Upregulation of serotonin-receptor-2a and serotonin transporter expression in the pulmonary vasculature of nitrofen-induced congenital diaphragmatic hernia

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Abstract

Purpose: Congenital diaphragmatic hernia (CDH) is attributed to severe pulmonary hypoplasia and pulmonary hypertension (PH). PH is characterized by structural changes resulting in vascular remodeling. Serotonin, a potent vasoconstrictor, plays a central role in the development of PH. It exerts its constricting effects on the vessels via Serotonin receptor 2A (5-HT2A) and induces pulmonary smooth muscle cell proliferation via the serotonin transporter (5-HTT). This study was designed to investigate expressions of 5-HT2A and 5-HTT in the pulmonary vasculature of rats with nitrofen-induced CDH.

Methods: Rats were exposed to nitrofen or vehicle on D9. Fetuses were sacrificed on D21 and divided into nitrofen and control group (n = 32). Pulmonary RNA was extracted and mRNA level of 5HT2A was determined by qRT-PCR. Protein expression of 5HT2A and 5-HTT was investigated by western blotting. Confocal immunofluorescence double-staining for 5-HT2A, 5-HTT, and alpha smooth muscle actin were performed.

Results: Pulmonary 5-HT2A gene expression levels were significantly increased in nitrofen-induced CDH compared to controls. Western blotting and confocal microscopy confirmed increased pulmonary protein expression in CDH lungs compared to controls.

Conclusion: Increased gene and protein expression of 5HT2A and 5-HTT in the pulmonary vasculature of nitrofen-induced CDH lungs suggest that 5HT2A and 5-HTT are important mediators of PH in nitrofen-induced CDH.

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It is widely accepted that the high morbidity and mortality of newborn infants diagnosed with congenital diaphragmatic hernia (CDH) is attributed to severe pulmonary hypoplasia and persistent pulmonary hypertension (PH) [1]. PH in patients with CDH results from morphological changes of the pulmonary artery wall. Proliferation of pulmonary arterial smooth muscle cells (PASMCs) and arterial fibroblasts causes muscularization of distal pulmonary arteries resulting in elevated pulmonary artery resistance [2–4]. However, the exact molecular mechanisms of increased media and adventitial thickening of the arterial wall causing persistent PH in CDH still remains unclear [5].

Serotonin, also known as 5-hydroxytryptamine (5-HT), is one of many potent vasoactive substances that has been associated with the development of clinical PH [6]. Human pulmonary vascular endothelial cells (ECs) were shown to produce 5-HT as well as its precursor tryptophan hydroxylase 1 (Tph1). In patients with idiopathic PH both 5-HT and Tph1 expression are known to be increased [7]. Furthermore, an increasing body of evidence implicates an important role of 5-HT in the development of experimental hypoxic induced PH, contributing to both hypoxia-induced acute vasoconstriction and chronic vascular remodeling by activating its cognate receptors and transporter [8,9]. In response to hypoxia, 5-HT is released from the pulmonary endothelium, neuroendocrine cells and neuroepithelial bodies distributed along the airways [10,11]. Following effects of released 5-HT have been described: (i) passage into the underlying PASMCs through the serotonin transporter (5-HTT) to initiate proliferation and/or (ii) activation of serotonin receptors on PASMCs inducing proliferation and/or contraction [12]. A variety of 14 different structurally distinct 5-HT receptors that are divided into seven families (5-HT1–7) are known. Especially the 5-HT2A receptor is known to mediate constriction in many arteries [13]. Experimental studies inhibiting the 5-HT2A prevented an increase in pulmonary artery pressure and pulmonary artery remodeling in rats stimulated by monocrotaline. This effect was accompanied by increased apoptosis of smooth muscle cells in the pulmonary artery suggesting that activation of 5-HT2A receptors by 5-HT could inhibit PASMCs apoptosis via its pathway [14]. Involvement of 5-HTT in the pathogenesis of PH was stated in a different experimental model demonstrating an overexpression of 5-HTT in mice after inducing PH by hypoxic conditions. These mice exhibited elevated pulmonary artery pressures as well as elevated...
pulmonary vascular remodeling and right ventricular hypertrophy [15]. On the contrary, 5-HTT deficient mice were less susceptible to hypoxia induced PH and demonstrated less pulmonary vascular remodeling than wild-type mice [16].

