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Endothelial nitric oxide synthase (NOS) is upregulated in rapid progressive pulmonary hypertension of the newborn

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Abstract *Objective:* To provide evidence for the upregulation of endothelial nitric oxide synthase (eNOS) or inducible nitric oxide synthase (iNOS) in the assumed imbalance in the pathophysiology of rapid progressive pulmonary hypertension of the newborn (RPPHN), which is characterized by abnormal hypertrophy of the pulmonary arterioles and arteries leading to increased pulmonary vascular resistance. Furthermore, to determine the cellular source and topographic distribution of eNOS and iNOS. *Material and Methods:* Lung biopsies were taken from two term neonates with clinical and echocardiographic evidence of RPPH and of three controls. Biopsies were obtained at an early stage of the disease as well as at post mortem and examined immunohistochemically for the presence of eNOS, iNOS and nitrotyrosine. *Results:* The endothelial cells of pulmonary arterioles stained significantly for eNOS protein in RPPHN patients. This was not the case in the control infants. There were no differences for nitrotyrosine or iNOS between RPPHN patients and controls. *Conclusion:* Rapid progressive pulmonary hypertension of the newborn leads to compensatory induction of eNOS synthesis specifically in endothelial cells of the pulmonary arterioles. This mechanism of compensation can lead to delayed presentation of RPPHN during the late neonatal period. Exogenous inhaled nitric oxide therapy does not lead to suppression of the endogenous synthesis of nitric oxide.

Keywords Persistent pulmonary hypertension of the newborn · Inducible nitric oxide synthase · Nitrotyrosine · Endothelial cells · Inhaled nitric oxide

Introduction

Successful postnatal transition from placental perfusion to the establishment of pulmonary gas exchange requires a significant fall in pulmonary vascular resistance and increased blood flow. This reduction in vascular resistance is mediated by nitric oxide (NO), which leads to synthesis of cGMP and subsequent vasodilation [1, 2]. There are three isoforms of NO synthase (NOS). Neuronal NOS (nNOS) and endothelial NOS (eNOS) are calcium-dependent [3, 4], whereas inducible NOS (iNOS) is calcium-independent and pro-

duced by macrophages or other cells following cytokine induction [5].

Besides several other functions, NO serves to maintain low pressure in the pulmonary circulation [6, 7]. Oxygen tension affects the activity of eNOS in pulmonary arteries [8, 9, 10, 11, 12]. NO production by endothelial cells from fetal pulmonary arteries [10, 11, 12], as well as eNOS expression in HUVEC cultures, can be reduced by hypoxia [8, 9, 13]. On the other hand there is convincing evidence from in vivo animal models showing increased eNOS expression following hypoxia in small, medium and large pulmonary arteries in the adult rat [14, 15], in rat conduit and resistance arteries [16] and, most prominently, in large vessels in mice [17]. Models of intrauterine ductus arteriosus ligation in the ovine fetus have shown reduced eNOS mRNA and protein content of the fetal lung [18].

Continuous inhalation of NO protects against the development of pulmonary hypertension in chronically hypoxic rats [19]. The reversal of hypoxic pulmonary vasoconstriction by inhaled NO therapy is well established as efficacious in term and near term neonates with persistent pulmonary hypertension of the newborn (PPHN) [20, 21, 22, 23, 24, 25]. A decrease of eNOS expression in pulmonary arteries has been shown in adult patients with pulmonary hypertension at an advanced stage of disease [26]. Reduced eNOS expression has been reported recently in umbilical vein endothelial cells cultured from infants who later developed PPHN [27]. RPPHN is characterized by the lack of a positive clinical response to pulmonary vasodilators—which generally occurs in cases of secondary PPHN—and a subsequent relentlessly progressive form of pulmonary hypertension. Our aim was, therefore, to investigate iNOS and eNOS expression with immunohistochemical methods in neonates during the clinical manifestation of pulmonary hypertension.

Methods

Case 1

This male infant was born after 37 weeks of gestation to a 24-year-old gravida V, para II, by section due to fetal retardation (APGAR 8/8/10, birth weight 2,270 g, umbilical artery pH 7.25). At the age of 3 weeks the infant was admitted to our level III Neonatal intensive care unit (NICU) in order to diagnose underlying causes of persistent thrombocytopenia further. On admission the infant's clinical condition was stable, the abdomen was grossly distended with liver and spleen being palpable below the rib cage (5 cm and 3 cm, respectively). Platelets were measured at 30,000/μl, the number of megakaryocytes in the bone marrow was within the normal range. While no apparent reason for the low number of platelets could be detected, clinical deterioration occurred within the next 48 h. Tachypnea with cyanosis indicated respiratory decompensation, minimal arterial pH was determined at 6.56. Radiologically there was evidence of pulmonary hypoperfusion; echocardiography showed tricuspid incompetence and an enlarged right atrium. The gradient across the pulmonary valve indicated pulmonary hypertension.

