, and tissue differentiation [3]. The major sources of vitamin A in the human diet are the provitamin A carotinoids in fruits and vegetables and retinyl esters found in food of animal origin. After absorption through the gut, retinyl esters are transported in chylomicrons to the liver for storage, where they are metabolized into retinol. To meet tissue needs for retinoid, the liver secretes retinol bound to retinol-biding protein (RBP) into the plasma, where it forms the main transporting complex with transthyretin. Circulating retinol is delivered to target tissues via a specific membrane receptor [4-6]. Within cells, retinol bound to cellular retinol-biding protein I (CRBP-I) is oxidized to retinal (Fig. 1). Retinal is oxidized to active metabolite, retinoic acid (RA), by retinal dehydrogenase. Currently, there are 3 known retinal dehydrogenases, RALDH1, RALDH2, and RALDH3, renamed as Aldh1a1, Aldh1a2, and Aldh1a3. Aldh1a2 and Aldh1a3 are essential for RA synthesis, whereas Aldh1a1 is involved in the catabolism of excess retinol [7]. Retinoic acid exerts its biologic effects through binding nuclear receptors, the retinoic acid receptor (RAR) and retinoid X receptor (RXR), of which there are 3 types of each; α, β, and γ. Most of the body's reserve of vitamin A is stored in the liver as retinyl esters; other sites of major vitamin A include the eye and the lung [6].

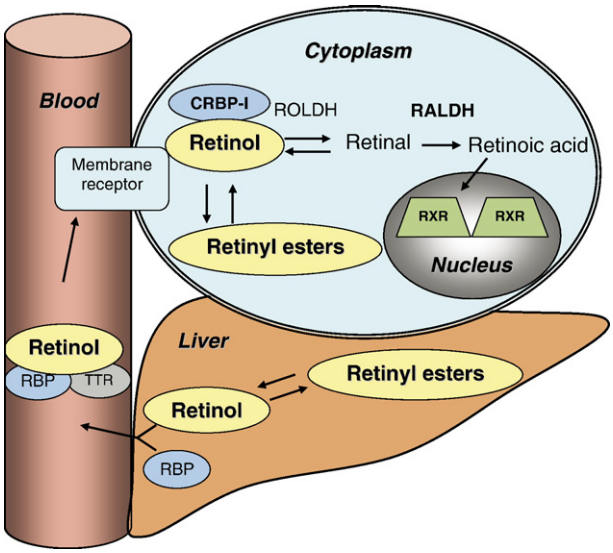

Fig. 1 Schematic overview of the retinoid pathway. TTR indicates transthyretin; ROLDH, retinol dehydrogenase.

Recent studies suggest that retinoid signalling pathway may be involved in pathogenesis of CDH and associated pulmonary hypoplasia. The first evidence linking retinoids with CDH was published in 1941. The authors found that there was 25% incidence of CDH in the litters born to dams with vitamin A–deficient diets [8]. A small clinical study revealed that the infants with CDH had lower plasma retinol levels compared with healthy infants [9]. A study using genetically engineered mice has shown a pronounced suppression of retinoic acid response element by nitrofen [10]. Various abnormalities reported in RARα/RARβ double knockout mice include diaphragmatic hernia [11]. An in vitro assay has demonstrated that nitrofen inhibits RALDH2, the enzyme catalyzing the final step in retinoic acid production [12]. Antenatal administration of vitamin A reduced the incidence of CDH and also restored lung maturation in the nitrofen rat model [13-15]. Furthermore, we have recently demonstrated that retinoic acid improves nitrofen-induced hypoplastic lung growth using fetal lung rat explants [16]. The above studies suggest that the retinoid pathway may be involved in the pathogenesis of CDH and associated pulmonary hypoplasia.

We hypothesized that nitrofen interferes with cellular uptake of retinol during lung morphogenesis and therefore designed this study to examine total retinol levels in lung, liver, and serum, and the gene expression of main components of the retinoid pathway in the nitrofen model of CDH.

