Journal of Pediatric Surgery 50 (2015) 1467–1471

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

Increased pulmonary RhoA expression in the nitrofen-induced congenital diaphragmatic hernia rat model

Hajime Takayasu *, Kouji Masumoto, Koki Hagiwara, Takato Sasaki, Kentaro Ono, Takahiro Jimbo, Toru Uesugi, Chikashi Gotoh, Yasuhisa Urita, Toko Shinkai, Hideaki Tanaka

Department of Pediatric Surgery, Faculty of Medicine, University of Tsukuba, 1-1-1, Tennoudai, Tsukuba, Ibaraki 305-8575, Japan

ARTICLE INFO

Article history:
Received 22 November 2014
Received in revised form 5 February 2015
Accepted 24 February 2015

Key words:
Congenital diaphragmatic hernia
Pulmonary hypertension
Nitrofen
RhoA/Rho-kinase
Vasoconstrictor

ABSTRACT

Purpose: Persistent pulmonary hypertension remains a major cause of mortality and morbidity in cases of congenital diaphragmatic hernia (CDH). Recently, RhoA/Rho-kinase-mediated vasoconstriction has been reported to be important in the pathogenesis of pulmonary hypertension (PH). Several recent reports have described that fasudil, a potent Rho-kinase inhibitor and vasodilator, could represent a potential therapeutic option for PH. We designed this study to investigate the hypothesis that the expression level of RhoA is increased in the nitrofen-induced CDH rat model. The expression level of Wnt11, an activator of RhoA, was also evaluated.
Methods: Pregnant rats were treated with or without nitrofen on gestational day 9 (D9). Fetuses were sacrificed on D17, D19 and D21 and were divided into control and CDH groups. Quantitative real-time polymerase chain reaction was performed to determine the pulmonary gene expression levels of both Wnt11 and RhoA. An immunofluorescence study was also performed to evaluate the expression and localization of RhoA.
Results: The relative mRNA expression levels of pulmonary Wnt11 and RhoA on D21 were significantly increased in the CDH group compared with the control group ($p = 0.016$ and $p = 0.008$, respectively). The immunofluorescence study confirmed the overexpression of RhoA in the pulmonary vessels of CDH rats on D21.
Conclusions: Our results provide evidence that the RhoA/Rho-kinase-mediated pathway is involved in the pathogenesis of PH in the nitrofen-induced CDH rat model. Our data also suggest that the fasudil, a Rho-kinase inhibitor, could represent a therapeutic option for the treatment of PH in CDH.

Congenital diaphragmatic hernia (CDH) still remains a major therapeutic challenge despite advances in neonatal medicine [1–3]. Children born with CDH suffer from a number of comorbidities. The most common and serious comorbidity is respiratory insufficiency resulting from a combination of lung hypoplasia and pulmonary hypertension (PH) [1–4]. High mortality of infants born with CDH is also attributed to lung hypoplasia and PH [1–5]. PH is characterized by structural changes in the pulmonary arteries, resulting in adventitial and medial thickening [5,6]. The nitrofen-induced rat CDH model has been widely used to investigate the pathogenesis of lung hypoplasia and PH associated with CDH. Recently, several investigators have shown that there are morphological changes and disruptions of several pathways associated with PH in CDH [7–9]. However, the exact mechanism(s) underlying the development of PH in CDH is still unclear.

There are several intracellular signaling pathways that can contribute to the morphological change of vessels and smooth muscle contractility. In the 1990s, Rho kinase was identified as an effector of the small GTPase, Rho, which plays an important role in various cellular functions, including smooth muscle contraction, actin cytoskeleton organization, cell adhesion and motility, cytokinesis and gene expression [10,11]. Rho and the Rho kinase pathway has been reported to play an important role in not only PH, but also vasospasm, ischemia–reperfusion injury, hypertension, stroke and heart failure [10,11]. Recently, a Rho kinase-mediated pathway was reported to be involved in the pathogenesis of monocrotaline-induced PH in a rat model [12]. In that study, treatment with fasudil, a Rho kinase inhibitor, improved the PH, right ventricular hypertrophy and pulmonary vascular lesions, along with suppression of the vascular smooth muscle cells (VSMC) proliferation and macrophage infiltration, enhanced VSMC apoptosis, and amelioration of the endothelial dysfunction and VSMC hypercontraction [10–12]. Another study has shown evidence of RhoA/Rho kinase-mediated vasoconstriction in patients with PH who received lung transplantation [13]. In children with PH associated with congenital heart disease, both the pulmonary arterial pressure and vascular resistance were significantly reduced by a Rho kinase inhibitor [14].

