CLINICAL AND MOLECULAR ASPECTS OF STRESS ON THE DEVELOPING LUNGS

Klinische en Moleculaire Aspecten van Stress op de Ontwikkelende Longen

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© Okazaki, Tadaharu

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Clinical and Molecular Aspects of Stress on the Developing Lungs

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Overige leden:	Prof. dr. W. Mooi Prof. dr. B. Lachmann Prof. dr. F.W.J. Hazebroek
Copromotor:	Dr. H.S. Sharma

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Introduction and Review of literature

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Chapter 1

1.1 Introduction

Growth and development are fundamental processes and understanding of the mechanisms resulting in abnormal growth and development form the core of pediatrics and pediatric surgery.

Many acute problems in the perinatal period arise from prematurity and congenital anomalies. Although mortality is steadily decreasing due to progress in treatment modalities and prenatal diagnosis, remaining life-long morbidity is increasingly important. In many of these children such as premature newborns with respiratory distress syndrome (RDS), abnormal pulmonary development in case of congenital diaphragmatic hernia (CDH) or oligohydramnios associated with prolonged rupture of membranes and renal anomalies (the so called Potter sequence) the lungs are the target organ for treatment.

1.2 Pathological aspects of CDH

It is generally accepted that the lung at the side of the defect in CDH is hypoplastic, although large variability exists in the amount of hypoplasia. The same holds through for the contralateral lung. Structural changes have been demonstrated in the pulmonary parenchyma of CDH patients such as delayed maturation of alveolar structures and a decreased number of bronchial branches [1,2]. At routine pathology a significant lower lung bodyweight ratio (< 0.012) and a decreased radial alveolar count (RAC) are common denominators of pulmonary hypoplasia.

Both in spontaneous human cases, as in experimentally induced CDH in a variety of animal models, contradictory results have been published related to the functional maturation of the lung in CDH. In the human these data are either based on measurement of surfactant components derived from bronchoalveolar lavage fluid [3] or consecutive lecithin/sphingomyelin (L/S) ratio in prenatally diagnosed congenital diaphragmatic hernia patients with or without polyhydramnios [3,4,5]. Prenatal ultrasound to predict the amount of pulmonary hypoplasia has not revealed simple measurements. Harrison et al. published a report on 44 fetuses with CDH, using a combination of sonographic parameters (lung/head ratio two dimensional area of right lung at the level of the right atrium / head circumference), which looked promising [6].

The structural changes in the lungs of CDH patients, are not restricted to the respiratory unit. Well determined pulmonary vascular abnormalities are present as well. These consist of a decrease in total size of the pulmonary vascular bed, increased thickness of the pulmonary arterial smooth muscle coat, a decreased number of vessels per unit of lung and an increased thickness of the adventitia.[7-9]

1.3 Clinical Consequences

One of the major problems following birth of a child with CDH is to predict outcome. The changed attitude towards timing of the operative repair of the diaphragmatic defect has revealed decreased compliance following repair of the diaphragm [10-12]. But an absolute value of compliance predicting 100% mortality is not available. The same holds through for the reaction of the lung on artificial ventilation for which a variety of treatment

modalities has been proposed to influence outcome ranging from gentle ventilation, high frequency oscillation with or without nitric oxide and extracorporeal membrane oxygenation with or without partial liquid ventilation [13-19].
Partial liquid ventilation is the latest therapeutic modality. No prospective randomized trials are available today on any of these treatment modalities proving one to be superior compared with other treatments. However we do know that following conventional artificial ventilation a high incidence of chronic lung disease in term born infants with CDH is documented [20].

1.4 Animal Models for CDH Mimicking the Human Situation

Various animals are used for surgical induction of CDH; the lamb is the most widespread used, but also monkeys and rabbits have been tested [21-25].
The timing of CDH induction in lambs is, as in rats, important; the earlier in fetal life the lesion is produced the more severe the hypoplasia. Because this model is based on penetration of a balloon or bowels through an already closed diaphragm technical problems restrict the earliest possible intervention. Adzick et al. created CDH in lambs at gestational day 60-63. In the lamb it is possible to evaluate hemodynamics and the influence of ventilation on blood gas values and morphology. Moreover the CDH lamb is surfactant deficient [26]. In the same model Wilcox et al. reported on the effect of exogenous surfactant replacement therapy on gas exchange; both lung mechanics and gas exchange improved markedly [27].
Harrison's group used the lamb model to study pulmonary hypoplasia that accompanies CDH and the possibility of reversing these changes by correcting the diaphragmatic defect in utero. In their view fetal therapy is the logical consequence of progress in fetal diagnosis. This influenced the ideas of Harrison's group on the embryological aspects of CDH: pulmonary hypoplasia was caused by migrated bowels during fetal development and could be corrected by retracting these loops out of the thoracic cavity in an as earliest possible stage of development. The lungs will show a compensatory growth which will beneficially influence survival. The results of the animal experiments conducted by Harrison et al. proved, at least partially, their ideas. In the meantime patient selection proved to be very difficult while no significant overall improvement of survival was documented based on a NIH supported trial of fetal correction in the human (28,29).
Pulmonary hypoplasia in humans can also be associated with other anomalies such as renal dysplasia and oligohydramnios. Animal experiments in sheep revealed a relation between pulmonary fluid dynamics and pulmonary growth [30-32]. Tracheal ligation in the fetus accelerates lung growth beyond normal limits, even in the absence of kidneys [33-35]. Ligation of the trachea, the so-called PLUG technique, of CDH lambs during fetal development, resulted in an improved survival of the lamb with CDH after birth [36,37]. This plugging is considered to provide a less invasive way of intra-uterine CDH treatment but negative effects on type II cell differentiation have been reported as well [38]. Although at least 20 successful cases in the human have been described, it is too early to judge upon this treatment modality. One has to realize that sham operation in the fetus has an adverse effect on lung growth resulting in a significant decrease in DNA, protein and

saturated phosphatidylcholine, but no significant change in lung volume [39].
In the rat model of CDH the lungs are biochemically immature or hypoplastic with regards to DNA and phospholipid levels but not in antioxidant enzyme activity [40-41]. Also morphologic measurements show hypoplasia: lowered lung weight, volumes and radial saccular count or immaturity. Using immunohistochemistry a retarded differentiation of cuboid type II cells into squamous type I cells was shown [42].

Whether these animal models really contribute to the understanding of the morphological and functional abnormalities of the lung in CDH remains questionable.
The rat model of CDH enables investigators to study the natural history of the defect. Moreover the biochemical maturation of the lung at different stages of development, with respect to type II cell differentiation, surfactant levels, anti oxidant enzyme activity, neuroendocrine cell body (NEB) distribution, nitric oxide synthase activity as well as eicasenoid levels has been investigated. The sequence of events related to the primary anomaly (diaphragmatic defect of pulmonary hypoplasia) in CDH is also investigated in this model. However it is very hard to perform interventions which mimic the clinical situation in the human, although short periods of artificial ventilation are possible [43-46].

In contrast to the rat model the sheep model offers great advantages in studying the pathophysiology of the lung and pulmonary vessels during different therapeutic interventions following birth [47]. Major differences exist between the lung developmental pattern in the sheep and the human while this animal model does not provide any insight into the pathogenesis of the defect or the natural history of the lung in CDH. Very few comparative studies are available in human cases of CDH describing surfactant levels, eicasenoid levels in BAL fluid, neuroendocrine body (NEB) distribution etc. ([48, 49]. For a better understanding of the specific aspects of the abnormal lung in CDH, a developmental biological approach is very important as well as studying the effects of "stress" on the abnormal lung.