1. Materials and methods

1.1. 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 2 groups. Animals in the experimental group received 100 mg

Table 1 Sequences of primers for PCR

Name of primer	Forward primer	Reverse primer	Product size (bp)
CRBP-I	AGTGCATGACCACAGTGAGC	ACCCTCTGCTCTCATCTCCA	128
Aldh1a2	ATGGGTGAGTTTGGCTTACG	AAGGAGGCCTGGTGATAGGT	134
Aldh1a3	TCGAGAGTGGGAAGAAGGAA	AGAAGACGGTGGGTTTGATG	90
RARα	CCTGCCTCGAATCTACAAGC	GATACTGAGTCGGAAGAAGC	109
RARβ	CCAGGTATACCCCAGAGCAA	GTCAGTCAGAGGACCGAAGC	98
RXRα	GGTACTTCGTGGGGTCTTCA	TGGGGGTACTCCAAACAGAGG	123
β-actin	TTGCTGACAGGATGCAGAAG	TAGAGCCACCAATCCACACA	108

of nitrofen (Wako Chemicals, Osaka, Japan) dissolved in 1 mL of olive oil via a stomach tube on day 9 of gestation (term = 22 days), whereas those in the control group received only vehicle. Fetuses were delivered by cesarean birth on day 21 of gestation. 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. The Department of Health and Children approved all the animal experiments (reference no. B100/3621) under the Cruelty to Animals Act 1876, as amended by European Communities Regulations 2002.

1.2. Blood and tissue collection

After sedation with isoflurane, blood was taken from dams by intracardiac puncture for serum retinol determination. Term fetuses were dissected free from the dams and blood was collected from the jugular vein under a dissecting microscope and centrifuged for 20 minutes at 1900*g*. Both lungs and liver were dissected from each fetus and weighed, respectively. All the samples for retinol analysis were snap frozen in liquid nitrogen and then stored at −80°C. Lung samples for reverse transcriptase–polymerase chain reaction (RT-PCR) were kept in RNA later (Ambion, UK) and stored at −20°C until analysis.

1.3. High-performance liquid chromatography analysis of total retinol

Preparation of samples was performed according to a previously described protocol [17]. Total retinol (retinol and retinol esters) concentrations of serum and tissue were analyzed by high-performance liquid chromatography provided with diode-array detector (Waters, Milford, Mass) on reversed-phase C18 column (Supelco, Bellefonte, Pa). The elution was acetonitrile/dichloromethane/methanol/1-octanol (90:15:10:0.1) and flow rate was 1.0 mL/min. Chromatograms were extracted at a wave lens of 325 nm. The concentration of each sample was extrapolated from calibration curve obtained with pure retinol samples (Sigma-Aldrich, Steinheim, Germany) of a concentration from 0.01 to 1.0 μg/mL.

1.4. RNA extraction and real-time RT-PCR

Total RNA was extracted from each lung using TRIZOL reagent (Life Technologies, Paisley, UK) according to the recommended protocol. DNA contamination was removed by a DNase treatment. Real-time RT-PCR was carried out to quantify the amounts of messenger RNA (mRNA) expression of CRBP-I, Aldh1a3, RARα, RARβ, and RXRα 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, Calif) using QuantiTect SYBR Green RT-PCR Kit (Qiagen, UK) according to the manufacturer's instruction. After reverse transcription at 50°C for 30 minutes, 45 cycles of amplification were carried out (denaturation at 95°C for 15 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds). Serial dilutions of 1-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 percentage of the control values that were set as 100%, after the normalization by the level of β-actin expression in each sample.

1.5. Statistical analysis

All numerical data are presented as mean ± SD. Differences between the 3 groups were tested by 1-way analysis of

Table 2 Total retinol concentration in lungs, liver, and serum

Total retinol	Lungs (μg/g)	Liver (μg/g)	Serum (μg/mL)
Controls	2.43 ± 0.31 (n = 28)	10.13 ± 2.35 (n = 44)	0.58 ± 0.12 (n = 38)
Nitrofen without CDH	1.61 ± 0.24* (n = 16)	9.26 ± 2.54 (n = 19)	0.75 ± 0.11* (n = 15)
Nitrofen with CDH	1.78 ± 0.37* (n = 31)	8.97 ± 2.27 (n = 38)	0.77 ± 0.13* (n = 25)

* $P < .001$ vs controls.