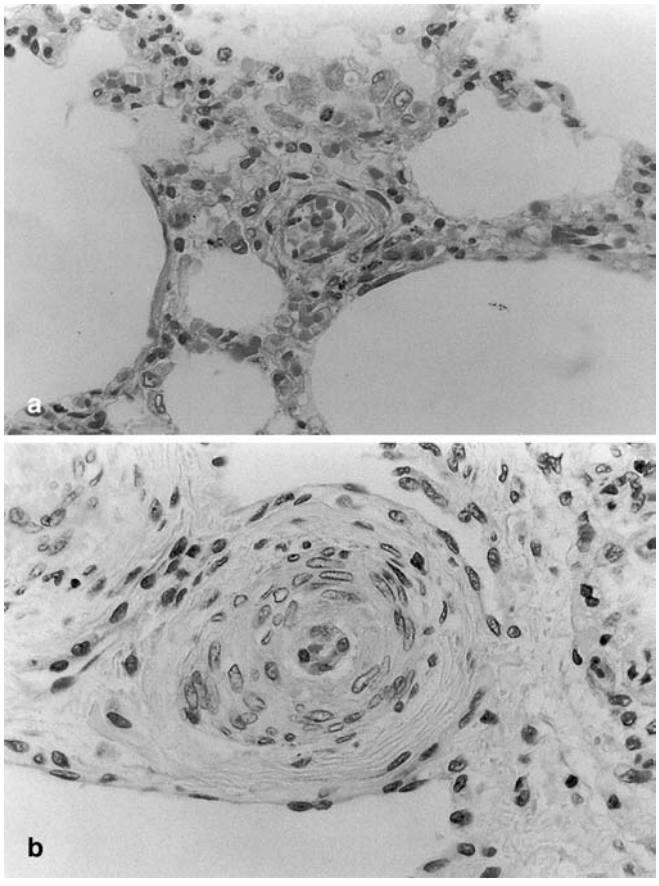

Fig. 1 Hematoxylin-eosin staining of small pulmonary arteries in **a** control infant (magnification ×400) **b** RPPHN patient 1 (magnification ×400)

Following intubation, ventilation and alkalization, the metabolic situation gradually improved. Adequate oxygenation was achieved by the combination of inhaled NO, intravenous prostacyclin and catecholamines. This steady state of normoxia and metabolic compensation was repeatedly disturbed by intermittent episodes of pulmonary hypertensive crisis. As the intensity and frequency of these pulmonary hypertensive crises increased, the option of increasing therapy further was abandoned in consensus with the infants' parents. Post mortem biopsies were taken of lung, liver and heart tissue for further investigation of the underlying causes of pulmonary hypertension (Figs. 1 and 2).

Case 2

This male infant was the first child of a 39-year-old gravida II, para I; delivery was performed after 39 weeks of gestation by cesarean section due to breech presentation (APGAR 9/10/10, birth weight 3,130 g, umbilical artery pH 7.32). At the age of 7 h the infant became cyanosed and dyspneic, and transfer to our level III NICU was instantly arranged. Rapid clinical deterioration was attributed to a newly developed tension pneumothorax, the drainage of which caused only transient improvement of oxygenation. Increased oxygen requirements were explained by excessive right-to-left-shunting across the arterial duct due to the development of