1.5 Developmental Biological Aspects of the Lung

Epithelial branching is one of the major events in lung morphogenesis. The branching process depends on the interaction of the epithelium with the mesenchyme. The amount of mesenchyme as well as its source (lung versus non-lung, terminal versus proximal) is shown to be of importance in a number of studies [50-53].

1.6 The significance of the mesenchyme

It seems likely that mesenchyme supports the morphogenesis and differentiation of the respiratory epithelium in part through the type of matrix that it synthesizes and deposits [54]. Among the components of the extracellular matrix that have been indicated as being necessary for branching are collagens III and IV, fibronectin and laminin [55-61]. Schuger et al. described that both lung epithelium and mesenchyme produce complete laminin molecules, shown by immunohistochemistry and in situ hybridization studies [62].
The expression of extracellular matrix components, transcription factors, growth factors,

and neuropeptides has hardly been studied in regard to the pathogenesis of abnormal lung development in CDH. The abnormal number of airway generations in the lung in CDH suggests a disturbed pattern of branching morphogenesis. However, it is not clear whether insufficient epithelial-mesenchymal interaction or insufficient stimulation by transcription factors or substances such a bombesin—a neuropeptide with growth factor-like properties—contibute to this feature. Immunohistochemical findings in Nitrofen-exposed rats indicate that the expression pattern of fibronectin, laminin, and collagens III and IV is not different between CDH and controls from gestational day 11 until day 21 (A.E. Brandsma, personal communication). In lungs of CDH rat pups, the expression of thyroid hormone receptors in the ipsilateral and contralateral lungs is similar to that of lungs in controls at all stages studied.
The expression of neuropeptides in CDH has been evaluated in only a few cases. In the rat model of CDH, increased calcitonin gene-related peptide (CGRP) immunoreactivity has been reported in pulmonary neuroendocrine cells towards the end of gestation, whereas a delay in CGRP expression was found on gestational day 18. In lungs of one infant with CDH the expression of gastrin-releasing peptide was lower than in gestational age-matched control lung. Increased bombesin immunoreactivity is documented to be present in lungs of some infants with CDH compared with infants with hydroplastic lungs due to other causes and controls.

1.7 Hormonal Effects on Lung Development Relevant to CDH

It has long been known that thyroid hormone and glucocorticoids influence pulmonary development, especially type II cell differentiation.
Because thyroid hormone acts via its receptors, it is necessary to demonstrate the tissue localization of the (nuclear) thyroid hormone receptor (THR) in early development. The spatio-temporal expression of the different isoforms of THR mRNAS both in the developing rat and mouse have been studied, using in situ hybridization techniques.
In rat THRalpha and THRbeta mRNAS were first detectable at embryonic day (ED) 13 and increased in cellular concentration up to ED 18, i.e. 4 days before birth, when organ maturation has come to proceed rapidly. THR-alpha is exclusively present in the mesenchyme. After ED 18, THRalpha and THRbeta mRNAS gradually decline so that by 1 week after birth, both receptors have reached their definitive (adult) levels. (Keijzer, unpublished results)
In both mouse and rat, an expression comparable to that of the THRs was found for one of the partners of the THRs with which they from heterodimers, the 9-cis retinoic acid receptors (RXRs), RXR-alpha being concentrated in the developing epithelium and RXR-beta in the supporting mesenchyme of the lung.
The importance of thyroid hormone for pulmonary development and the structural resemblance of Nitrofen and thyroid hormone originated research into a possible inhibition of T3 receptor-binding by Nitrofen showing a non-competitive inhibition at receptor level [63].
Subsequently the spatio-temporal expression pattern was investigated of the THR, GCR (glucocorticoid) and RXR (retinoic acid) receptor during abnormal pulmonary

development following induction of CDH by Nitrofen. No differences were observed suggesting that abnormal development is not mediated by a change in receptor expression in the developing lung. In other words, differences in expression pattern of thyroid hormone receptor can not be held responsible for the abnormal development of the lung in CDH.
Amongst the other known growth factors in the developing rat lung, mRNA expression of all components of the IGF system, (IGFs, IGFBPs and the type I IGF receptor) have been reported [64-67]. In addition, gene disruption strategies have demonstrated that both IGFs and the type I IGF receptor are indispensable for normal embryonic and postnatal growth. Type I IGF receptor mutants and some IGF-I mutants died at birth of respiratory failure [68]. This suggests that these proteins may participate in lung development in a paracrine or autocrine way.

1.8 Vascular Development in CDH

From a pathological point of view the morphological abnormalities in the pulmonary vasculature are well documented (7,8,9). The question remains whether these morphological features are directly correlated to the response of the pulmonary vasculature on hypoxia, metabolic acidosis and other stressful events. Moreover the reaction on the variety of vasoactive drugs including inspiratory NO-therapy used in a clinical practice is highly unpredictable.
Much attention has been paid to the increased muscular coat observed at birth and the peripheral extension of the muscularity in CDH [7,8]. In contrast detailed descriptions on the development of the vasculature in CDH are almost non existing. This means that no direct correlation can be made between a well characterized stage in development in which this type of muscularization is a normal feature and the morphology of the pulmonary vasculature in CDH. In other words, vascular balance with regards to tone of the vessel wall is hardly understood. It is uncertain whether the described abnormalities in the CDH lung represent a developmental delay only.
For the development of the blood vessels in the lung, angiogenesis and vasculogenesis are important morphogenetic processes [69]. A close correlation is observed between the development of the airways and arteries. The synchronization of airway and vessel branches suggests that there is a response to common mediators or that they exchange messenger molecules [for review, see 70].
Following the initial stage of angiogenesis and vasculogenesis, control of vascular proliferation is determined by the local production and action of a variety of growth factors.
In this context TGB-beta1; the platelet derived growth factor receptor ligand system and the insulin-like growth factors (IGFs) have been documented especially in in vitro culture systems as well as in tissue sections using in situ hybridization techniques and RT-PCR.
Much interest is directed nowadays towards vascular endothelial growth factors (VEGF), one of the potent angiogenic factors. Its mitotic activity is restricted to vascular endothelial cells in contrast to others such as TGF, PDGF and IGF [71,72]. VEGF is an important regulator of endothelial cell proliferation during the extensive tissue growth and

remodeling that occurs in utero [73]. In addition, hypoxia has been shown to be a potent inducer of VEGF expression implicating its direct role in hypoxia mediated angiogenesis [74,75]. Regarding the role of VEGF in abnormal vascular development in the CDH lung, however, no studies are available so far.
Besides endothelial cell growth, normal vascular development consists of the development of a muscular coat represented by smooth muscle cells and extracellular matrix. The smooth muscle cell is essential for the development of vascular tone. Especially the group of Stenmark has contributed to the understanding of the interaction between the medial smooth muscle cells and the extracellular matrix using the high altitude calf as an animal model to study the cell biological features underlying pulmonary hypertension, review Stenmark [70].
Smooth muscle cells do contain different forms of myosin heavy chain molecules. In a number of species, including rabbit, rat and human, at least three types of MHC are isolated. Using cDNA probes and isoform specific isoforms Sm1 (204kD), SM2 (200 kD) and SM embryo (200kD) are known to be developmentally regulated [76]. It is of relevance to study the expression pattern of these isoforms during abnormal lung development in CDH. Although the morphological abnormalities in the pulmonary vascular layers have been documented, it is not known which development stage of vascular muscular differentiation the lungs in CDH represent. Following the description of morphological changes in the developing pulmonary vasculature, more recently the role of growth factors has been investigated, especially the family of fibroblast growth factor (FGF), transforming growth factor β (TGF- β) and isoforms of platelet derived growth factor (PDGF). this chapter for detailed description.