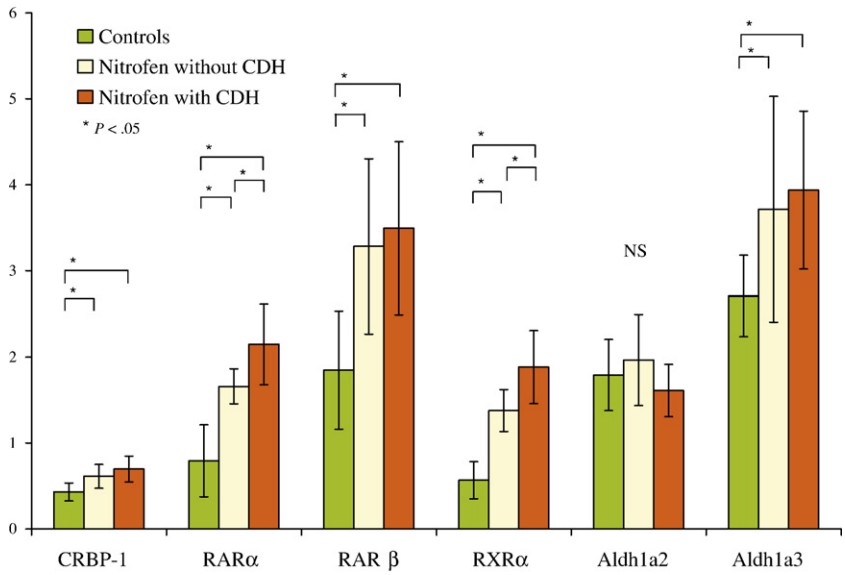

Fig. 2 Relative expression levels of mRNA of components of the retinoid signalling pathway.

variance. Differences between 2 groups were tested by unpaired *t* test. Statistical significance was defined as less than .05.

2. Results

Total retinol concentration in the lung, liver, and serum are shown in Table 2. Lung retinol levels were significantly lower in both nitrofen groups compared with controls, whereas serum retinol was significantly higher in nitrofen groups than in controls. There were no significant differences in total liver retinol levels between 3 groups. We did not find any significant difference in serum retinol level between nitrofen-exposed mothers (n = 4, 0.660 ± 0.073 μg/mL) and control mothers (n = 4, 0.654 ± 0.151 μg/mL).

Relative mRNA expression of CRBP-I, Aldh1a3, RARα, RARβ, and RXRα were significantly up-regulated in the lungs of nitrofen-exposed groups compared with controls (Fig. 2). There were no significant differences in relative levels of Ald1a2 between the 3 groups.

3. Discussion

It is well known that prenatal administration of nitrofen to pregnant mice or rats induces CDH and pulmonary hypoplasia in their litters. This experimental model has been widely used and has vastly contributed to our understanding of this congenital malformation. Using this model, some investigators have shown that hypoplasia of the lung precedes the closure of the diaphragm and led to the concept of a "dual-hit hypothesis" that explains pulmonary hypoplasia by 2 developmental insults. The first insult occurs before diaphragm development and affects equally the both lungs. After the occurrence of diaphragmatic defect, the second insult affects ipsilateral lung because of compression by herniated abdominal viscera [18]. This concept suggests that pulmonary hypoplasia in CDH could be a disease of impaired lung development associated with, but not necessarily caused by, a structural defect of the diaphragm [19].

