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Decreased expression of monocarboxylate transporter 1 and 4 in the branching airway epithelium of nitrofen-induced congenital diaphragmatic hernia

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ABSTRACT

Background/Purpose: Monocarboxylate transporters (MCTs) are crucial for the maintenance of intracellular pH homeostasis in developing fetal lungs. MCT1/4 is strongly expressed by epithelial airway cells throughout lung branching morphogenesis. Functional inhibition of MCT1/4 in fetal rat lung explants has been shown to result in airway defects similar to pulmonary hypoplasia (PH) in congenital diaphragmatic hernia (CDH). We hypothesized that pulmonary expression of MCT1/4 is decreased during lung branching morphogenesis in the nitrofen model of CDH-associated PH.

Methods: Timed-pregnant rats received nitrofen or vehicle on gestational day 9 (D9). Fetuses were harvested on D15, D18, and D21, and divided into control and nitrofen-exposed group. Pulmonary gene expression levels of *MCT1/4* were analyzed by qRT-PCR. Immunofluorescence staining for MCT1/4 was combined with E-cadherin in order to evaluate protein expression in branching airway tissue.

Results: Relative mRNA levels of *MCT1/4* were significantly reduced in lungs of nitrofen-exposed fetuses on D15, D18, and D21 compared to controls. Confocal laser scanning microscopy confirmed markedly decreased immunofluorescence of MCT1/4 in distal bronchial and primitive alveolar epithelium of nitrofen-exposed fetuses on D15, D18, and D21 compared to controls.

Conclusion: Decreased expression of MCT1/4 in distal airway epithelium may disrupt lung branching morphogenesis and thus contribute to the development of PH in the nitrofen-induced CDH model.

Congenital diaphragmatic hernia (CDH) is a relatively common malformation, affecting 1 in 2500 live births [1]. The main cause of high mortality and morbidity in newborns with CDH is severe pulmonary hypoplasia (PH) and persistent pulmonary hypertension [2]. Hypoplastic lungs are characterized by immaturity and smaller size with a decreased number of terminal airways, thickened alveolar walls, increased interstitial tissue, diminished alveolar airspaces and reduced gas-exchange surface area [3,4].

In general, fetal lung development takes place in a relatively hypoxic environment, thus generating intracellular acids, which in turn leads to a decrease in intracellular pH [5,6]. The low oxygen environment of the fetus has been shown to be essential for normal lung branching morphogenesis [7]. Furthermore, it has been reported that disruption of the cellular response to oxygen alterations results in a significantly reduced distal airway branching [8]. Although the pathological mechanisms of PH have been extensively studied, the molecular basis of disrupted lung branching morphogenesis in CDH remains largely unclear.

Monocarboxylate transporters (MCTs) have been demonstrated to be crucial for the maintenance of intracellular pH homeostasis in developing fetal lungs by transporting intracellular acids across the plasma membrane [9–11]. MCT1 and MCT4 are strongly expressed in distal bronchial and primitive alveolar epithelial cells during lung branching morphogenesis, indicating their important role in the formation of fetal airways [12]. In addition, functional inhibition of MCT1 and MCT4 in fetal rat lung explants has been shown to result in severe defects in lung branching with a reduced number of distal airway buds similar to the phenotype of PH in human CDH [12].

Most of our current knowledge about the structural and molecular changes in CDH derives from experimental animal models [13]. Administration of the herbicide nitrofen (2,4-dichloro-phenyl-p-nitrophenyl ether) to pregnant rats on gestational day 9 (D9) has been demonstrated to result in PH and diaphragmatic defects in the offspring, both remarkably similar to human CDH [14,15].

We designed this study to investigate the hypothesis that pulmonary expression of MCT1 and MCT4 is decreased during lung branching morphogenesis in the nitrofen model of CDH-associated PH.

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1. Material and methods

1.1. Animals, drugs and experimental design

Pathogen-free adult Sprague–Dawley rats® (Harlan Laboratories, Shardlow, UK) were mated overnight, and females were checked daily for spermatozoids in their vaginal smear. The day of observation was considered as proof of pregnancy and was defined as embryonic day 0.5 (D0.5). Timed-pregnant animals were randomly divided into two experimental groups: "Nitrofen" and "Control". On gestational day 9 (D9), dams were briefly anesthetized with 2% volatile isoflurane (Piramal Healthcare Ltd., Morpeth, UK) and either 100 mg of nitrofen (WAKO Chemicals GmbH, Neuss, Germany), dissolved in 1 ml of olive oil, or vehicle alone was administered *via* oral-gastric lavage. On the selected time-points D15, D18 and D21, animals were anesthetized and their fetuses were delivered *via* caesarean section. After laparotomy, fetal diaphragms were inspected under a Leica S8AP0 stereomicroscope (Leica Microsystems AG, Heerbrugg, Switzerland) for CDH (Fig. 1). Whole lungs of nitrofen-exposed fetuses with a diaphragmatic defect ($n = 12$ per time-point) and controls ($n = 12$ per time-point) were dissected under sterile conditions *via* thoracotomy and stored either in a TRIzol® reagent (Invitrogen, Carlsbad, USA) for total RNA isolation or fixed in 10% paraformaldehyde (PFA) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) for immunofluorescence-double-staining. All animal procedures were carried out according to the current guidelines for management and welfare of laboratory animals and the experimental protocol was approved by the local research ethics committee (REC668b) and the Department of Health and Children (Ref. B100/4378) under the Cruelty to Animals Act, 1876 (as amended by European Communities Regulations 2002 and 2005).