Two morphogenic processes contribute to the development of the lung vasculature: vasculogenesis and angiogenesis [77-82]. In vasculogenesis, blood vessels develop de novo. The pre-existing endothelial cell precursors or angioblasts form primitive vascular channels, which subsequentlly remodel so that arteries, veins and lymphatics are produced, depending on local stimuli from the surrounding mesoderm [77-81]. In contrast, in angiogenesis, blood vessels develop from pre-existing ones by a process of budding and sprouting [78,79,81]. Angiogenesis is thought to be responsible for the formation of axial arteries [78, 81].

The structure of the pulmonary arteries varies with vessel size and developmental stage of the lung. The muscular coat of the artery firstly becomes apparent in the canalicular stage. Axial arteries from the hilum to the 7th generation are elastic; more peripheral arteries are muscular, partial muscular or, at the level of intraacinar artery, predominantly non-muscular. By definition, an elastic artery has more than two elastic laminae in its media, while a muscular artery has only two elastic laminae [81-82]. A partially muscular artery has smooth muscle cell tissue in only one part of its circumference; at this level the continuous muscular coat has been replaced by a spiral of smooth muscle cells (SMC). A non-muscular artery (arteriole) is similar in structure to an alveolar capillary, except for (larger) diameter [81,82]. Small muscular and probably the partially muscular arteries

represent the -so called- *resistance arteries*. Muscularization decreases towards the lung periphery in the normal fetus. A newborn has one artery for every 20 alveoli. In humans, due to formation of new alveoli postnatally, this ratio is reduced to 8:1 [81-83].

Two types of pulmonary arteries can be distinguished: axial arteries, which accompany airways and additional or supernumerary arteries. The latter are small lateral branches that arise from axial arteries and run a short course to supply the capillary bed of alveoli immediately adjacent to the pulmonary artery at the peribronchial parenchyma [80]. The latter are considerably more numerous and contribute in a significant way to the cross-section of the total recruited vascular bed. Supernumerary arteries constitute about 25% of the cross-sectional area at preacinar level, whereas at the intraacinar level they make up about 33%. According to Hislop and Reid in the normal lung, there are 23 generations of conventional arteries along the posterior basal axial pulmonary artery branch with 64 supernumerary branches, giving a ratio of 1:2.8 between conventional and supernumerary arteries for one axial branch [77,83]. Supernumerary arteries facilitate blood oxygenation by allowing passage of venous blood to the more remote alveoli adjacent to large arteries, veins and airways [80,83,84]. The intraacinar arteries represent an important part of the resistance arteries in the pulmonary vascular bed. The external diameter (ED) of preacinar arteries usually exceeds 200 μm; while the arteries running with the respiratory bronchioli represent the intraacinar arteries and have a ED of 50-200 μm [85]. These intraacinar arteries together with the supernumerary arteries increase rapidly in number and dilate near term to accommodate the postnatal demands of the pulmonary circulation [85,86].

1.9 Stress and Injury in the Developing Lung

Prolonged exposure to hyperoxia and barotrauma causes acute lung injury that leads to an inflammatory reaction. The initial phase of the injury process is characterized by an influx of cells, mainly neutrophils and an elevation of the release of various inflammatory mediators [87]. This early inflammatory phase is followed by a subacute fibroproliferative response with fibroblast and smooth muscle proliferation, this leads to interstitial and perialveolar fibrosis [88]. Recent evidence suggests that the fibroproliferation response may be due to exaggerated expression of the fibroproliferation cytokines such as platelet-derived growth factor-BB (PDGF-BB), transforming growt factor-ß (TGF-ß) and interleukin 1b by locally accumulated alveolar macrophages and probably epithelial cells [89-91]. In some cases the inflammatory and fibroproliferation response can lead to development of chronic lung disease (CLD).

The potential role of inflammation and infection in the process of CLD is suggested by cytological, histopathological and clinical studies [92-94]. Several of these studies show that artificial ventilation or ECMO treatment lead to a high incidence of CLD, which occuurs mainly in prematurely born infants with respiratory distress syndrome (RDS) and surfactant deficiency. CLD has been described in 33% of the CDH survivors, despite a mean birth weight of nearly 3000 grams [20]. Patients who are treated with ECMO for respiratory insufficiency have an 11.5 -fold increased risk for development of CLD,

especially when ECMO is started 96 hours after birth [87,95].
Immaturity of the lung, barotrauma and oxygen toxicity all contribute to the lung injury and thepathogenesis of CLD, but the exact mechanisms by which the lung undergoes severe disruption in structure and function is not fully understood. The same hold true for mechanisms preventing lung injury [20,87]. The ungoing tissue damage can be diminished or prevented by a number of molecules. Antioxidant enzymes (AOE) scavenge or detoxify the highly reactive oxygen metabolites. In the rat model of CDH, AOE activities increased gradually in normal rat pups during 5 hours of artificial ventilation but not in CDH rats. Another system of cellular defense mechanisms under stress are the so called heat shocks proteins (HSPs). HSPs are a group of highly conserved proteins that can be induced by a variety of pathophysiological phenomena such as hypoxia, oxidatives and metabolic stress [96-99]. We are not fully aware of the exact mechanisms of the cellular defense system under stress in normal and CDH lungs.

1.10 Concluding Remarks

Many questions are unanswered at this moment. The significance of growth factors revealed by Northern blot analysis or differences in distribution pattern as shown by in situ hybidization or immunohistochemistry are hard to interpret either as cause or consequence of abnormal lung growth. The same holds true for the effects of hormones, especially the significance of thyroid hormone for (normal) lung growth, and differentiation of type II cells in particular. The transgenic mice models such as (conditional) knock out mutants for different genes relevant for lung development, such as THR alpha and the different surfactant proteins (surfactant protein B) are experimental approaches nowadays performed in different laboratories. In the meantime experimental application of hormones like corticosteroids leads to an increase in SP A levels. Consequently prospective randomized trials are in the last phase of preparation evaluating the effect of prenatal administration of corticosteroids following prenatal diagnosis of CDH in humans.

The release of vasoactive substances and the unpredictable reaction of the pulmonary vessels on inhaled nitric oxide is hardly understood. Whether the lungs are primarily abnormal in their vasoactive response or these responses result from the insult of the lung during artificial ventilation is largely unknown.
Moreover, the significance of the described pathological features and the translation towards functional abnormalities is unclear. Especially the understanding of the reaction of the lung following the "insult" of pre or postnatal treatment modalities, such as different forms of artificial ventilation, the application of vasoactive drugs and the operative procedure is hardly investigated.

1.11 Specific Aims

The specific aims of the studies described in this thesis are:

1. to investigate differences in pulmonary vascular development between healthy and CDH lungs (chapter 2,3).
2. to investigate the expression patterns of vaso-active mediators in CDH lungs; in other

words "pulmonary vascular balance in CDH" (chapter 4).