Presently, the nitrofen-induced CDH is the most widely accepted animal model to study the pathogenesis and clinical consequences of CDH in accordance with the striking similarities to the human condition [17–19]. We hypothesized that 5-HT plays an important role in the pathogenesis of CDH-associated PH and therefore designed this study to investigate the gene and protein expression of the 5-HT2A receptor and the 5-HTT.

1. Methods

1.1. Animals and drugs

Timed-pregnant, adult Sprague–Dawley rats (Harlan Laboratories, Shardlow, UK) were randomly divided into two experimental groups (“CDH” and “control”). The presence of spermatozoïdes in the vaginal smear was considered as proof of pregnancy; the day of observation was determined as day 0 (D0). On D9, animals in the CDH group received 100 mg of nitrofen (2,4-dichloro-p-nitrophenyl ether, WAKO Chemicals, Osaka, Japan) intra-gastrically dissolved in 1 ml of olive oil. Animals in the control group received only vehicle. On D21, dams were anesthetized with 2% volatile isoflurane (Piramal Healthcare UK, Morpeth, UK), followed by delivery of the fetuses via caesarean section. The fetuses were sacrificed by decapitation and divided according to the two groups CDH and control. In the CDH group laparotomy was performed for inspection of CDH. Left lungs with a diaphragmatic defect (n = 16) and controls (n = 16) were dissected via thoracotomy and stored native at −80 °C (n = 8), in formalin (n = 8) or in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) at −20 °C. All animal experiments were carried out according to the current guidelines for management and welfare of laboratory animals. The experimental protocol was 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. RNA isolation from left lungs (D21)

TRIzol reagent (Invitrogen) was used for the acid guanidinium thiocyanate–phenol–chloroform extraction method to isolate total RNA from D21 lungs according to the manufacturer’s protocol. Spectrophotometrical quantification of total RNA was performed using a NanoDrop ND–1000 UV–vis Spectrophotometer (Thermo Scientific Fisher, Wilmington, USA). The RNA solution was stored at −20 °C until further use.

1.3. cDNA synthesis and quantitative 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 Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, West Sussex, UK) 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 (Roche Diagnostics, Mannheim, Germany) in a total reaction mix of 20 μl per well. Gene-specific primer pairs are listed in Table 1. After 5 min of initial denaturation at 95 °C, 45 cycles of amplification for each primer were carried out. Each cycle included denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 10 s. Relative mRNA levels of gene expression were determined using a LightCycler 480 System (Roche Diagnostics) and the relative changes in gene expression level of 5-HT2A was normalized against the level of GAPDH gene expression in each sample (ΔΔCt—method). Experiments were carried out in duplicate for each sample and primer.

1.4. Western blot

Fresh frozen whole lungs (n = 8) were thawed, sonicated and proteins were isolated in lysis buffer containing 25 mM Tris–HCl, 50 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1% NP-40, 10% glycerol and 1% protease inhibitor cocktail (Sigma-Aldrich Ireland, Wicklow, Ireland). Protein concentrations were determined by Bradford assay (Sigma-Aldrich Ireland). Protein concentrations were equalized by dilution with distilled water. For gel electrophoresis, a concentration of 20 μg of total protein was used for every specimen and denatured in Laemmli sample buffer (Sigma-Aldrich Ireland). Gel electrophoresis for protein separation was performed using precast 10% SDS polyacrylamide gels (NuPAGE Novex Bis-Tris gels, Invitrogen) in NuPAGE MES SDS running buffer (Invitrogen). Proteins were then transferred to 0.45 μm nitrocellulose membranes (Millipore Corporation, Billerica, USA) by Western blotting. Following Western blotting, the membranes were blocked in 3% BSA + 0.05% Tween for 30 min or overnight before antibody detection. Primary antibodies against 5-HT2A (sc-15073, dilution 1:500, Santa Cruz Biotechnology, Santa Cruz, USA) and 5-HTT (sc-1458, dilution 1:500, Santa Cruz Biotechnology) were incubated overnight at 4 °C. On the following morning, followed by extensive washing (4 hrs), the membranes were incubated with the secondary antibodies in a dilution of 1:5000 (donkey antigoat—sc-2020, Santa Cruz Biotechnology) followed again by extensive washing. Detection was performed with the Pierce chemiluminescence kit (Thermo, Fisher Scientific, Dublin, Ireland).

