Increased uptake of dietary retinoids at the maternal-fetal barrier in the nitrofen model of congenital diaphragmatic hernia

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A R T I C L E   I N F O

Abstract

Background/Purpose: The retinol signaling pathway is disrupted in congenital diaphragmatic hernia (CDH). Since there is no fetal retinol synthesis, maternal retinol has to cross the placenta. Nitrofen interferes with the retinol-binding protein (RBP) transfer pathway in CDH. However, in RBP knockout mice, retinol has been shown to be present. In this model, increased uptake of maternal dietary retinyl ester (RE) bound in low-density-lipoprotein (LDL) through low-density-lipoprotein-receptor 1 (LDLR) and increased activity of RE hydrolysis by lipoprotein-lipase (LPL) have been found. The aim of this study was to investigate the RE transfer pathway in the nitrofen CDH model.

Methods: Pregnant rats were treated with nitrofen or vehicle on gestational day (D9) and sacrificed on D21. Immunohistochemistry was performed to evaluate LRP1 and LPL protein expression. Serum LDL levels were measured by ELISA. Pulmonary and serum retinoid levels were measured using HPLC.

Results: Markedly increased trophoblastic and pulmonary LRP1 and LPL immunoreactivity were observed in nitrofen treated rats in comparison with controls. Serum LDL levels were increased in nitrofen treated rats in comparison with controls.

Conclusions: The increased uptake of dietary retinoids at the maternal-fetal barrier in the nitrofen CDH model suggests that the RE transfer pathway may be the main source of retinol in this model.

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retinol delivery to the fetus [7]. It has been demonstrated that maternal dietary RE bound in LDL through low-density lipoprotein-receptor 1 (LRP1) can be transferred to the placenta [9]. The RE in the placenta can be either hydrolyzed into retinol and transferred to the fetal circulation by lipoprotein-lipase (LPL), or can be released to the fetus via scavenger-receptor class B-1 (SR-B1) receptor as LDL containing RE [8,12]. In RBP knockout mice, increased placental activation of the alternative retinol transfer (increased LRP1 and LPL activation) has been found [12]. Moreover, it has been recently shown that lungs are able to take in retinol in RE formation from serum LDL through LRPI receptor[13] and lungs can hydrolyze RE to retinol by LPL [14]. Therefore, we hypothesized that in the nitrofen model of CDH during lung morphogenesis the alternative retinol transfer is activated in the placenta and then the lungs can take in dietary retinol for lung development. Thus, we designed this study to investigate the uptake of dietary retinoids at the maternal-fetal barrier in the nitrofen model of CDH.

1. Material and methods

1.1. Animals and drugs

Adult Sprague–Dawley rats were mated, and the females were checked daily for pluggings. The presence of spermatozoïds in the vaginal smear was considered as a proof of pregnancy: the day of observation determined gestational day 0 (term, 22 days). Pregnant females rats were then randomly divided into two groups. At day 9 of gestation (D9), animals in the experimental group received intragastrically 100 mg of nitrofen (WAKO Chemicals, Osaka, Japan) dissolved in 1 ml of olive oil under short anesthesia, whereas those in the control group received only the vehicle. Fetuses were harvested by cesarean section on D21 and divided into two groups: control (n = 8) and nitrofen with CDH (n = 8).

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, all animals were treated according to the current guidelines of animal care.

1.2. Tissue collection

After sedation with isoflurane, term fetuses were harvested free from the dams. Placentas and lungs were dissected from each fetus. Blood was taken from the dams by intracardiac puncture for serum LDL and retinol determination. Freshly prepared serum samples for enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC) were stored in aliquots at −80 °C after clotting for two hours and centrifugation for 10 minutes at 1,000×g. Lung samples for quantitative real-time polymerase chain reaction (qRT-PCR) were kept in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Total RNA quantification was performed spectrophotometrically (NanoDrop ND–1000 UV–vis Spectrophotometer). Total RNA (1 μg) was reverse-transcribed using Transcriptor High Fidelity cDNA Synthesis Kit® (Roche Diagnostics, West Sussex, UK) according to manufacturer’s instruction. Following reverse transcription at 44 °C for 60 minutes, qRT-PCR was performed using a LightCycler® 480 SYBR Green I Master® (Roche Diagnostics, West Sussex, UK) according to the manufacturer’s protocol. Gene-specific primer pairs are listed in Table 1. After initial denaturation step of 5 minutes at 95 °C, 45 cycles of amplification for each primer pair were carried out. Each cycle included a denaturation step (10 seconds at 95 °C), an annealing step (15 seconds at 60 °C) and an elongation step (10 seconds at 72 °C). Final elongation temperature was 65 °C for 1 minute. Relative levels of gene expression were measured using a LightCycler® 4800® (Roche Diagnostics, West Sussex, UK) according to the manufacturer’s instructions. The relative changes in the expression levels of LRP1 and LPL 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.