3. to evaluate the effects of "insults" such as new forms of artificial ventilation ("partial liquid ventilation") and exogenous stimuli (NO_2 exposure) on stress response in neonatal healthy and CDH lungs (chapter 5,6).

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Part II

Pulmonary Vascular Development

Chapter 2

The Role of Vascular Endothelial Growth Factor and Myosin Isoforms during Pulmonary Angiogenesis in a Rat Model of CDH

Based on the article:

Okazaki T, Sharma HS, Aikawa M, Yamataka A, Nagai R, Miyano T and Tibboel D: Pulmonary expression of vascular endothelial growth factor and myosin isoforms in rats with congenital diaphragmatic hernia. *J. Ped. Surg* 32 (3): 391-394, 1997

2.1 Summary

Abnormalities of the pulmonary vasculature are well documented in congenital diaphragmatic hernia (CDH). Vascular endothelial growth factor (VEGF), a novel angiogenic factor, is a recently described endothelial cell specific growth factor. Myosin heavy chain (MHC) isoforms such as SMemb, SM1 and SM2 are important molecular markers to study vascular muscle cell differentiation. SMemb is expressed only in immature smooth muscle cells(SMC), while SM2 is expressed in mature SMCs. We investigated the expression of VEGF and SMC differentiation in pulmonary vessels in CDH rat lungs and controls. The lungs of Nitrofen induced CDH rat fetuses (N=16, gestational age E16, E18, E20, E22) were stained immunohistochemically using antibodies against VEGF, SMemb and SM2, while alpha-actin was used as a general marker of smooth muscle.

In the CDH group VEGF expression was negative in pulmonary vessels before birth, while in the control group VEGF was positive in smooth muscle cells in vessel walls from E20 in both vessels at the hilum and in pulmonary parenchyma. In both control and CDH groups SMemb expression was positive from E16. SM2 expression was negative in vessel walls during the prenatal period in both groups. Alpha-actin was determined both in control and CDH lung in the lung hilum from E16 and around peripheral vessels from E18. Differences in vascular smooth muscle cell differentiation were not observed between control and CDH lung. These findings suggest that differences in pulmonary vascular development exist between control and CDH rats for VEGF expression, while maturational differences in smooth muscle cell differentiation are not present. This role of altered endothelial cell growth might be related to the different pulmonary vascular reactivity present in CDH lungs.

2.2 Introduction

Congenital diaphragmatic hernia(CDH) is a serious anomaly with a high mortality and morbidity due to the presence of pulmonary hypoplasia and pulmonary hypertension[1,2]. The high mortality rate of 40 to 50 % has not changed significantly during the past decennium despite changing concepts in treatment such as delayed surgery, nitric oxide and ECMO[3,4]. As pulmonary vessel abnormalities are well documented in CDH[2], recently several investigators have examined vasoactive factors such as endothelin[5] and nitric oxide synthase[6] in CDH. However, detailed descriptions on the natural history of pulmonary vascular development, especially in the embryonic and fetal phase, are not existing.

Vascular endothelial growth factor(VEGF), a novel angiogenic growth factor, has been described since 1989 as a specific endothelial cell growth promoter[7,8]. It is an

important regulator of endothelial cell replication during the extensive tissue growth and remodeling that occurs in utero[9]. Moreover, myosin heavy chain (MHC) isoforms, such as SMemb, SM1 and SM2, are important molecular markers to study vascular muscle cell differentiation[10,11,12]. SMemb is expressed only in immature smooth muscle cells(SMC) while SM2 is expressed in mature SMCs. SM1 is considered as a general marker of smooth muscle cells because it is constitutively expressed at all stages.

Because the interrelationship of endothelial cell growth and SMC differentiation in pulmonary vessel development has not been studied in CDH so far, we examined the expression of VEGF and MHC isoforms immunohistochemically in a rat model of CDH.

2.3 Materials and Methods

Experimental Animals

Adult female Sprague-Dawley rats were mated overnight. Observation of positive smears was considered a proof of pregnancy (day 0 of pregnancy). To induce CDH, 100 mg of 2,4-dichloro-phenyl-p-nitrophenylether (Nitrofen) dissolved in 1 ml olive oil was given on day 10 of gestation[13]. In control animals, the same dose of olive oil was given without Nitrofen. Water and food were supplied ad libitum during the whole period of the experiment. At gestational age 16 (E16), 18 (E18), 20 (E20) and 22 (term) (E22) the mother was anesthesized by inhalation of ether and cesarean section was performed. The fetuses were removed and killed before any breathing occurred. Age-matched normal fetuses were obtained from control animals.

Immunohistochemistry

The lungs from CDH and control fetuses (n=16 in each gestational age group) were fixed in a mixed solution of 95 vol% ethanol 100% and 5% acetic acid 96%. They were embedded with paraffin and sectioned in 3-μm slices. Immunohistochemical studies were performed with immunoenzymatic method. To reduce nonspecific reactions, sections were preincubated with 0.3% hydrogen peroxidase and normal goat serum. Antibodies (Ab) against VEGF (diluted 1:100), SMemb (diluted 1:200) and SM2 (diluted 1:100) were applied as primary antiserum. Antibody against VEGF was purchased from Santa Cruz Biotechnology Inc.(Germany). Antibodies against SMemb and SM2 were developed by one of the authers from two oligopeptides specifying the carboxyl terminal end of SMemb (Thr-Ser-Asp-Val-Asn-Glu-Thr-Gln-Pro-Pro-Gln-Ser-Glu) and SM2 (Gly-Pro-Pro-Pro-Gln-Glu-Thr-Ser-Gln) as described before[12]. Instead of SM1, alpha-actin (diluted 1:100) was used as primary antiserum because our

antibody of SM1 stained negative with smooth muscle in the rat. The slides were couterstained with hematoxyline solution. Negative controls were obtains by applying nonimmune goat serum in place of antibodies against VEGF, SMemb, SM2 and alpha-actin. The expression of these proteins were evaluated as positive (+) or negative (-).

2.4 Results

The results are summarized in Table 1. In the CDH group VEGF expression was negative in vessel walls during fetal development (Fig 1 a), while in the control group VEGF was positive in SMCs around large and peripheral vessel walls from E20 (Fig. 1 b).

In both control and CDH animals SMemb expression was positive in large vessel walls in the lung hilum from E16 (Fig. 2) and in peripheral vessels from E18. SM2 expression was negative in vessel walls during the prenatal period in both groups.

Alpha-actin was determined both in CDH and control lung in large vessels in the lung hilum from E16 and around peripheral vessels from E18. Differences in vascular SMC differentiation were not observed between control and CDH groups (Fig. 3).
See color pictures on page 122.

Table 1. Expression of VEGF, Smemb, SM2, and Alpha-Actin in Pulmonary Vessels in CDH and Control Rat Lungs.

	Day 16	Day18	Day 20	Day 22
VEGF				
CDH	-	-	-	-
Control	-	-	+	+
Smemb				
CDH	+*	+	+	+
Control	+*	+	+	+
SM2				
CDH	-	-	-	-
Control	-	-	-	-
Alpha-Actin				
CDH	+*	+	+	+
Control	+*	+	+	+

* positive only in large vessels in lung hilium.

