

Prenatal retinoic acid upregulates pulmonary gene expression of PI3K and AKT in nitrofen-induced pulmonary hypoplasia

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Abstract

Background The precise mechanism of pulmonary hypoplasia associated with congenital diaphragmatic hernia (CDH) still remains unclear. Recently, prenatal treatment with retinoic acid (RA) has been reported to stimulate alveologenesis in hypoplastic lungs in the nitrofen model of CDH. The serine/threonine protein kinase B (AKT) plays a key role in lung morphogenesis through epithelial–mesenchymal interaction in phosphatidylinositol 3-kinase (PI3K)-dependent manner. It has been reported that the lung morphogenesis in explants in mice is interfered by inhibitors of PI3K–AKT signaling pathway. Furthermore, we have recently shown that nitrofen inhibits PI3K–AKT signaling during mid-to-late lung morphogenesis in the nitrofen-induced hypoplastic lung. We hypothesized that prenatal administration of RA upregulates pulmonary gene expression of PI3K and AKT in the nitrofen-induced hypoplastic lung.

Methods Pregnant rats were exposed to either olive oil or nitrofen on day 9 of gestation (D9). 5 mg/kg of RA was given on D18, D19 and D20. The fetuses were harvested on D21, and fetal lungs were obtained and divided into four groups: control, control + RA, nitrofen, nitrofen + RA. The mRNA expression levels of PI3K and AKT were analyzed in each lung by real-time RT-PCR and

statistically analyzed. Immunohistochemistry was also performed to evaluate protein expression of PI3K and AKT in the fetal lungs at D21.

Results The pulmonary gene expression levels of PI3K and AKT were significantly upregulated in nitrofen + RA group compared to nitrofen group and control + RA group ($p < 0.05$), whereas there were no significant differences between controls and control + RA group. Immunoreactivity of PI3K and AKT was markedly increased in nitrofen + RA lungs compared to nitrofen-induced hypoplastic lungs.

Conclusions Upregulation of PI3K and AKT genes after prenatal treatment with RA in the nitrofen-induced hypoplastic lung suggests that RA may have a therapeutic potential in modulating lung alveologenesis by stimulating epithelial–mesenchymal interaction via PI3K–AKT signaling.

Keywords Retinoic acid · Pulmonary hypoplasia · Nitrofen · PI3K · AKT

Introduction

Despite significant advances in prenatal diagnosis and improved neonatal resuscitation and intensive care, the mortality and morbidity in patients with congenital diaphragmatic hernia (CDH) remains high [1, 2]. Pulmonary hypoplasia in CDH, characterized by immaturity and small lung size, produces respiratory failure that is considered to be the principle contributor to the high mortality [1, 3]. Much of the current understanding of pathogenesis of pulmonary hypoplasia in CDH originates from experimental studies. Maternal exposure of nitrofen (2,4-dichlorophenyl-*p*-nitrophenyl ether) in rodents during specific

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time in gestation results in a high rate of CDH and associated pulmonary hypoplasia to their fetuses, which is strikingly similar to the condition seen in the human [4]. Several studies from our laboratory using nitrofen-induced CDH rodent model have provided important insights into the molecular mechanisms of pulmonary hypoplasia associated with CDH [5–7]. However, the exact molecular mechanism by which nitrofen induces hypoplastic lung in this model still remains unclear.

In the embryogenesis and fetal development, retinoids, the family of molecules derived from vitamin A, are essential for normal development of various organs including the lungs and diaphragm [8]. Retinoic acid (RA) is the active metabolite of vitamin A that is essential in each of the lung developmental stages [9]. Recent studies have suggested that the retinoid signaling pathway (RSP) is disrupted in the nitrofen-induced hypoplastic lung [10–13]. Work from our laboratory has shown that retinoic acid rescues lung hypoplasia in nitrofen-induced hypoplastic fetal rat lung explants [14, 15]. We have further reported that prenatal treatment with RA can upregulate the expression level of genes, which are involved in RSP and known to be downregulated in the nitrofen-induced pulmonary hypoplasia [16].