1.6. Enzyme-linked immunosorbent assay (ELISA)

Fetal serum LDL levels were measured with a rat LDL ELISA kit (E91107RA, USCN, China) according to the manufacturer’s protocol.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>5′-tgg cta aca gga tgg aga ag-3′</td>
<td>5′-tag agc ccc caa tcc caa ca-3′</td>
</tr>
<tr>
<td>LRP1</td>
<td>5′-ctt cgc aag acc tgt ac-3′</td>
<td>5′-aca gac ccc aca ttt acc ac-3′</td>
</tr>
<tr>
<td>LPL</td>
<td>5′-aca ctt gaa aag ctc tgt -3′</td>
<td>5′-ctg cct aat aat cgt tct c-3′</td>
</tr>
</tbody>
</table>
The results were measured at 450 nm with Synerg Mx microplate reader (BioTek, Winooski, USA) immediately after adding the stop solution. Experiments were carried out at least in duplicate for each data point.

1.7. Statistical analysis

All numerical data are presented as mean ± standard error of the mean (SEM). Differences between two 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.

2. Results

2.1. Immunohistochemical staining of LRP1, LPL and SR-B1

Immunohistochemistry showed markedly increased LRP1, LPL and SR-B1 immunoreactivity in CDH placenta compared to controls.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 8)</th>
<th>CDH (n = 8)</th>
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<tbody>
<tr>
<td>LRP1</td>
<td>48.93 ± 5.09</td>
<td>68.12 ± 3.39*</td>
</tr>
<tr>
<td>LPL</td>
<td>1.94 ± 0.40</td>
<td>4.05 ± 0.70*</td>
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*p<0.05 vs Control

Fig. 1. Trophoblastic LRP1, LPL and SR-B1 protein expression (magnification 40×).

Fig. 2. Relative mRNA expression levels and pulmonary protein expression of LRP1 and LPL.
Within the intestinal mucosa the retinol, regardless of its dietary small amount of RA) or as carotenoids from vegetables and fruits [8].

2.4. LDL levels in maternal and fetal serum

There were no significant differences in maternal serum LDL levels between nitrofen-exposed mothers (n = 4, 24.4 ± 2.5 ng/mL) and control mothers (n = 4, 29.0 ± 2.1 ng/mL). Significantly increased serum LDL levels were detected in CDH group (19.5 ± 1.4 ng/mL) compared to controls (14.9 ± 1.9 ng/mL, p < 0.05).

3. Discussion

It is well understood that retinoids, vitamin A and its derivates are essential for the morphogenesis of most developing organs and tissues, including lungs [4]. All retinoids are derived from diet either as preformed vitamin A from animal products (retinol, RE and very small amount of RA) or as carotenoids from vegetables and fruits [8]. Within the intestinal mucosa the retinol, regardless of its dietary origins, is re-esterified with long-chain fatty acids primarily [8]. Together with other dietary lipids, the newly synthesized REs are packaged into chylomicrons and secreted into the lymphatic system [8]. Once in the general circulation, LPL, which is bound to the luminal surface of the vascular endothelium, catalyzes the lipolysis of triglycerides to generate free fatty acids and chylomicron remnants [8]. After chylomicron remnants acquire apolipoprotein E, either in plasma or in the hepatic space of Disse, approximately 75% of chylomicron remnant–RE is cleared by the liver, the major site of retinol storage and metabolism [8]. Once taken up by the hepatocytes, REs are hydrolyzed again to retinol either to be transferred to stellate cells and then re-esterified for storage; or retinol can bind to its sole specific serum transport protein, RBP, to be secreted into the bloodstream [7]. The major function of RBP is to mobilize hepatic retinoid stores and deliver retinol to peripheral tissues such as embryos [7]. In the fasting circulation, retinol-RBP accounts for approximately 95-95% of all serum retinoids [8]. However, even in the fasting state there are always low concentrations of RE associated with circulating lipoproteins [8].