1.2. Total RNA isolation from fetal rat lungs

Total RNA was isolated from fetal lungs with the acid guanidinium thiocyanate-phenol-chloroform extraction method using a TRIzol® reagent according to the manufacturer's protocol. Spectrophotometrical quantification of total RNA was performed with a NanoDrop ND-1000 UV–Vis® Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA).

1.3. Complementary DNA synthesis and quantitative real-time polymerase chain reaction

Reverse transcription of total RNA was carried out at 85 °C for 3 min (denaturation), at 44 °C for 60 min (annealing), and at 92 °C for 10 min (reverse transcriptase inactivation) using a Transcript High Fidelity cDNA Synthesis Kit® (Roche Diagnostics, Grenzach-Whylen, Germany) according to the manufacturer's instruction. The resulting cDNA was used for quantitative real-time polymerase chain reaction (qRT-PCR) using a LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Gene-specific primer pairs are listed in Table 1. After an initialization phase at 95 °C for 5 min, 55 amplification cycles were carried out. Each cycle included an initial denaturation step at 95 °C for 10 s, an annealing step at 60 °C for 15 s and an elongation step at 72 °C for 10 s. The final elongate temperature was 65 °C for 1 min. Relative mRNA expression levels of *MCT1* and *MCT4* were measured with a Light Cycler® 480 instrument (Roche Diagnostics, West Sussex, UK) and gene levels were normalized to the housekeeping gene *β-actin*. All experiments were run duplicated for each sample and primer pair.

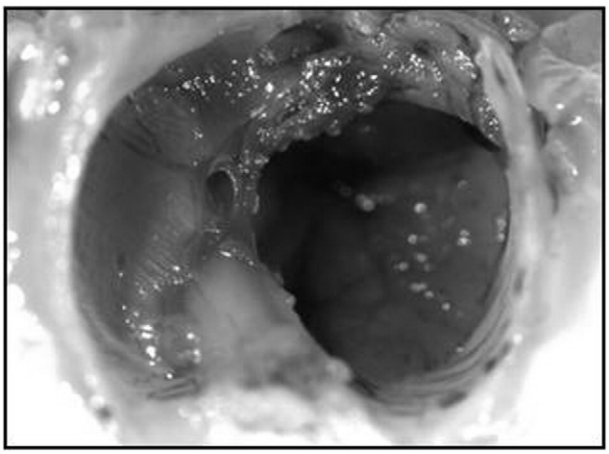

Fig. 1. Diaphragmatic defect in the nitrofen-induced rat on gestational day 21.

1.4. Immunofluorescence double staining and confocal laser scanning microscopy

Following overnight fixation in 10% PFA, fetal lungs were paraffin-embedded, transversely sectioned at a thickness of 5 μm, and mounted on polylysine-coated slides (VWR International, Leuven, Belgium). Tissue sections were deparaffinized with xylene and rehydrated through ethanol and distilled water. To improve cell permeabilization, sections were incubated with phosphate-buffered saline (PBS) containing 1.0% Triton X-100 (Sigma Aldrich Ltd., Arklow, Ireland) for 20 min at room temperature. Sections were then washed in PBS + 0.05% Tween (Sigma Aldrich, Saint Louis, USA) and subsequently blocked with 3% bovine serum albumin (Sigma Aldrich, Saint Louis, USA) for 30 min to avoid non-specific absorption of immunoglobulin. The blocking solution was rinsed off and sections were incubated with primary antibodies either against MCT1 (goat polyclonal, sc-14,917, 1:100) or MCT4 (rabbit polyclonal, sc-50,329, 1:100) and E-cadherin (mouse polyclonal, sc-8426, 1:100) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) overnight at 4 °C. On the next day, sections washed in PBS + 0.05% Tween and incubated with corresponding secondary antibodies (donkey anti-goat Alexa 555-A21432, 1:250, donkey anti-rabbit Alexa 647-A150067, 1:250 and donkey anti-mouse Alexa 488-A150109, 1:250) (Abcam plc, Cambridge, UK) for 1 h at room temperature. After another washing step in PBS + 0.05% Tween, sections were counterstained with a DAPI antibody (10,236,276,001, 1:1000) (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min, washed again, and mounted with glass coverslips using Sigma Mounting Medium (Sigma-Aldrich, St. Louis, MO, USA). All sections were scanned with a ZEISS LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) and independently evaluated by two investigators.

1.5. Statistical analysis

All numerical data are presented as means ± standard error of the mean. Differences between two groups were tested using an unpaired Student's *t* test when the data had normal distribution or a Mann–Whitney *U* test when the data deviated from normal distribution. Statistical significance was accepted at *P* values of less than 0.05.

2. Results

2.1. Relative mRNA expression levels of MCT1 and MCT4 in fetal rat lungs

Following qRT-PCR, the relative mRNA expression levels of *MCT1* and *MCT4* were significantly reduced in CDH lungs of nitrofen-exposed fetuses on D15 (0.23 ± 0.10 *vs.* 0.67 ± 0.37; $P < 0.05$ and 0.17 ± 0.04 *vs.* 0.25 ± 0.08; $P < 0.05$), D18 (0.44 ± 0.23 *vs.* 0.69 ± 0.23; $P < 0.05$ and 0.10 ± 0.07 *vs.* 0.18 ± 0.04; $P < 0.05$) and D21 (0.24 ± 0.13 *vs.* 0.61 ± 0.37; $P < 0.05$ and 0.10 ± 0.05 *vs.* 0.19 ± 0.06; $P < 0.05$) compared to controls.