The serine/threonine protein kinase B (also known as AKT) is involved in multiple cellular functions, such as cell proliferation, differentiation, and survival [17]. The activity of AKT is regulated by phosphatidylinositol 3-kinase (PI3K) [18]. PI3K–AKT signaling mediates growth and survival signals through epithelial–mesenchymal interaction during development of fetal mouse lung [19]. We have recently reported that pulmonary gene expression of PI3K and AKT is downregulated during late stages of lung development in the nitrofen-induced hypoplastic lung [20]. Although it has been reported that RA activates PI3K/AKT signaling pathway in vascular endothelial cells [21], little is known about the effect of RA on lung morphogenesis through activation of PI3K–AKT signaling pathway.

We therefore designed this study to investigate the hypothesis that the expression of PI3K and AKT is upregulated after prenatal treatment with RA in the nitrofen-induced hypoplastic lung.

Materials and methods

Animals and drugs

Adult Sprague-Dawley rats were mated and the presence of spermatozooids in the vaginal smear was verified and was considered as gestational day 0. Pregnant female rats were then randomly divided into two groups. Animals in the experimental group received intragastrically 100 mg of

nitrofen (Wako Chemicals, Osaka, Japan) dissolved in 1 ml of olive oil on day 9 of gestation (D9), whereas those in the control group received only vehicle. On D18, the rats were randomly injected intraperitoneally with all *trans*-retinoic acid 5 mg/kg in cottonseed oil (Sigma, St Louis, MO, USA) with 100% ethanol as diluents. The injections were repeated on D19 and D20, and then the rats were sedated with isoflurane and killed by cervical dislocation on D21. The fetuses were recovered by cesarean section and harvested the left lungs of fetuses from four treatment groups: control, control + RA, nitrofen and nitrofen + RA ($n = 8$ for each group). The control group consisted of fetuses that only received olive oil, and the control fetuses that exposed to RA were defined as the control + RA group. Fetuses treated with nitrofen were defined as the nitrofen group and additionally exposed to RA were defined as the nitrofen + RA group. In order to obtain representative numbers, the fetuses in each group came from at least three different dams. The Department of Health and Children approved all the animal experiments (ref. B100/4022) under the Cruelty to Animals Act, 1876; as amended by European Communities Regulations 2002 and 2005.

RNA isolation and real-time reverse transcription polymerase chain reaction

The peripheral region of left lungs dissected from the thoracic cavity were immediately suspended in RNAlater solution (Ambion/Applied Biosystems, TX, USA), and stored at -20°C . The total RNA of each lung derived from fetuses was isolated using TRIZOL[®] reagent (Invitrogen, Carlsbad, CA, USA), according to recommended protocol. As reverse transcription (RT), first strand cDNA was synthesized from RNA by using RETROscript[®] (Ambion/Applied Biosystems) according to manufacturer's instruction. Following RT at 44°C for 60 min, polymerase chain reaction (PCR) was performed using a LightCycler[®] 480 SYBR Green I Master (Roche Diagnostics, West Sussex, UK) according to the manufacturer's protocol. Gene-specific primers are listed in Table 1. The specific primer pairs used in this study are same sequences as described in our previous paper. After initial denaturation step of 5 min at 95°C , 45 cycles of amplification for each primer pair were carried out. Each cycle included a denaturation step; 10 s at 95°C , an annealing step; 15 s at 60°C and an elongation step; 10 s at 72°C . Final elongation temperature was 65°C for 1 min. Relative levels of gene expression was measured using a LightCycler[®] 480 (Roche Diagnostics) according to the manufacturer's instructions. The relative changes in the expression levels of PI3K and AKT 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.