Since there is no de novo fetal synthesis of retinol, to meet its requirement for retinoids, the developing mammalian embryo relies on circulating maternal retinol that reaches the embryo through the maternal-fetal barrier [8]. Quadro et al. [16] have demonstrated that two major retinoid forms can be identified in the maternal bloodstream: retinol bound to RBP secreting from liver stores and RE packaged in chylomicrons upon dietary retinol intake. It has been shown in mice that maternal RBP does not cross the placenta [10]. Therefore, to enter the fetal circulation, maternal retinol bound to maternal RBP must be released at the maternal-fetal interface and trophoblasts have to produce their own RBP for the retinol transfer from placenta to fetus [10]. Interestingly, Wendler et al. [17] have found only a slightly diminished overall fetal size with a mild version of retinol deficiency in RBP knockout mice. They have also found high levels of RE incorporated in maternal circulating LDL in mice lacking retinol-RBP. These findings suggested that the failure of RBP ablation to recapitulate severe retinol deficiency phenotypes may reflect compensation by dietary retinoids. Quadro et al. [16] have demonstrated that RBP−/− fetuses from RBP knockout dams have pulmonary hypoplasia with an overall reduction in lung size, thus suggesting an impaired lung formation due to RBP deficiency. They have further shown that RBP−/− mice are highly dependent on dietary retinol to support pregnancy and fetal development. Even a small deprivation of maternal dietary retinoids can therefore cause severe PH and lung agenesis [16]. Recently it has been demonstrated that maternal dietary RE bounded in LDL through LRP1 can be transferred to the fetal circulation by LPL, or can be released it to the fetus via SR-B1 receptor as LDL containing RE [8]. In RBP knockout mice, increased placental activation of LRP1 and LDL has been found [9]. Therefore, Spiegler et al. [8] have concluded that under normal circumstances the retinol-RBP pathway is the primary contributor to fetal development, while in mice lacking retinol-RBP, the RE levels incorporated in maternal circulating chylomicron remnants provide the embryos with sufficient amounts of retinol for survival.

Several authors have shown that the retinoid signaling pathway is disrupted in both humans and animal models of CDH, and decreased pulmonary retinol levels have been associated with the development of PH in CDH [5,6]. Beurskens et al. [5] have found decreased serum RBP and retinol levels in human newborns with CDH compared to controls, whereas mothers of both have comparable levels of RBP and retinol. These authors concluded that the maternal-fetal transport may be disrupted, leading to CDH and PH. The importance of the retinoid signaling pathway during fetal development and especially in lung morphogenesis is further supported by a study showing that RA
administration attenuates the development of PH in the nitrofen-induced CDH model [18]. The nitrofen CDH model is one of the most widely used animal models to study the pathogenesis of PH in CDH [19,20]. However, the exact molecular mechanism underlying nitrofen-induced PH in CDH still remains unclear. It has recently been reported that nitrofen does not directly interfere with RA signaling but induces a dose-dependent apoptosis [21]. Clugston et al. [22] demonstrated that the levels of nitrofen estimated to reach the embryo would be too low to induce apoptosis directly. Recently, we showed that nitrofen disturbs the expression of trophoblastic RBP, resulting in PH in the nitrofen CDH model [12].

There are conflicting findings in serum retinol levels in CDH between human and animal studies [5,6,23]. Significantly decreased serum retinol levels have been found in human newborns with CDH [5,23]. In contrast, our previous work [6] and the present study have demonstrated significantly increased serum total retinol levels in CDH. This difference may be due to the different detection methods employed. Beurskens et al. [5] and Major et al. [23] measured only the retinol levels and they did not investigate RE levels in CDH. However, in our study we used that detection method which measured retinol as well as RE and RA. By using simultaneous measurement of retinol and RE, we demonstrated in the present study that the serum retinol level is significantly decreased in nitrofen-induced CDH fetuses. Moreover, we found that the most of the circulating retinoids in CDH are RE. At the same time significantly increased serum LDL levels were observed, thus suggesting that the circulating RE is bound to LDL. Therefore, the increased RE levels in serum of CDH fetuses may represent a potential mechanism of dietary retinoids to compensate the low retinol levels in serum. It has been shown that the lungs are able to take in retinol in RE form from serum LDL through the LRP1 receptor [13] and lungs can hydrolyze RE to retinol by LPL [14]. The increased pulmonary activation of LRP1 receptor may suggest that nitrofen-induced hypoplastic lungs increase their RE uptake. The comparable pulmonary retinol levels in CDH and controls together with the increased pulmonary LPL activation in CDH suggests, that CDH lung hydrolyze RE into retinol during lung morphogenesis. Therefore, it is tempting to speculate that the RE pathway is the main source of retinol in CDH during lung morphogenesis in the nitrofen model.

References