In this study, we hypothesized that the RhoA/Rho kinase-mediated vasoconstriction contributes to the pathogenesis of PH in CDH. If RhoA/Rho kinase is involved in the pathogenesis of PH in CDH, then fasudil, a Rho-kinase inhibitor, could be a novel therapeutic option for this condition. To investigate this hypothesis, we designed this study to investigate the expression levels of RhoA and Wnt11, which is one of Rho/Rho-kinase activator, in the nitrofen-induced CDH rat model.

* Corresponding author. Tel.: +81 29 853 3094; fax: +81 29 853 3149.
E-mail address: hajimeta@md.tsukuba.ac.jp (H. Takayasu).

http://dx.doi.org/10.1016/j.jpedsurg.2015.02.063
0022-3468/

1. Materials and Methods

Pathogen-free, timed-pregnant, adult *Sprague–Dawley* rats (Charles River Laboratories, Yokohama, Japan) were randomly divided into two experimental groups: "CDH" and "control". The observation of spermatozoids in the vaginal smear was considered proof of pregnancy, and the day of observation was determined to be D0. Under anesthesia, the animals in the CDH group received 100 mg of nitrofen (2,4-dichloro-*p*-nitrophenyl ether) (WAKO Chemicals, Osaka, Japan) intragastrically that had been dissolved in 1 ml of olive oil on D9. The animals in the control group received only vehicle (olive oil). On D17, D19, and D21, selected dams were anesthetized with 2% volatile isoflurane (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) in an induction chamber, followed by delivery of the fetuses by cesarean section. In the CDH group, laparotomy was performed for inspection of the fetuses for CDH. Left lungs with a diaphragmatic defect ($n = 8$ at each time point) and controls ($n = 8$ at each time point) were dissected via thoracotomy and stored in RNA Later solution (Ambion, Life Technologies, Tokyo, Japan) at −20 °C.

After thawing and homogenizing the samples, total RNA and protein of the diaphragmatic tissue were extracted using the TRIzol® Reagent (Life Technologies, Tokyo, Japan) according to the manufacturer's protocols. Afterwards, total RNA quantification was performed spectrophotometrically (NanoDrop® ND-1000 UV–vis Spectrophotometer, Wilmington, USA). cDNA synthesis was performed using the GeneAmp RNA PCR Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Reverse transcription was carried out at 65 °C for 3 min (denaturation), 37 °C for 5 min (annealing) and 98 °C for 10 min (reverse transcriptase inactivation). Quantitative PCR was performed using THUNDERBIRD™ SYBR® qPCR Mix (TOYOBO, Tokyo, Japan). The gene-specific primer sets used are shown in Table 1. The relative mRNA expression levels were measured by the Applied Biosystems® 7500/7500 Fast Real-Time PCR system® (Applied Biosystems, Tokyo, Japan) and the levels of the target genes were normalized to those of the housekeeping gene, β-actin (ΔΔCT method). After initialization at 95 °C for 20 s, 40 RT-PCR cycles for amplification were carried out at 95 °C for 3 s (denaturation) and 60 °C for 30 s (annealing). The mRNA levels of controls were assumed to be normal. Experiments were carried out in triplicate for each data point.

1.1. Immunofluorescence study

Fetal left lungs were washed in PBS, embedded in Tissue Tech O.C.T. compound (Funakoshi, Tokyo, Japan) and frozen at −80 °C. Frozen blocks were sectioned transversely at a thickness of 7 μm and mounted on Matsunami Adhesive Slide (MAS)-coated Superfrost glass microslides (Matsunami, Osaka, Japan). The sectioned lung tissue for immunohistochemistry was fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 4 h. After washing them with PBS, the sections were subjected to cell membrane permeabilization with 0.5% Triton X-100 for 5 min at room temperature. Sections were then washed and subsequently incubated with blocking solution (2% bovine serum and 0.3 M glycine) for 10 min to avoid nonspecific absorption of immunoglobulin. The blocking solution was then rinsed off, and sections were incubated with antibodies against RhoA (mouse monoclonal, sc-418, 1:50 dilution in PBS, Santa Cruz Biotechnology, USA) and von Willebrand Factor (VWF) (rabbit polyclonal, A0082, 1:400 dilution in PBS, Dako, USA) simultaneously for 1 h at room temperature. VWF was used to identify pulmonary arteries.