1.5. Immunofluorescence staining and confocal microscopy

Fetal left lungs (n = 8) were fixed with 10% buffered formalin (Santa Cruz Biotechnology) overnight. Whole organs were washed overnight in PBS, embedded in O.C.T. Mounting Compound (VWR International, Leuven, Belgium) and frozen at −80 °C. Frozen blocks were sectioned transversely at a thickness of 10 μm and mounted on SuperFrost Plus slides (VWR International). After washing with PBS, sections underwent cell membrane permeabilization with 1% Triton X-100 for 20 min at room temperature. Sections were then washed and subsequently blocked with 3% BSA for 30 min to avoid non-specific absorption of immunoglobulin. Blocking solution was rinsed off and sections were incubated with primary antibodies against 5-HT2A (goat polyclonal, sc-15073, 1:1000 dilution in PBST, Santa Cruz Biotechnology), 5-HTT (goat polyclonal, sc-1458, 1:1000 dilution in PBST, Santa Cruz Biotechnology) and alpha smooth muscle actin (mouse monoclonal, M0851, 1:200 dilution in PBST, DAKO Diagnostics Ireland, Dublin, Ireland) overnight at 4 °C. After washing, 5-HT2A as well as 5-HTT sections were incubated with corresponding secondary antibodies (rabbit antigoat Alexa—A21446 and goat anti-mouse Alexa 488—A11029; respectively donkey antigoat Alexa 488–A11055 and donkey antimouse Alexa 647—A11571) for 30 min at room temperature. After washing, sections were counterstained with DAPI antibody (1:1000 in PBST, Roche), washed again and mounted using Sigma Mounting Medium (Sigma-Aldrich, St. Louis, MO, USA). Sections were scanned with a ZEISS LSM 700 confocal microscope (Carl Zeiss Microlmaging GmbH, Jena, Germany) and evaluated independently by two investigators.

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Product size (bp)</th>
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pulmonary tissue. First noticeable difference was the increased medial and adventitial thickness of pulmonary arteries. Confocal microscopy validated the qRT-PCR and the Western blot findings showing increased vascular 5-HT_{2A} and 5-HTT expression in PASMCs of nitrofen-exposed lungs compared to normal lung tissue (Fig. 3).

3. Discussion

To our knowledge this is the first study demonstrating upregulation of 5-HT_{2A} and 5-HTT in the pulmonary vasculature of nitrofen-induced CDH in rats on D21. Upregulation of 5-HT_{2A} and 5-HTT protein expression was accompanied by significantly upregulated gene expression of 5-HT_{2A} in nitrofen-induced hypoplastic lungs. Confocal microscopy revealed markedly increased 5-HT_{2A} and 5-HTT expression in the pulmonary vasculature of hypoplastic lungs.

In this study we used the well established nitrofen-induced CDH model. Some authors have argued that nitrofen model is a toxicological model and therefore does not link directly to the human situation [1]. However of all the animal models of CDH (surgical, transgenic and toxicological) the nitrofen model appears to be the best available model presently. Maternal exposure of nitrofen in both mouse and rat models during a specific time in gestation results in a high rate of CDH with associated pulmonary hypoplasia and pulmonary vasculature abnormalities, strikingly similar to the human situation [1,20].