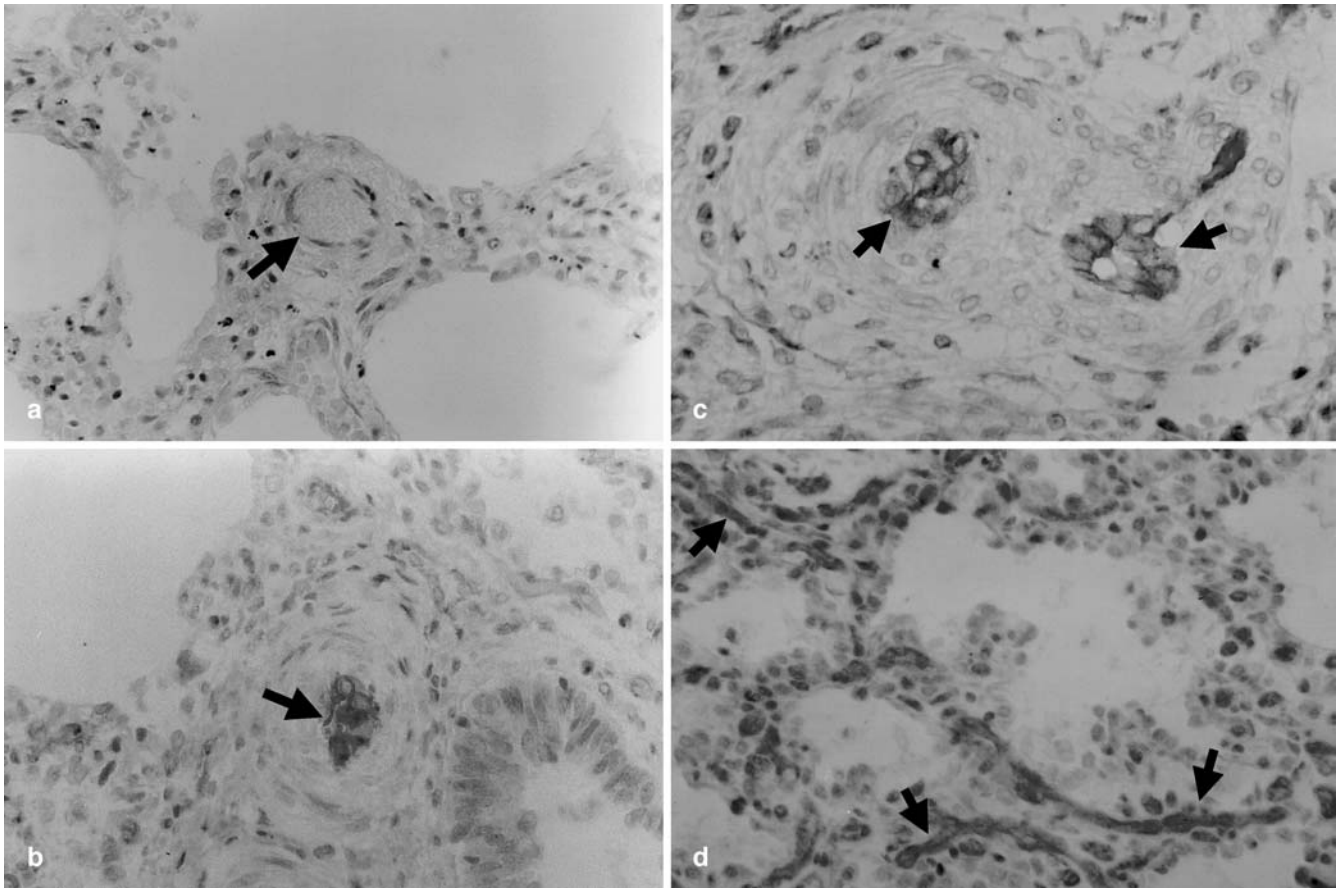

Fig. 2 Endothelial nitric oxide synthase immunostaining of **a** small pulmonary arteries in control infant (magnification ×400), **b** small pulmonary arteries in RPPHN patient 1 (magnification ×400), **c** small pulmonary arteries (autopsy) in RPPHN patient 2 (magnification ×400), **d** pulmonary capillaries (biopsy) in RPPHN patient 2 (magnification ×400)

PPHN. Treatment consisted of oxygen treatment including ventilation, inhaled NO, exogenous surfactant, tolazoline, prostacyclin, sedation and relaxation, and catecholamine therapy.

Despite these therapeutic options, the oxygenation index reached 25 on postnatal day 12. At that stage open lung biopsy was performed, the results of which were still pending when the infant was referred to the local extracorporeal membrane oxygenation (ECMO) center within the city. Veno-arterial ECMO was run for approximately 10 days, thereafter oxygen requirements were still high and the infant could not be weaned off the ventilator. Echocardiography showed persistent dilatation of the right ventricle, which did not improve during the course of the next 9 days. Since clinical deterioration occurred again, no further resuscitative measures were taken, in agreement with the parents, and the infant died at the age of 5 weeks. Partial autopsy of the thoracic organs was agreed to by the parents and performed immediately after death.

Controls

Control infants were chosen from those who had died due to sudden infant death syndrome (SIDS) and had an autopsy performed at our Department of Pathology. None of the infants was resuscitated by medical personnel, there were no therapeutic interventions like mechanical ventilation nor was there evidence of pulmonary infection in any of the infants.