Table 1 Primers for quantitative real-time RT-PCR

Gene	Sequence (5'–3')	Product size (bp)
β -actin		
Forward	TTG GAT GCC TGT GGT CTG TC	108
Reverse	TAG AGC CAC CAA TCC ACA CA	
PI3K		
Forward	ATG GGT TGG AAG ATC TGC TG	113
Reverse	TGG AAA CTT CAC CAC ACT GC	
AKT		
Forward	TGT GAT GGA GTA TGC CAA CG	214
Reverse	TTT GCA CAA GCC AAA GTC AG	

Immunohistochemistry

The paraffin-embedded fetal lungs were sectioned at a thickness of 5 μ m, and the sections were deparaffined with xylene and then rehydrated through ethanol and distilled water. Tissue sections were immersed in target retrieval solution (DAKO, Cambridgeshire, UK) in a microwave oven (at 750 W) for 10 min followed by 10 min incubation in 0.3% H₂O₂ to block endogenous peroxidase activity. Sections were incubated at 4°C overnight with each of the primary antibodies including a 1:100 dilution of rabbit polyclonal antibodies against PI3K p110 α (Lot sc-7174; Santa Cruz Biotechnology, CA, USA) and AKT1/2/3 (Lot sc-8312; Santa Cruz Biotechnology). Sections were then treated in horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibodies and then processed using a kit (DAKO) developed with a diaminobenzidine–H₂O₂ substrate complex, and counterstained with hematoxylin.

Statistical analysis

All numerical data are presented as means \pm standard deviation. Differences between four groups were tested by Tukey–Kramer post hoc test following one-way ANOVA or Scheffe's *F* test following Kruskal–Wallis test. Statistical significance was accepted at *P* values <0.05.

Results

Relative mRNA expression levels of PI3K and AKT at D21 in fetal rat lungs

The pulmonary gene expression levels of PI3K and AKT were significantly increased in nitrofen + RA group (14.43 \pm 3.08 and 1.41 \pm 0.17, respectively) compared to nitrofen group (6.25 \pm 1.01 and 1.18 \pm 0.27, respectively) (*p* < 0.05) (Table 2). Furthermore, pulmonary expression

Table 2 The relative mRNA expression levels of PI3K and AKT in fetal lung on D21

	Control	Control + RA	Nitrofen	Nitrofen + RA
PI3K	8.95 \pm 3.22	7.78 \pm 1.43	6.25 \pm 1.01	14.43 \pm 3.08*
AKT	1.22 \pm 1.07	1.10 \pm 0.14	1.18 \pm 0.27	1.41 \pm 0.17**

* *p* < 0.0001, ** *p* < 0.05 vs. control + RA and nitrofen

levels of PI3K and AKT gene were also significantly upregulated in nitrofen + RA group (14.43 \pm 3.08 and 1.41 \pm 0.17, respectively) compared to control + RA group (7.78 \pm 1.43 and 1.10 \pm 0.14, respectively) (*p* < 0.05). There were no significant differences in pulmonary PI3K and AKT gene expression between controls and control + RA group.

Immunohistochemical study of PI3K and AKT at D21 in fetal rat lungs

To determine whether the increased amounts of PI3K and AKT transcripts after prenatal treatment with RA were reflected in the amount of the protein themselves in fetal rat lung, immunohistochemical study was performed (Fig. 1). Although there was no remarkable difference in immunoreactivity of PI3K between control lung (a) and control + RA lung (c), markedly increased expression of PI3K was observed in nitrofen + RA lung (g) compared to nitrofen-induced hypoplastic lung (e). Immunoreactivity of AKT was also markedly increased in nitrofen + RA lung (h) compared to nitrofen-induced hypoplastic lung (f), whereas there were no differences in intensity of protein expression between control + RA lung (d) and controls (b) Table 2.