2.5 Discussion

Our findings show that differences in pulmonary vascular development exist between control and CDH rats for VEGF expression from E20, while differences in expression of MHC isoforms, which are useful markers for SMC differentiation[10,11,12], are not present. So far several investigators[1,2,14-16] reported pulmonary vascular abnormalities in CDH such as an increase in muscle mass and decrease in the size of the pulmonary vascular bed. However, our results suggest that maturational differences in SMC differentiation are not present between control and CDH rats at least at MHC isoform level. In addition, because SMemb and alpha-actin expression are observed in large vessel walls from E16 and in peripheral lung fields from E18 in both control and CDH rats, these findings suggest that there is no differences in the developmental sequence of SMC differentiation.

VEGF is one of the potent angiogenic factors and it's mitotic activity is restricted to vascular endothelial cells in contrast to others such as fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF)[17,18]. VEGF is also important in the regulation of angiogenesis during fetal development. As VEGF is localized primarily to myocytes and epithelial cells but not to endothelial cells in the human fetus, it is considered that VEGF has a paracrine mechanism of action[10]. Our result that VEGF is expressed in SMCs in vessel wall and in the airway epithelium (data not shown) from E20 in control rat lung is comparable with these findings. In contrast to control rat lung, VEGF expression is negative in CDH rat lung. This suggests that the differences of VEGF expression in prenatal vessels might play an important role in abnormal pulmonary vascular development in the CDH lung.

Recently some authors reported that inhaled nitric oxide (NO) improved oxygenation and decrease pulmonary artery pressure in experimental[19] and clinical[20] cases of CDH. NO is a potent pulmonary vasodilator and it is produced by the enzyme nitric oxide synthase (NOS) in the pulmonary vascular endothelium. North et al.[7] described that endothelial NOS mRNA in the lung was decreased on day 20 of gestation in the rat model with CDH. Our results suggest that an altered endothelial cell growth resulting in a different VEGF expression might form the background of a changed pulmonary vascular reactivity in the absence of significant abnormalities of SMC differentiation. This also might be relevant considering the role of NO and NOS as modulators of pulmonary vascular tone.

As the mode of action of VEGF is directed through receptor-affinity and at least two different receptors are identified[21,22], further elucidation of the exact change of VEGF in abnormal lungs of CDH is warranted.

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Chapter 3

The Role of Vascular Endothelial Growth Factor in the Vascular Abnormalities of Human CDH

Based on Article:

Shehata. SMK, Mooi. WJ, Okazaki. T, El-Banna. I, Sharma. HS, and Tibboel. D.: Enhanced Expression of Vascular Endothelial Growth Factor in Lungs of Newborn Infants with Congenital Diaphragmatic Hernia and Pulmonary Hypertension. *Thorax*. 54; 427-431, 1999.

3.1 Summary

Pulmonary hypoplasia accompanied by therapy resistant pulmonary hypertension (PH) is an important feature of congenital diaphragmatic hernia (CDH). The pathogenesis of the pulmonary vascular abnormalities in CDH remains to be elucidated at the molecular level. Vascular endothelial growth factor (VEGF), an endothelial cell specific mitogen, is known to play a role in pulmonary angiogenesis and vascular remodeling, however no data on VEGF expression are available in CDH patients.

Twenty-one lung autopsy specimens of human CDH patients with lung hypoplasia and of seven age-matched control newborns without lung hypoplasia were processed for immunohistochemistry using affinity purified anti-human VEGF antibodies. All CDH cases had pulmonary hypoplasia, as evident from a lung/body weight index ≤ 0.012 and pulmonary hypertension documented by repeated cardiac ultrasound. Cellular localization of VEGF was semiquantitatively analyzed using a staining score ranging from 0 (no staining) to 4 (very strong staining). The results were statistically evaluated with accepted significance at P level of ≤ 0.05.

Significantly elevated levels of VEGF immunoreactivity were observed in CDH lungs as compared to the controls. VEGF was mainly detected in bronchial epithelium and in medial smooth muscle cells (SMC) of large (> 200 μm) and small (< 200 μm) pulmonary arteries, with the most intense staining of the pulmonary vasculature in medial SMC of small pulmonary arteries. In CDH patients, but not in controls, endothelial cells were positive for VEGF staining.

This is the first study on VEGF expression in human CDH neonates. Elevated expression levels of VEGF, especially in the small, pressure-regulating pulmonary arteries, point to a potential role in vascular remodeling. Perhaps this may reflects an unsuccessful attempt of the developing fetus to increase the pulmonary vascular bed in the CDH hypoplastic lungs in order to alleviate the associated pulmonary hypertension.

3.2 Introduction

Congenital diaphragmatic hernia (CDH) remains one of the major challenges in pediatric surgery and neonatology. Despite recent developments in therapeutic modalities such as delayed surgery, exogenous surfactant therapy, nitric oxide (NO) inhalation, extracorporeal membrane oxygenation (ECMO), and partial liquid ventilation, the mortality rate remains around 40% in high-risk cases[1-3]. The main documented pathological findings in CDH lungs are lung hypoplasia and pulmonary vascular abnormalities. The latter consist of: a) reduced total pulmonary vascular bed and decreased number of vessels per volume unit of lung, b) medial hyperplasia of pulmonary arteries together with peripheral extension of the muscle layer into small arterioles[4-6]. The most common cause of the unfavorable outcome in human CDH is persistent pulmonary hypertension (PH)[2-5]. Indeed, follow up of surviving patients with CDH has revealed that the pulmonary perfusion scan does not improve although the

ventilation scan improves towards nearly normal level[7].

Recently a developmental study of the lung vasculature showed that in early gestation the pulmonary vasculature develops by a combination of central angiogenic sprouting and the formation of peripheral vasculogenic lakes, which progressively communicate with each other as the gestation advances[8]. A number of growth factors with a proven or potential role in vascular development and remodeling in health and disease conditions have been identified[9,10]. Vascular endothelial growth factor (VEGF), a potent angiogenic growth factor, has been reported to have a narrow target cell specificity to endothelial cells. Moreover, VEGF regulates vasculogenesis and postnatal vascular remodeling[11-13]. VEGF binds to high affinity cell surface receptors, KDR/flk and flt, which are predominantly expressed in endothelial cells[13]. Expression of VEGF is up regulated under a variety of pathophysiological conditions, including pulmonary hypoxia[12-14].
In order to investigate the pathogenesis of the underlying vascular abnormalities in CDH lungs, we investigated the cellular localization of VEGF in pulmonary autopsy specimens obtained from human CDH and age-matched control neonates.

3.3 Materials and Methods

Tissue Specimens:

Lung tissue specimens used in this study were obtained from our archival collection at the department of Pathology. These specimens represent the available material from patients with CDH, who were treated in Sophia Children's Hospital, died and parents' consent for autopsy was obtained during the period 1981-1997. Twenty-one CDH cases were identified. All cases were associated with lung hypoplasia, as evident from a lung/body weight ratio index ≤ 0.012[15]. The control group consisted of 7 age-matched neonates who died in the first 24 hours of extrauterine life because of neonatal asphyxia or placental insufficiency. These control cases did not have lung hypoplasia on histological screening. The CDH group had a gestational age varying from 35 weeks to term, with a mean of 38.4 weeks, while that of the control group was 35.1 weeks. Neither the CDH nor the control group was subjected to ECMO treatment. We examined randomly either side of lung in this study, since on histological screening we did not find significant differences between the two lungs, as all cases represent the high-risk group of CDH. Lung tissue specimens were fixed in formalin by immersion-fixation and embedded in paraffin for histopathological examination and immunohistochemistry.

