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

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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) bounded in low-dense-lipoprotein (LDL) through low-density-lipoprotein-receptor 1 (LRP1) 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 CDH compared to controls. Significantly increased serum LDL and RE levels were observed in CDH compared to 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.

Despite prenatal diagnosis and improved postnatal treatment strategies, the mortality rate of infants born with congenital diaphragmatic hernia (CDH) remains high [1]. The high mortality is mainly attributed to pulmonary hypoplasia (PH) and associated persistent pulmonary hypertension [2]. Much of our current understanding of the pathogenesis of PH in CDH originates from experimental studies. Maternal exposure of nitrofen (2,4-dichlorophenyl-*p*-nitrophenyl ether) to rodents during mid-gestation results in a high rate of CDH and associated PH to their fetuses, which is strikingly similar to the condition seen in humans [3]. However, the exact molecular mechanism by which nitrofen induces hypoplastic lungs in this model still remains unclear.

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]. Human [5] and animal [6] studies have found that the retinoid signaling pathway is disrupted in CDH, thus contributing to the development of PH. Recent work from our laboratory has shown that pulmonary retinol levels are significantly decreased in nitrofen-induced hypoplastic lungs during late lung morphogenesis, supporting the hypothesis that a disturbed retinol status is involved in the pathogenesis of CDH [6].

Within the maternal circulation, approximately 95–99% of retinol is bound to its sole specific carrier retinol-binding protein (RBP), which is the most abundant retinoid form [7]. It has been demonstrated that even in the fasting state there are always low concentrations of dietary retinyl ester (RE) associated with circulating low-density lipoprotein (LDL) and small amounts of circulating retinoid acid (RA) bound to albumin [8]. The placenta has a major role in the retinol homeostasis during fetal life [9]. Since there is no fetal retinol synthesis, the fetus relies on circulating maternal retinol that reaches the embryo through the maternal-fetal barrier in the placenta [8]. Recently, it has been demonstrated that maternal RBP does not cross the placental barrier [10]. Therefore, to enter the fetal circulation, maternal retinol bound to maternal RBP must be released at the maternal-fetal interface; and trophoblast have to produce their own RBP for retinol transfer from the placenta to the fetus [10,11]. It has been shown that this is the primary retinol contributor during fetal development [10]. In human newborns with CDH, both retinol and RBP in serum has been reported to be decreased, whereas maternal levels were comparable between mothers of CDH patients and mothers of healthy children in a case–control study [5]. The above findings suggested that maternal-fetal retinol transport by placenta may be disrupted and thus contribute to the development of PH in CDH. Recently, we demonstrated that nitrofen alters the trophoblastic RBP expression in the nitrofen model of CDH [12]. However, in RBP knockout mice retinol has been shown to be present and this reflects the existence of an alternative pathway of

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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 LRP1 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 plugging. The presence of spermatozoids in the vaginal smear was considered as a proof of pregnancy; the day of observation determined gestational day 0 (term, 22 days). Pregnant female 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, USA) and stored at −20 °C until further analysis. Placenta and lung samples for immunohistochemistry were fixed in 4% formalin and embedded in paraffin.

1.3. Immunohistochemistry

The paraffin-embedded lungs and placentas were sectioned at a thickness of 5 μm, and the sections were 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 monoclonal primary antibody against LRP1 (ab92544; Abcam, Cambridge, UK), 1:100 dilution of rabbit polyclonal primary antibody against SR-B1 (ab24603; Abcam, Cambridge, UK), and 1:50 dilution of rabbit polyclonal primary antibody against LPL (sc32885; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, sections were incubated with horseradish peroxidase-labeled anti-rabbit secondary antibodies and processed using a DAKO EnVision kit® (DAKO Ltd, Cambridgeshire, UK), developed with a diaminobenzidine-H_2O_2 substrate complex, and counter-stained with hematoxylin.

1.4. High-performance liquid chromatography (HPLC) analysis

Preparation of samples for measurement of total retinol concentration (including retinol, RE, RA and other metabolites of vitamin A) was performed by modification of a previously described protocol [6]. Total retinol concentrations of lungs and serum were analysed by HPLC with a SPD-10A Shimadzu UV–vis detector on a 3.9 × 150 mm, 5 μm reverse phase resolve C18 column (Waters, Milford, UK). The elution phase was acetonitrile/methanol/DMSO (90:10:1) and 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, Steinheim, Germany) between 0.1 and 10 μg/mL in concentration.

Preparation of samples for simultaneous determination of retinol and RE was performed by modification of a previously described protocol [15]. Retinol and RE concentrations of serum and lungs were analysed by HPLC as described above.

1.5. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA of each lung derived from fetuses was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to recommended 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 480® (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
Primers for quantitative real time PCR. Gene-specific primer pairs for qRT-PCR.

Gene	Sequence	
β actin	Forward	5′-ttg ctg aca gga tgc aga ag-3′
	Reverse	5′-tag agc cac caa tcc aca ca-3′
LRP1	Forward	5′-ctt tcg aag acc ctg agc ac-3′
	Reverse	5′-aca gag ccc aca ttt tcc ac-3′
LPL	Forward	5′- aca ctg gaa acg ctg ttg tg -3′
	Reverse	5′-ttc cgg ata aaa cgt tct cg-3′