The data derived from vitamin A–deficient diet and retinoid receptor null mutant studies provided the foundation for the hypothesis stating that nitrofen is acting to perturb the retinoid signalling pathway [8,11,20]. Chinoy et al [21] suggested that nitrofen and its metabolites may competitively bind with RARs during early embryonic exposure and thus lead to abnormal development of lungs and diaphragm. On the other hand, Chen et al [10] suggested that nitrofen could interact with events upstream of nuclear retinoid receptors, such as interference with the transport of retinol or its enzymatic conversion to retinoic acid. An in vitro assay has demonstrated that nitrofen inhibits RALDH2, which catalyzes retinal to retinoic acid [12].

Retinoic acid exerts its biologic effects through binding nuclear receptors, RARs and RXRs (isotypes α, β, and γ), which heterodimerizes to form the functional unit that transduces RA signalling. In the lung, these receptors are expressed from the earliest developmental stages throughout embryonic and postnatal life. Retinoic acid receptor α and all RXRs are ubiquitously expressed and do not change over time, whereas RARβ is excluded from the distal epithelium during the period of branching, but maintained in the epithelial cells of proximal and midsized airway [22,23]. Dramatic abnormalities have been reported in RARα/RARβ double knockout mice, which include unilateral lung agenesis and contralateral lung hypoplasia, strongly supporting a role for these receptors in lung morphogenesis [11].

In the 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 early weeks after

prenatal life as the lungs grow and develop. A sufficient and continuous availability of retinol (either on the blood pathway or by local storage sides) is pivotal, especially for a time-dependent regulation of the lung development and related formation of the active metabolite RA [24,25].

Retinoids in circulation are found predominately as retinol bound to RBP and as retinyl ester incorporated in lipoprotein of dietary origin. Retinol bound to RBP is considered to be the most physiologically important retinoid form transported from mother to fetus and within the fetus [26]. In the present study, we found that the retinol levels in nitrofen-induced hypoplastic lungs on day 21 of gestation were significantly decreased compared with those in controls. We also measured total retinol levels in hypoplastic lungs in the nitrofen model on day 17 and 19 of gestation and found strikingly similar result as on day 21 of gestation (unpublished data). This is of particular significance because days 17 to 21 are the most crucial period of retinol utilization in lung development. In contrast, we found that serum retinol levels were significantly higher in the nitrofen-exposed groups compared with those in controls. The serum retinol levels in dams demonstrated no significant difference among 3 groups. Based on the above findings, it is clear that the decreased lung retinol levels in the nitrofen-induced hypoplastic lungs are neither a consequence of insufficient retinol supply from the dams nor a lack of circulating retinol in the fetus. In addition, relative expression levels of CRBP-I, Aldh1a3, RARα, RARβ, and RXRα were significantly up-regulated in the lungs of nitrofen-exposed groups. The altered gene expressions of most downstream components of the retinoid signalling pathway may be a reaction to the deficiency of lung retinol. Although the mechanism by which the transfer of retinol form from extracellular RBP to intracellular CRBP is affected is not clearly understood, our results imply that nitrofen may act by interfering with cellular uptake of retinol in the lung.

4. Q&A

Kim, Toronto

In the context of diaphragmatic hernia model, is there a difference in the left side vs right side cellular uptake of the interaction between nitrofen and retinols?

A.

In this study we did not divide between right and left.

References

[1] Shinkai M, Shinkai T, Pirker ME, et al. Effect of nitric oxide on the development of nitrofen-induced fetal hypoplastic lung explants. J Pediatr Surg 2005;40(1):17-21.

[2] Kluth D, Kangah R, Reich P, et al. Nitrofen induced diaphragmatic hernia in rats: an animal model. J Pediatr Surg 1990;25(8):850-4.

[3] Blomhoff R, Blomhoff HK. Overview of retinoid metabolism and function. J Neurobiol 2006;66(7):606-30.

[4] Harrison EH. Mechanisms of digestion and absorption of dietary vitamin A. Annu Rev Nutr 2005;25:87-103.

[5] Paik J, Vogel S, Quadro L. et al. Vitamin A: overlapping delivery pathways to tissues from the circulation J Nutr 2004;134(1):276S-80S.

[6] Mactier H, Weaver LT. 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 2005;90:F103-8.