Immunohistochemistry:

Paraffin sections (6 μm thickness) of the lung tissues were cut and mounted on 3-amino-propyl-trioxysilane (Sigma, St Louis, MO, USA) coated glass slides. Immunohistochemistry was performed using a standard avidin-biotin complex (ABC) method as described earlier[16,17]. In brief, after deparaffinization in xylene and rehydration through graded alcohol, the slides were rinsed with water and phosphate buffered saline (PBS) and placed in a Sequeza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor, Runcorn). Slides were preincubated for 15 minutes with normal goat serum to block non-specific binding, then incubated for 30 minutes at room

temperature with affinity-purified rabbit polyclonal antibodies in a dilution of 1:200. The anti-VEGF antiserum used was raised against a 20 amino acid synthetic peptide corresponding to residues 1-20 of the amino terminus of human VEGF[18] (Santa Cruz Biotechnology, Inc., Santa Cruz, USA).

The optimal dilution was identified by examining the intensity of staining obtained with a series of dilutions of the antiserum from 1:50 to 1:500. The dilution (1:200) resulted in specific and easily visible signals in paraffin sections of a capillary hemangioma. The hemangioma sections served as a positive control in the study. After washing with PBS, the test and control slides were incubated for 30 minutes with biotinylated secondary antibody (Multilink, 1:75 dilution, Biogenex, San Ramon, MO, USA). After two washes in PBS, slides were incubated for 30 minutes with alkaline phosphatase conjugated streptavidin (Biogenex) in a dilution of 1:50. Finally, the slides were rinsed with 0.2 M TRIS-HCL pH 8.0, Levamizole (Sigma) was used to block the endogenous alkaline phosphatase activity, and stained for 30 minutes with 0.3% New Fuchsin/TRIS-HCL (Sigma) as color enhancement system. Negative controls were prepared by omission of the primary antiserum. Slides were lightly counterstained with Mayer's hematoxylin for 10 seconds.

Immunolocalization of VEGF in endothelial cells as well as SMC was verified by staining these cells with specific markers. Endothelial cells were identified by CD31 immunostaining[19]. Staining was done by the ABC method, but using 0.025% 3,3-diaminobenzidine (DAB) as chromogen. Slides were incubated for 20 minutes in methanol with 0.3% H_2O_2 to block the endogenous peroxidase activity. Slides were incubated with the primary anti-human CD 31 monoclonal antibody in a dilution of 1:80 (Dako Corporation, Glostrup, Denmark) at room temperature for 30 minutes and subsequently visualized after developing the color using 0.025% 3,3-diaminobenzidine (DAB) (Sigma). Employing DAB based color development method, consecutive tissue sections were stained with anti-human mouse monoclonal alpha-smooth muscle actin (α-SMA) antibody (clone 1A4: Biogenex) in a dilution of 1:200.

Semiquantitative Analysis:
Prior to screening sections were coded, so that the observers were unaware of the clinical details of the case under study. Expression of VEGF was analyzed semiquantitatively, using a semiquantitative visual scale, ranging from 0-4: grade 0 = no staining, grade 1 = focal staining, grade 2 = diffuse faint, grade 3 = diffuse moderate and grade 4 = diffuse strong staining, respectively[20]. The entire slide of a tissue block, taken from the mid-lung area was investigated and scored at the same magnification by three independent observers. The average of the three scores used for subsequent analysis. Sections were graded from 0-4 for the localization of VEGF in the bronchial epithelium, endothelium and medial SMC in small (50-200 μm external diameter ED) and large (> 200 μm ED) pulmonary arteries[21]. This scoring method has previously been shown to allow the detection of differences in expression level as small as 1.5 times[20,22].

Statistical Analysis:
The VEGF staining score was calculated from the two groups, and the results were expressed as Median. Before ranking score values were rounded to the complete number. Statistical analysis was performed after ranking using one of the non- parametric tests either, Mann–Whitney test or Fisher's exact test according to what is appropriate to the compared groups. Significance of the results for probability value was accepted at $P \leq 0.05$.

3.4 Results

Clinical and Autopsy Data:
Thirteen of the 21 diaphragmatic hernias were left sided; the remaining 8 were right sided. All patients presented in the first six hours of life and were treated according to a standard protocol including conventional mechanical ventilation, cardiac ultrasound, and delayed surgery[1]. Clinical evidence of right-to-left shunting was obtained by preductal and postductal transcutaneous O_2-saturation differences of > 10% in CDH cases[23]. Echocardiography documented the right to left shunt and pulmonary hypertension. In three cases, associated major congenital anomalies were found: Fallot's tetralogy, tracheo-esophageal cleft, and trisomy 21, respectively (one each). Nineteen of the CDH cases died within 48 hours postnatally, five of these cases died in the first hour. In 3 instances hyaline membrane disease was observed, whereas one patient developed pulmonary bleeding. All seven age-matched controls died in the first 24 hours after birth. Control cases were subjected to ventilatory therapy in settings similar to that of CDH cases, including inspiratory oxygen fraction of 1.0 for variable periods of time up to 16 hours postnatally.

Localization of VEGF:
We detected VEGF in the bronchial epithelium and medial arterial SMC in control cases. Distinct VEGF immunostaining was identified in the bronchial epithelium and in the medial SMC of pulmonary arteries in tissue specimens from the CDH group, as verified with the immunolocalizaton of αSMA. VEGF immunoreactivity in bronchial epithelium and arterial medial SMC was more intense in the CDH cases than in the controls (Fig 1. A& B).
See color pictures on page 124.

In the CDH cases, VEGF staining in pulmonary vasculature was most intense in the medial SMC of small pulmonary arteries, with an ED under 200 μm. High VEGF expression levels were noticed also in the supernumerary arteries of CDH cases. Furthermore, VEGF expression was detected in the endothelium of pulmonary arteries only in CDH cases (Fig 1.C). This endothelial staining was colocalized with consecutive sections stained with the endothelial cell marker, CD 31 (Fig 1.D). No VEGF immunopositivity was detected in the endothelium of control cases (Fig 1.B). Weak VEGF expression was observed in the medial SMC of large pulmonary veins in CDH cases.

There were no differences in VEGF expression pattern between CDH cases that were artificially ventilated up to 48 hours and the five cases that died in the first hour after birth despite maximal attempted resuscitation, even without receiving vigorous ventilatory support. No VEGF expression was observed in the arterial medial SMC of pulmonary veins in the control group.

Mean graded score values of the semiquantitative analysis for the VEGF expression in CDH cases showed a maximal expression score value of 3.38 in the bronchial epithelial cells. Endothelial and medial SMC of large pulmonary arteries (ED > 200 μm) depicted low staining values of 0.5 and 1.43, respectively. Statistical analysis of VEGF expression scores in the two groups; CDH and controls, showed significantly higher levels ($P \leq 0.05$) in the bronchial epithelium, medial SMC of large and small pulmonary arteries where P values were 0.001, 0.027, and 0.002, respectively, using the non-parametric Mann-Whitney U test as shown in Table I.