The slides were then washed in PBS and sections were incubated with corresponding secondary antibodies (goat antimouse Alexa Fluor 488, swine antirabbit TRITC, Dako, USA) for 30 min at room temperature. After extensive washing, the sections were counterstained with Hoechst antibody (Hoechst33342, 1:100 in PBS, Invitrogen, USA), washed again, mounted and coverslipped with FluorSave Reagent (Calbiochem, USA). Sections were scanned with a fluorescent microscope (BZ-9000, KEYENCE, Tokyo, Japan) and evaluated independently by three investigators.

All animal experiments were carried out according to the current guidelines of the University of Tsukuba's Regulations of Animal Experiments, and the experimental protocol was approved by the Animal Experiment Committee, University of Tsukuba (No. 13-355).

Table 1
Gene-specific primer sequences used for qRT-PCR.

Gene	Sequence (5′–3′)	Product size (bp)
Wnt11 fw	gcccccaactacctgctt	60
Wnt11 rv	ggcatacacgaaggctgact	
RhoA fw	ggcatacacgaaggctgact	60
RhoA rv	cctgcagtccaggtgagaa	
β-Actin fw	cgtcatccatggcgaact	71
β-Actin rv	cccgcgagtacaaccttct	

1.2. Statistical analysis

The statistical analyses were performed using the JMP software program (version 9.02; SAS Institute, Inc, Cary, NC, USA). After real-time PCR and ELISA, the target genes' mRNA and protein levels in the nitrofen group were compared to those in controls. All numerical data are presented as the means ± standard deviation. Differences on each gestational day were tested using the unpaired Student's *t* test if the data were normally distributed or the Mann–Whitney *U* test if they deviated from a normal distribution. The confidence interval was set at 95%, and statistical significance was defined by values of $p < 0.05$.

2. Results

The levels of Wnt11 and RhoA mRNA expression increased and peaked on D19 and decreased on D21 in the control group (Fig. 1). On the other hand, the mRNA expression level of Wnt11 showed no significant changes between D19 and D21 in the nitrofen-induced CDH group (Fig. 1a). In contrast, the mRNA expression level of RhoA was increased on D21, compared to D19 in the nitrofen-induced CDH group (Fig. 1b). The relative mRNA expression levels of pulmonary RhoA and Wnt11 on D21 were significantly increased in the CDH group ($p < 0.05$) compared to controls of equivalent age (Figs. 1a and b).

An immunofluorescence study corroborated the qRT-PCR results showing increased pulmonary protein expression of RhoA in the lungs of nitrofen-induced CDH fetuses, compared to controls on D21 (Fig. 2). To study the localization of RhoA expression, we used VWF as a marker for vessels. RhoA was expressed especially in the endothelial layer of peripheral vessels of the lung (Fig. 2). The medial and adventitial thickness of pulmonary arteries was significantly increased in the CDH model on D21. The RhoA immunoreactivity was very weak in control group. In contrast, the RhoA immunoreactivity in the peripheral pulmonary vessels was increased in CDH model on D21 (Fig. 2).

3. Discussion

The disruption of the Wnt signaling pathway has recently been shown to be one of the mechanisms contributing to the pathogenesis of lung hypoplasia in a nitrofen-induced model of CDH [15]. The Wnt family of secreted glycoproteins comprises 19 known ligands that control a broad variety of biological processes, including cell fate specification, polarity, migration and proliferation. Wnt signaling was also shown to be involved in lung development [16], as well as in the pathogenesis of pulmonary diseases like fibrosis [17] and tumor formation [18]. Recently, the upregulation of Wnt11 gene expression was reported using real-time PCR of laser microdissected pulmonary peripheral arteries from idiopathic PH patients [19]. In the present study, the expression of RhoA was also upregulated as a result of the upregulation of Wnt11. Our immunohistochemical staining results