[7] Everts HB, Sundberg D, Ong E. Immunolocalization of retinoic acid biosynthesis systems in selected sites in rat. Exp Cell Res 2005; 308:309-19.

[8] Andersen DH. Incidence of congenital diaphragmatic hernia in the young of rats bred on a diet deficient in vitamin. Am J Dis Child 1941;62:888-9.

[9] Major D, Cadenas M, Leclerc S. Retinol status of newborn infants with congenital diaphragmatic hernia. Pediatr Surg Int 1998;13:547-9.

[10] Chen MH, MacGowan A, Ward S, et al. The activation of the retinoic acid response element is inhibited in an animal model of congenital diaphragmatic hernia. Biol Neonate 2003;83(3):157-61.

[11] Mendelsohn C, Lohnes D, Decimo D, et al. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development 1994;120(10):2749-71.

[12] Mey J, Babiuk RP, Clugston R, et al. Retinal dehydrogenase-2 is inhibited by compounds that induce congenital diaphragmatic hernias in rodents. Am J Pathol 2003;162(2):673-9.

[13] Thebaud B, Tibboel D, Rambaud C, et al. Vitamin A decreases the incidence and severity of nitrofen-induced congenital diaphragmatic hernia in rats. Am J Physiol 1999;277:L423-9.

[14] Thebaud B, Barlier-Mur AM, Chailley-Heu B, et al. Restoring effects of vitamin A on surfactant synthesis in nitrofen-induced congenital diaphragmatic hernia in rats. Am J Respir Crit Care Med 2001; 164:1083-9.

[15] Babiuk RP, Thebaud B, Greer JJ, et al. Reductions in the incidence of nitrofen-induced diaphragmatic hernia by vitamin A and retinoic acid. Am J Physiol Lung Cell Mol Physiol 2004;286(5):L970-3.

[16] Montedonico S, Nakazawa N, Puri P. Retinoic acid rescues lung hypoplasia in nitrofen-induced hypoplastic foetal rat lung explants. Pediatr Surg Int 2006;22(1):2-8.

[17] Hosotani K, Kitagawa M. Improved simultaneous determination method of beta-carotene and retinol with saponification in human serum and rat liver. J Chromatogr B 2003;791:305-13.

[18] Keijzer R, Liu J, Deimling J. Dual-hit hypothesis explains pulmonary hypoplasia in the nitrofen model of congenital diaphragmatic hernia. Am J Pathol 2000;156(4):1299-306.

[19] Gallot D, Marceau G, Coste K, et al. Congenital diaphragmatic hernia: a retinoid-signaling pathway disruption during lung development? Birth Defects Res A Clin Mol Teratol 2005;73(8):523-31.

[20] Greer JJ, Babiuk RP, Thebaud B. Etiology of congenital diaphragmatic hernia: the retinoid hypothesis. Pediatr Res 2003;53(5):726-30.

[21] Chinoy MR, Chi X, Cilley RE. Down-regulation of regulatory proteins for differentiation and proliferation in murine fetal hypoplastic lungs: altered mesenchymal-epithelial interactions. Pediatr Pulmonol 2001;32(2):129-41.

[22] Wongtrakool C, Malpel S, Gorenstein J, et al. Down-regulation of retinoic acid receptor alpha signaling is required for sacculation and type I cell formation in the developing lung. J Biol Chem 2003; 278(47):46911-8.

[23] Roth-Kleiner M, Post M. Similarities and dissimilarities of branching and septation during lung development. Pediatr Pulmonol 2005; 40(2):113-34.

[24] Biesalski HK, Nohr D. Importance of vitamin-A for lung function and development. Mol Aspects Med 2003;2:431-40.

[25] Shenai JP, Chytil F. Vitamin A storage in lung during perinatal development in the rats. Biol Neonate 1990;57(2):126-32.

[26] Quadro L, Hamberger L, Gottesman ME, et al. Pathways of vitamin A delivery to the embryo: insights from a new tunable model of embryonic vitamin A deficiency. Endocrinology 2005;146(10):4479-90.