Control 1. The infant of a 15-year-old primigravida who was a cigarette smoker. The infant had a birth weight of 3,000 g, APGAR 9/10, arterial cord pH: 7.29. Ten days prior to death diarrhea was noted, the infant died on day 73 of life.

Control 2. This infant was born to a 22-year-old primigravida with a history of smoking. The birth weight was 2,740 g, APGAR 8/10, arterial cord pH: 7.24. The infant died on day 86 of life.

Control 3. This was the second pregnancy of a 24-year-old mother who smoked. The infant was delivered at 39 weeks of gestation with a birth weight of 2,610 g. The amniotic fluids were meconium-stained; APGAR was 9/9, arterial cord pH: 7.29. There was local fungal infection of mouth and skin at 8 weeks of age. The infant died on day 137 of life.

Methodology for immunocytochemistry

Lung tissue was supplied as paraffin-embedded tissue blocks. The sections (4 μm) were cut from the blocks and rehydrated, and this was followed by antigen retrieval (microwave pressure cooking or trypsin incubation). The sections were treated to block non-specific binding of primary and secondary antibodies and non-specific reaction with chromogens as described previously [16]. The sections were then incubated with the specific antibody for 60 min at room temperature (eNOS: Cat.-No. N30020, BD Transduction, Lexington, USA, 1:200, pretreatment: pressure cook; iNOS: Cat.-

No. N32020, BD Transduction, 1:500, pretreatment: pressure cook; nitrotyrosine: Cat.-No. 06-284, Upstate Biotechnology, Lake Placid, USA, 1:200, pretreatment: trypsin). Nitrotyrosine staining was included in order to verify whether or not sufficient NO was formed to induce the formation of peroxynitrite as indicated by tyrosine nitration. Bound antibody was detected using goat anti-mouse IgG conjugated with horseradish peroxidase using a streptavidin-biotin link, and visualized with diaminobenzidine. In negative controls the primary antibody was replaced with pre-immune serum. Sections were counterstained using hematoxylin and viewed by light microscopy.

Vessels of an internal diameter of less than 250 μm were regarded as small pulmonary arteries, whereas vessels greater than 250 μm were regarded as large pulmonary arteries.

Results

General morphology

The lungs of patient 1 were congested with thickened alveolar walls, collections of intra-alveolar macrophages and a mild interstitial chronic inflammatory infiltrate. No acute inflammatory changes were present in relation to the large or small airways. The smaller pulmonary artery branches were very thickened with additional layers of smooth muscle and the endothelial cells were enlarged and active looking (Fig. 1b). Patient 2 exhibited similar changes, there were no differences between the left and the right lungs.

Control specimens were congested and edematous with collections of intra-alveolar macrophages and patchy interstitial chronic inflammation. No thickened pulmonary artery branches were seen (Fig. 1a).

Endothelial nitric oxide synthase

In patient 1, specific staining was found for eNOS in the endothelial layer of the large arteries and was particularly intense in the small pulmonary arteries (Fig. 2b). Autopsy samples of patient 2 showed eNOS to be increased in the endothelium of pulmonary arteries (Fig. 2c), especially in the arterioles with thickened walls, compared to the control patients. Biopsy samples of patient 2 also showed increased eNOS in muscularized pulmonary arteries. In addition, pulmonary capillaries were strongly positive for eNOS, and this was more evident than in the autopsy samples (Fig. 2d). This finding in the biopsy samples suggests that eNOS upregulation at the level of the capillaries is an early event in the development of pulmonary hypertension.

In the controls there was slight positivity in the endothelium of the large arteries, but none was visible in the small arteries (Fig. 2a).

Inducible nitric oxide synthase

In both patients, immunoreactivity for iNOS was found in the macrophages and in the apical portions of the epithelial cells (figures not shown). The endothelium of both large and small pulmonary arteries was very positive and the smooth muscle cells were also positive. The controls had a similar staining pattern, and thus there was no overall upregulation of iNOS in patients compared to the controls.

Nitrotyrosine

The media of pulmonary arteries stained lightly for nitrotyrosine in both patients and controls (figures not shown).

Discussion

Endothelial cells of small pulmonary arteries expressed markedly more eNOS protein in RPPHN patients as compared to healthy controls. This was most pronounced in small arterioles, less so in large vessels. To our knowledge this is the first report of an increase in eNOS expression during conditions of increased pulmonary resistance in the human neonate during the neonatal period.