Discussion

Retinoids, vitamin A and its derivatives, are essential for growth, development, and tissue differentiation in various organs including lungs [8]. RA, active metabolite of retinoids, is necessary for the lung development in each developmental stage [9]. RA exerts its biologic effects through binding nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which heterodimerizes to form the functional unit that transduces RA signaling. During the saccular to the alveolar period, there is a decrease of retinyl ester storage and production of RA [9]. It has been reported that postnatal treatment with retinoic acid increases the number of pulmonary alveoli in rats [22]. It has further been demonstrated that RA can induce alveolar regeneration in the adult mice [23]. Recent studies suggest that RSP may be involved in pathogenesis of CDH and associated pulmonary hypoplasia. Studies

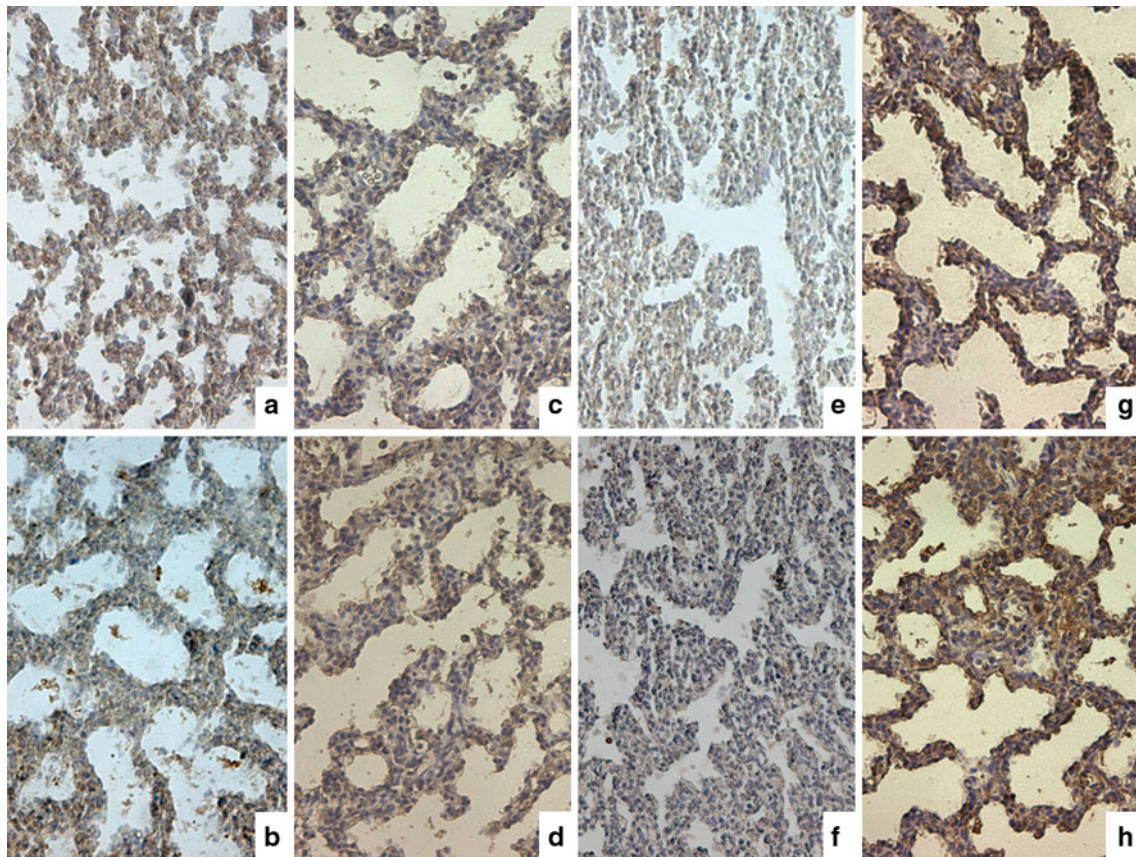


Fig. 1 PI3K and AKT immunohistochemical study. Although there was no remarkable difference in immunoreactivity of PI3K between control lung (a) and control + RA lung (c), increased expression of PI3K was observed in nitrofen + RA lung (g) compared to nitrofen-induced hypoplastic lung (e). Immunoreactivity of AKT was also

