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Spatiotemporal alterations in Sprouty-2 expression and tyrosine phosphorylation in nitrofen-induced pulmonary hypoplasia☆

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Abstract

Background/Purpose: Pulmonary hypoplasia (PH) is a life-threatening condition of newborns presenting with congenital diaphragmatic hernia (CDH). Sprouty-2 functions as a key regulator of fibroblast growth factor receptor (FGFR) signalling in developing foetal lungs. It has been reported that FGFR-mediated alveolarization is disrupted in nitrofen-induced PH. *Sprouty-2* knockouts show severe defects in lung morphogenesis similar to nitrofen-induced PH. Upon FGFR stimulation, Sprouty-2 is tyrosine-phosphorylated, which is essential for its physiological function during foetal lung development. We hypothesized that Sprouty-2 expression and tyrosine phosphorylation are altered in nitrofen-induced PH.

Methods: Time-pregnant rats received either nitrofen or vehicle on gestation day 9 (D9). Foetal lungs were dissected on D18 and D21. Pulmonary Sprouty-2 gene and protein expression levels were analyzed by qRT-PCR, Western blotting and immunohistochemical staining.

Results: Relative mRNA expression of *Sprouty-2* was significantly decreased in hypoplastic lungs without CDH (0.1050 ± 0.01 vs. 0.3125 ± 0.01; $P < .0001$) and with CDH (0.1671 ± 0.01 vs. 0.3125 ± 0.01; $P < .0001$) compared to controls on D18. Protein levels of Sprouty-2 were markedly decreased in hypoplastic lungs on D18 with decreased tyrosine phosphorylation levels on D18 and D21 detected at the molecular weight of Sprouty-2 consistent with Sprouty-2 tyrosine phosphorylation. Sprouty-2 immunoreactivity was markedly decreased in hypoplastic lungs on D18 and D21.

Conclusion: Spatiotemporal alterations in pulmonary Sprouty-2 expression and tyrosine phosphorylation during the late stages of foetal lung development may interfere with FGFR-mediated alveolarization in nitrofen-induced PH.

☆ Conflict of interest: The authors declare that there are no conflicts of interest.

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Congenital diaphragmatic hernia (CDH) is a relatively common congenital malformation. EUROCAT, a network of population-based registries for the epidemiologic surveillance of congenital anomalies in Europe, has recently reported a CDH prevalence rate of 2.55 per 10,000 births [1]. Pulmonary hypoplasia (PH) is considered to be one of the major contributors of high morbidity and mortality in newborn infants with CDH [2]. Despite recent improvements in antenatal diagnosis and postnatal intensive care, CDH remains a major life-threatening cause of severe respiratory failure, which is mainly attributed to PH [3].

Most of our current understanding of the pathophysiological mechanisms of CDH originates from experimental animal models [4]. The nitrofen-induced rat model of CDH is the most widely used animal model to investigate the pathogenesis of PH in CDH. Administration of the herbicide nitrofen (2,4-dichloro-*p*-nitrophenyl ether) to pregnant rats on day 9 (D9) of gestation induces PH and diaphragmatic defects in the offspring, both remarkably similar to the pathologic findings in the posterolateral or Bochdalek CDH in humans [5,6]. Furthermore, it has been shown that the disruption of alveolarization and airway maturation contribute to the development of PH in the nitrofen-induced rat model [7]. Although several studies have recently provided new insights into the pathogenesis of PH associated with CDH [8,9], the exact molecular mechanisms underlying nitrofen-induced PH remain unclear.

Sprouty-2 is involved in many biological processes, including cell growth, differentiation, and lung morphogenesis [10]. Sprouty-2 functions as a key regulator of fibroblast growth factor receptor (FGFR) signalling pathway, which plays an essential role in foetal lung development [11]. Moreover, it has recently been reported that FGFR-mediated alveolarization is disrupted in nitrofen-induced PH [12].

During embryogenesis, Sprouty-2 is widely expressed in several developing organs, including the lung [13]. In foetal rat lungs, expression of Sprouty-2 is mainly observed in epithelial cells of the distal alveolar buds, indicating its important role during alveolar maturation [14]. In addition, it has been shown that *Sprouty-2* knockouts exhibit severe defects in lung morphogenesis similar to nitrofen-induced PH with a significantly reduced number of distal airways [15]. Newly synthesized Sprouty-2 proteins undergo posttranslational modification such as tyrosine phosphorylation [16]. It has been reported that upon FGFR stimulation, Sprouty-2 proteins are tyrosine phosphorylated, suggesting that tyrosine phosphorylation plays an essential role for the physiological function and activity of pulmonary Sprouty-2 proteins during foetal lung development by regulating their interaction with a subset of partner proteins [17,18].

This study was designed to investigate the hypothesis that pulmonary Sprouty-2 expression and tyrosine phosphorylation status are altered during the late stages of foetal lung development in nitrofen-induced PH.

1. Material and methods

1.1. Animals, drugs and experimental design

Pathogen-free, timed-pregnant (D0 = sperm in vaginal smear) adult *Sprague–Dawley* rats were randomly divided into two groups ("nitrofen" and "control"). On D9, animals received intragastrically under short anaesthesia either 100 mg nitrofen (2,4-dichloro-*p*-nitrophenyl ether) (Wako Chemicals GmbH, Neuss, Germany), dissolved in 1 mL olive oil, or only vehicle. Dams were sacrificed on selected end points D18 and D21, and foetuses were delivered by caesarean section. In total, 144 foetal lungs were dissected via thoracotomy. Nitrofen-exposed lungs were divided into two subgroups: hypoplastic lungs without a diaphragmatic defect (CDH^-) and hypoplastic lungs with a diaphragmatic defect (CDH^+). Foetal lungs of animals that received only vehicle constituted the control group. The lungs of each experimental group (control, CDH^- and CDH^+) were either snap-frozen in liquid nitrogen (and stored at −70 °C) for RNA isolation (n = 8 in each group at each end point) and protein extraction (n = 8 in each group at each end point), or were fixed in 4% paraformaldehyde for 24 h until immunohistochemical staining was carried out (n = 8 in each group at each end point). All animal procedures were performed following current guidelines for management and welfare of laboratory animals and were approved by 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 and Complementary DNA Synthesis

Total RNA was isolated from snap-frozen whole lungs of each experimental group (n = 8 in each group at each end point) with the acid guanidinium thiocyanate-phenol-chloroform extraction method using a TRIzol reagent (Invitrogen by Life Technologies, Carlsbad, USA) according to the manufacturer's protocol. Concentration and purity of total RNAs were determined spectrophotometrically using a NanoDrop ND-1000 UV–vis (Thermo Scientific Fisher, Wilmington, USA). 1 μg of total RNA was retroscripted to complementary DNA (cDNA) by reverse transcription reaction using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. All cDNA samples were stored at 4 °C until further use.

1.3. Quantitative Real-Time polymerase chain reaction

Sprouty-2 mRNA expression was quantified with a LightCyler 480 system using a SYBR Green I Master Mix (Roche Diagnostics GmbH, Mannheim, Germany) according