There is an increasing body of evidence that 5-HT plays a central role in the pathogenesis of both experimental and human PH. Pulmonary hypertension is characterized by adventitial and medial hypertrophy and intimal thickening [21,22]. Although the process of vascular remodeling in PH involves all layers of the vessel wall and each cell type (ECs, SMCs and fibroblasts), hypertrophy of PASMCs is the most striking underlying pathological change in PH [23]. Serotonin has long been recognized as one of the most potent naturally occurring pulmonary vasoconstrictors and it exerts its mitogenic and comitogenic effects on PASMCs. The mitogenic action of 5-HT on PASMCs is mediated by 5-HTT which induces internalization of 5-HT [24]. Mice lacking the 5-HTT are less sensitive to hypoxia induced PH and hypoxic induced pulmonary vascular remodeling is less distinct than in wild type mice [9]. On the other hand, mice overexpressing 5-HTT demonstrate elevated pulmonary pressures and exaggerated hypoxia induced PH resulting in increased vascular remodeling when compared with their wild-type controls [25]. Citalopram and fluoxetine, both inhibitors of the 5-HTT, proved to be protective against PH secondary to hypoxia in mice [26]. Additionally fluoxetine was shown to protect against monocrotaline-induced upregulation of 5-HTT resulting in PH in rats [9]. Eddahibi et al. reported increased 5-HTT expression in the pulmonary vasculature of nitrofen-induced CDH lungs. Molecular expression in the pulmonary vasculature of hypoplastic lungs.

While the mitogenic action of 5-HT on PASMCs is mediated by 5-HTT, the constricting action of 5-HT on PASMCs is mainly mediated by 5-HT receptors (5-HT_{2A}, 5-HT_{2B}, 5-HT_{1B}). Of the various 5-HT receptors, 5-HT_{2A} is dominantly expressed in the pulmonary vasculature and plays an important role in pulmonary artery remodeling [14]. Inhibition of the 5-HT_{2A} receptor results in reduction of pulmonary arterial pressure and improvement of pulmonary remodeling [13,21]. In our study western blot and confocal microscopy revealed markedly increased 5-HT_{2A} protein expression in the pulmonary vasculature of nitrofen-induced CDH lungs. Molecular
studies confirmed that 5-HT_{2A} gene expression was upregulated in the hypoplastic lungs in our model.

The above observations suggest a close correlation between increased pulmonary vascular expression of 5-HTT and 5-HT_{2A} and pulmonary vascular remodeling in the nitrofen-induced CDH lungs. Altered 5-HTT and 5-HT_{2A} expression may have direct effect on pulmonary vasculature smooth muscle cell function resulting in PH in our model. Future studies aimed at better understanding the role of 5-HT in PH may lead to therapeutic strategies to inhibit the action of 5-HT in PH complicating diaphragmatic hernia.

**Fig. 3.** Immunofluorescence evaluation of pulmonary tissue for (top) 5-HTT (1:500), alpha smooth muscle actin (1:500) and (bottom) 5-HT_{2A} (1:500) and alpha smooth muscle actin (1:500). α-Smooth muscle actin was used to identify pulmonary arteries. Confocal microscopy revealed increased vascular 5-HTT and 5-HT_{2A} expression of nitrofen-exposed lungs compared to control lung tissue (top and bottom).

**Discussion**

**Discussant: Dr. Joel Shilyansky (Iowa City, IA):** Can you block the receptor or can you treat these normal animals with serotonin analog and see if you would induce similar response?
Response: Dr. Hofmann: This study was performed to confirm the hypothesis that the serotonin receptor and transporter are involved in pulmonary hypertension in the nitrofen-induced CDH model. Up to now functional pharmacological studies regarding the serotonin receptor and the serotonin transporter have been performed in adult experimental models of pulmonary hypertension. Our future experiments will focus on investigating whether a prenatal administration of pharmacological agents may attenuate the vascular remodeling in the nitrofen model of CDH.