Since no expression of VEGF in endothelium of pulmonary arteries in control cases was observed and the score was always zero, Fisher's exact test was considered more appropriate for the comparison of endothelial staining scores[24,25]. Significantly higher expression was observed in the endothelial cells of CDH cases using Fisher's exact test, with P values of 0.01 and < 0.001 for large and small pulmonary arteries, respectively, as compared to controls.

Table I: *Table of ranking data for Mann-Whitney U test*

Tissue examined	N	Mean rank	Sum of ranks	P value
Bronchial epithelium				
CDH	21	17.38	365.0	0.001*
control	7	5.86	41.0	
Large artery medial SMC				
CDH	21	16.45	345.0	0.027*
control	7	8.64	60.5	
Small artery medial SMC				
CDH	21	17.12	359.5	0.002*
control	7	6.64	46.5	

Where: CDH = congenital diaphragmatic hernia, SMC = smooth muscle cells, N = number and (*) indicates statistical difference at $p \leq 0.05$.

3.5 Discussion

We have found increased VEGF immunoreactivity in medial SMC and endothelium of pulmonary arteries in CDH cases with pulmonary hypoplasia. The highest levels of expression in the pulmonary vasculature were observed in the medial SMC of arteries with a diameter of less than 200 μm, and especially in the supernumerary arteries, which are known to play an important role in pulmonary blood pressure regulation and vascular resistance[26].

Our results are in agreement with a previous experimental report confirming that, a source of VEGF is the arterial medial SMC[27]. The increased VEGF expression detected in CDH cannot be due to artificial ventilation, since we did not find any difference in VEGF expression between the patients ventilated for short (up to 1 hour) or longer (up to 48 hours) periods. In addition, no differences in the degree of VEGF expression were found in bronchial epithelium in the CDH group, regardless whether or not these lungs were exposed to high levels of inspiratory oxygen or volume trauma and shear forces related to variable periods of artificial ventilation. Since no significant differences were observed among either lung side in case of high-risk group of CDH regarding lung hypoplasia as descriped previously[28,29], so we have examined randomly either side of lungs from CDH group and also we have found no differences.

VEGF is recognized as an endothelial cell mitogen and angiogenic inducer with activity restricted to the vascular endothelial cells[30,31]. VEGF is expressed in a variety of cells, and a paracrine mechanism of action has been suggested, whereby non-endothelial cells secrete VEGF, which modulates the vasculogenesis and angiogenesis in the adjacent vascular endothelium[27]. This important angiogenic role of VEGF is evidenced from the fact that abnormal vessel development leading to death occurred in embryos lacking a single VEGF allele[32], or followed the experimental inactivation of the VEGF gene by replacing the coding sequence of exon-3 of the VEGF gene in embryonic stem cells[33]. We report endothelial reactivity for VEGF in the pulmonary vasculature of human CDH hypoplastic lungs and not in the control cases, the latter being in accordance with a previous study report that VEGF is not expressed in the normal endothelium of the developing fetus[27]. However, it has been previously documented that in endothelial cells derived from microvessels, VEGF expression can be up regulated in vitro by hypoxia and adenosine[34,35].

There is much similarity between the structural changes in the pulmonary vasculature in CDH hypoplastic lungs and that of another pediatric form of pulmonary hypertension, namely, persistent pulmonary hypertension of neonates[9,36]. It is unclear whether similar growth factors and cytokines contribute to these vascular abnormalities[37]. Furthermore, it is of note that increased VEGF expression has been reported in lungs of patients with primary pulmonary hypertension[37].

Our knowledge of vascular development in congenital diaphragmatic hernia and/or vascular remodeling following postnatal interventions is far from complete, and it is too

early to speculate in detail about the pulmonary vascular abnormalities at the molecular level. No doubt, growth factors play an essential role in the pulmonary and vascular development and maturation[38,39]. The increased VEGF expression in small-diameter and supernumerary pulmonary arteries in CDH cases complicated by PH, may reflect an - apparently unsuccessful- attempt of the developing fetus and the neonate to compensate for the stunted lung vessel growth and/or to stimulate the arterial angiogenesis of the pulmonary pressure-regulating arteries caused by a mechanism which remains to be identified.

Acknowledgements
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Part III

Control of Pulmonary Vascular Tone

Chapter 4

Pulmonary Vascular Balance in Congenital Diaphragmatic Hernia: Enhanced Endothelin-1 Gene Expression as a possible cause of Pulmonary Vasoconstriction

Based on:

Okazaki T, Sharma HS, McCune SK and Tibboel D: Pulmonary vascular balance in congenital diaphragmatic hernia: Enhanced endothelin-1 gene expression as a possible cause of pulmonary vasoconstriction. *J Ped Surg* 33(1): 81-85, 1998.

4.1 Abstract

Pulmonary hypoplasia and persistent pulmonary hypertension (PPH) are the principal causes of the ongoing mortality in congenital diaphragmatic hernia (CDH) presenting with respiratory insufficiency within 6 hours after birth. Endothelin-1 (ET-1) is an endothelial-derived vasoconstrictor, which could play an important role in modulating pulmonary vascular tone in PPH. ET-1 exerts its role in controlling vascular tone through two different subtype receptors, endothelin-A receptor (ET_A) which is responsible for vasoconstriction and endothelin-B receptor (ET_B) which is responsible for vasodilatation by induction of nitric oxide synthase. We examined the pulmonary expression of ET-1, ET_A and ET_B mRNAs in a rat model of CDH. CDH was induced in rats by administration of 100 mg of Nitrofen dissolved in olive oil on day 10 of gestation. Fetal lungs were collected following cesarean section on gestational day 22 (term) and processed for Northern blot analysis and quantitative PCR. Significantly ($p<0.05$) enhanced levels of ET-1 mRNA were observed in CDH rats as compared to control rats. In contrast to equal levels of ET_B mRNA, a 2-4 fold increase in ET_A mRNA levels were observed in CDH as compared to control rats. The upregulated expression of ET-1 and ET_A receptor mRNA before birth strongly support the reason for pulmonary vasoconstriction and altered pulmonary vascular muscularization in CDH. Consequently in the clinical setting the use of endothelin receptor blockade for the treatment of PPH may be considered against the background of the unpredictable and variable response to inhaled nitric oxide in newborns with CDH.

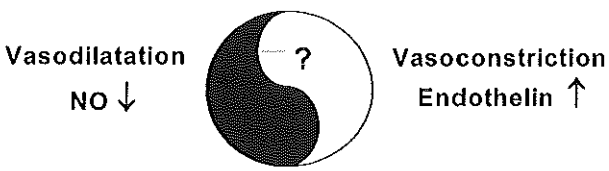
4.2 Introduction

The high mortality and morbidity in children with congenital diaphragmatic hernia (CDH) is largely determined by the severity of lung hypoplasia and by the therapy resistant persistent pulmonary hypertension (PPH).[1] Histopathologically, abnormalities in the pulmonary vasculature in CDH are well documented.[2,3] However, the question remains whether these morphological features are directly correlated to the response of the pulmonary vasculature during the perinatal period. Clinical observasions show the reaction on a variety of vasoactive agents including inhaled nitric oxide (NO) to be highly unpredictable.[4]

Recently two factors, NO and endothelin-1 (ET-1), have been put forward as essential vasoactive mediators in the perinatal pulmonary circulation.[5,6] Nitric oxide, produced in vascular endothelial cells by nitric oxide synthase (NOS) during the conversion of L-arginin to L-citrulline, is a potent pulmonary vasodilator, which increases the concentration of cGMP and results in smooth muscle cell relaxation.[7] There are three types of NOS, the endothelial (eNOS), inducible (iNOS) and neuronal (nNOS).