markedly increased in nitrofen + RA lung (h) compared to nitrofen-induced hypoplastic lung (f), whereas there were no differences in intensity of protein expression between control + RA lung (d) and controls (b)

from our laboratory have previously demonstrated that prenatal treatment with RA in late gestation upregulates expression of genes involved in lung morphogenesis and promotes pulmonary alveologenes in the nitrofen model of CDH [15, 16]. Although there is only one clinical study that reported that there was a 50% reduction in plasma retinol and retinal-binding protein levels in a group of 11 CDH newborn compared to matching controls [24], this clinical data along with the extensive experimental evidence in the animal model of CDH strongly support the concept of a therapeutic potential of RA in reverting lung hypoplasia in CDH.

PI3K–AKT signaling pathway plays a pivotal role in fetal lung development through regulating epithelial cell proliferation, differentiation and cell survival [19]. In vitro study has demonstrated that all-*trans* retinoic acid (ATRA) activates RAR-mediated PI3K–AKT pathway [21]. In the nitrofen-induced CDH rodent model, it has been considered that RSP is inhibited in the nitrofen-induced hypoplastic lung. We have recently shown that pulmonary PI3K and AKT gene expression is downregulated in later

gestational days in the nitrofen CDH model [20]. These studies suggest that, in the nitrofen CDH model, reduced activity of RAR-mediated PI3K–AKT pathway during later stages of lung development may induce disruption of various pivotal biological processes in fetal lung development, thus causing pulmonary hypoplasia. In the present study, we clearly demonstrated that prenatal RA treatment upregulated pulmonary gene expression of PI3K and AKT in the nitrofen-induced hypoplastic lung. Upregulation of PI3K and AKT may rescue lung hypoplasia in nitrofen CDH model by activating lung epithelial cell proliferation and differentiation through RAR-mediated PI3K–AKT pathway.

Cellular retinol-binding protein-I (CRBP-I) has been described as an inhibitor of the PI3K–AKT pathway in a RAR-dependent manner [25]. It has been previously shown from our laboratory that mRNA expression levels of CRBP-I in the nitrofen-induced hypoplastic lung are significantly upregulated on D21 compared to controls [12]. In the nitrofen-induced hypoplastic lung, downregulation of PI3K and AKT genes during later stages of lung

development is consistent with upregulation of CRBP-I on D21. We showed in this study that the gene expression levels of PI3K and AKT are significantly upregulated in the nitrofen-induced hypoplastic lung after prenatal treatment with RA. Thus, our data suggest that prenatal treatment with RA may modulate lung growth in the nitrofen-induced hypoplastic lung, preventing CRBP-I-mediated inhibition of PI3K–AKT pathway.

In the present study, although the pulmonary gene expression levels of PI3K and AKT were significantly upregulated in nitrofen + RA group compared to nitrofen group, there were no significant differences between control + RA group and controls. The significant upregulation of PI3K and AKT gene expression in nitrofen + RA group may be a reaction to the deficiency of RA in the nitrofen-induced hypoplastic lung.

In summary, we provide evidence for the first time that prenatal treatment with RA in hypoplastic lung during late gestation upregulates the pulmonary gene expression levels of PI3K and AKT in the nitrofen rodent model of CDH. Furthermore, we also observed that the immunoreactivity of PI3K and AKT is markedly increased after prenatal treatment with RA in the nitrofen-induced hypoplastic lung. It is therefore tempting to speculate that upregulation of PI3K and AKT genes during the alveolar period of lung morphogenesis may rescue lung hypoplasia and enhance lung growth in the nitrofen CDH model, by stimulating RAR-mediated PI3K–AKT signaling. Our findings suggest that RA may have a therapeutic potential in modulating lung alveologenesis by stimulating epithelial–mesenchymal interaction via PI3K–AKT signaling.

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