Support for the role of an imbalance between vasoactive substances in PPHN patients is derived from a comparison of total NO compounds (NOx) and endothelin-1 (ET-1) concentrations in term neonates [28]. Initially comparable levels of NOx for PPHN patients and controls exist, which increase during the course of pulmonary hypertension to be significantly higher at the age of 5 days. Variations of inspiratory oxygen concentrations (both hypoxia and hyperoxia) have been shown to affect eNOS expression in the pulmonary tissue of adult rats. Hypobaric hypoxia for a duration of 3–4 weeks increased eNOS mRNA, protein and NO production in adult rat lungs [29]. Similar findings were obtained in a model of chronic hypoxia in the adult rat at 2 weeks [16]. Our present findings are well in line with the results of animal data, mostly derived from rat models, which support the concept of hypoxia-induced eNOS-upregulation in pulmonary endothelial cells [14, 15, 17].

Differences between in vitro and in vivo findings may be explained by variations in the duration of exposure to hypoxia and also by varying degrees of maturity from fetal to adult tissue and enzyme systems. However, in a different model of oxygen exposure, hyperoxia led to a significant increase in the number of eNOS positive endothelial cells in adult rat lungs [30]. Whereas, during hypoxia, pulmonary vasoconstriction with subsequent reduced pulmonary perfusion results, hyperoxia leads to

increased pulmonary blood flow, which will be further augmented by additional eNOS-induced synthesis of NO.

Newborns with the classical form of PPHN were found to have decreased gene expression of eNOS in cultures of umbilical vein endothelial cells collected immediately after birth [27]. Mice with congenital deficiency of eNOS exhibited a significantly higher proportion of muscularized small pulmonary vessels than in wild-type mice [31]. Taken together, these results indicate that eNOS-induced NO production is required for the postnatal reduction of pulmonary arterial pressures in the neonate. Hypoxia, which is a frequent event during the clinical course of PPHN, represents an important trigger for the induction of eNOS expression. The duration of exposure to hypoxia may be an important variable for the upregulation of eNOS, which was never present postnatally in the above-mentioned investigation of Villanueva [27]. Since umbilical cords were collected immediately postnatally and endothelial cells were only analyzed when respiratory failure occurred, eNOS expression cannot be connected to postnatal hypoxia. Our finding of eNOS upregulation at the level of pulmonary capillaries at an early stage (biopsy specimen) suggests that this may be an early event in the development of pulmonary hypertension. Specifically in pulmonary capillaries, eNOS expression was less evident at the second investigation during autopsy. The fact that eNOS expression decreases again with longstanding pulmonary hypertension [26] may be explained by the continuous damage to the endothelium, which, in turn, might compromise NO synthesis.

Additionally there was evidence of increased iNOS expression and nitrotyrosine staining as a marker for peroxynitrite in RPPHN and control tissue, both of which have been shown following hypoxia in the rodent model [16, 17]. The fact that there were no significant differences between RPPHN patients and controls in our study might be related to the difficulty of finding adequate controls for human studies. SIDS patients may very well suffer from hypoxia in the hours prior to death, which could affect oxygen-dependent gene regulation and lead to mRNA expression and protein synthesis. The fact that eNOS immunostaining was not increased in our controls made us confident to assume that hypoxia in the controls was not sufficiently severe and/or long enough to upregulate eNOS expression.

Another important issue is the effect of maturation on patterns of NOS expression. While intrauterine data show increases of eNOS expression with gestational age until around the expected date of delivery [32], postnatal data indicate a decrease of eNOS expression at day 7, levels of which return to normal adult levels at day 14 [33]. Since the autopsy specimens in both of our RPPHN patients were taken beyond the postnatal age of 14 days, we are confident the controls are comparable with the patients, at least with regard to the maturational effects of eNOS expression.

An inevitable limitation of our study consists in the lack of mRNA or protein data in our patients, which would further support our findings. Unfortunately neither DNA samples nor fresh lung tissue were available when our hypothesis was established. Since RPPHN is a rare disease, we would like to encourage other researchers to collect specimens in order to perform these investigations.

While pulmonary hypertension due to secondary PPHN is generally rapidly reversed by adequate treatment of the underlying disease, RPPHN progresses relentlessly towards pulmonary vasoconstriction and hypoxemic pulmonary failure. Immunohistochemical data point towards an upregulation of eNOS in our RPPHN patients; this could explain the delay in clinical presentation of the first patient presented. Since both RPPHN patients were treated with inhaled NO for variable amounts of time, we speculate that exogenous NO does not suppress endogenous synthesis by endothelial NOS.

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