Endothelial cells express eNOS and iNOS.[8,9] In the nitrofen induced rat model of CDH, decreased eNOS protein and mRNA on gestational day 20 have been reported.[10] Endothelin-1, a 21-amino acid polypeptide, also produced in endothelial cells, is a potent vasoconstrictor[11] and a mitogen for vascular smooth

Fig. 1: Vascular balance hypothesis



muscle cells.[12] The effect of ET-1 is directed through at least two distinct receptor subtypes; the ET_A receptor localized on vascular smooth muscle cells mediates vasoconstriction, whereas the ET_B present on the vascular endothelial cells mediates vasodilatation via the induction of NOS.[5,13] Although, high levels of circulating immnunoreactive ET-1 have been reported in human neonates with PPH[14] and CDH,[15] the pulmonary expression and the exact mechanisms how ET-1 and its receptors interact to regulate pulmonary vascular tone in CDH are not fully understood (Fig. 1).

We hypothesized that in CDH altered pulmonary vascular reactivity might be related to differential expression of ET-1 and its receptors. Therefore, the present study was undertaken to examine the pulmonary expression of ET-1, ET_A and ET_B receptor mRNAs in a rat model of CDH and to compare the expression pattern with age matched controls.

4.3 Materials and Methods

Experimental protocol

Adult female Sprague-Dawley rats were mated overnight. Observation of positive smears was considered as a proof of pregnancy (day 0 of pregnancy). To induce CDH, 100 mg of 2,4-dichloro-phenyl-p-nitrophenylether (nitrofen) dissolved in 1ml olive oil was given on day 10 of gestation as described before.[16] In control animals, 1 ml of olive oil was given without nitrofen. At term (gestational age 22 days), the mothers were anesthetized by inhalation of ether and cesarean section was performed. The lungs from CDH and control fetuses were removed and snap frozen in liquid nitrogen and stored at -80°C until analyzed.

Extraction of RNA and Northern blot analysis

Total cellular RNA was extracted from 100 mg of frozen lung tissue by the method of Chomzynski and Sacchi.[17] Ten g of total RNA was denatured and size fractionated on 1% agarose gel containing 2.2M farmaldehyde, subsequently transferred to Hybond-N membrane (Amersham Nederland B.V., 's-Hertogenbosch). Filters were hybridized with a 32p-labeled cDNA probe specific for the rat ET-1[18] and washed under stringent condition and exposed to Kodak x-omat® films. Hybridization conditions were carried out as described before.[19] For reference purpose, a glyceraldehyde-3-phosphate dehydregenase (GAPDH) cDNA probe was used to rehybridize membranes. After autoradiography and densitometric measurement of signals, the optimal density (OD) of the ET-1 signal was divided by OD of the corresponding GAPDH signal and relative mRNA levels were calculated (mean ± S.E.). Expression was statistically analyzed using student's t-test and significance was accepted at $p<0.05$.

Quantitative RT-PCR

Using specific oligonucleotide primers, ET_A receptor, ET_B receptor[20] and cyclophilin cDNA fragments were selectively amplified in a competitive reverse transcriptase polymerase chain reaction (RT-PCR). Synthesis of cDNA mimic templates for competitive RT-PCR was carried out using deletion primer, providing amplified cDNA with priming sites identical to those of the gene of interest, but with a shorter intervening sequence to allow for separation by gel electrophoresis. Bands were quantitated on the basis of ^{32}P-dCTP incorporation. Relative intensities of amplified product and mimic were plotted on a semi-log scale to obtain the "equivalence point" - the point at which the concentration of the cDNA of interest was equal to the known concentration of mimic cDNA template. Results for CDH lungs are expressed as percent of control after normalization to cyclophilin levels.

4.4 Results

Nitrofen induced CDH in rats is a well established model in our laboratory. In this study, more than 80 % of fetuses developed left sided CDH. Both left and right lungs from only left sided CDH fetuses were examined for the expression of ET-1 and its receptor at mRNA level. Significantly ($p<0.05$) enhanced levels of ET-1 mRNA were observed in CDH rat lungs as compared to controls (Figure 2). No significant differences in the expression of ET-1 mRNA between right and left lung (the most hypoplastic) in CDH rats were observed (fig 2A and B). A 3.0±0.9 fold increase in ET_A mRNA was observed in CDH as compared to controls (Figure 3, 4), whereas ET_B mRNA levels remained unchanged in both CDH and control rats (Figure 4, 5).

4.5 Discussion

In the present study, we found enhanced levels of ET-1 and ET_A receptor mRNAs in CDH rat lungs on gestational day 22, indicating for a potential role of ET-1 and ET_A receptor system in regulating the pulmonary vascular tone. Based on their specific biological properties, recent interest has focused on endothelin and NO pathways in the fetal and perinatal pulmonary circulation. NO produced in vascular endothelial cells by NOS is a potent pulmonary vasodilator.[7] ET-1 increases pulmonary vascular resistance through its interaction with the ET_A receptor which is localized on vascular smooth muscle cells,[21] whereas ET-1 induces NOS using the ET_B receptor which is present on the vascular endothelial cells.[22] It has also been reported that administration of a selective ET_A receptor antagonist (BQ 123) decreases pulmonary vascular resistance in fetal lambs.[23]

Enhanced mRNA levels of ET-1 and ET-A for the regulation of pulmonary vascular tone in CDH

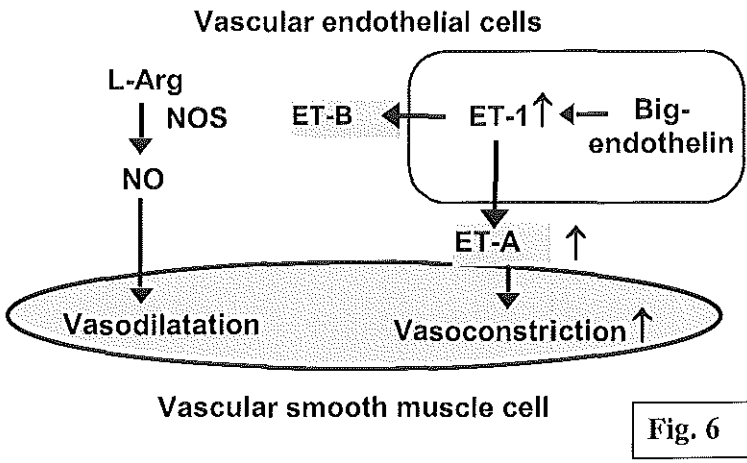


Fig. 6

Furthermore, ET_B receptor stimulation by sarafotoxin S6c results in vasodilatation through endothelial cell NO release.[5] Moreover, application of a nonselective ET_A and ET_B receptor antagonist (Bosentan®) has shown to attenuate the development of hypoxic pulmonary hypertension in rats.[24] In experimental rat and lamb models of CDH, differential expression patterns of NOS mRNA and protein have been reported so far.[10,25,26] On the other hand, responses to inhaled NO are divergent and unpredictable in human cases of CDH.[4] Our findings suggest that in addition to the altered NO pathway, the ET-1 - ET_A receptor system is upregulated which may contribute to altered pulmonary vascular reactivity in CDH (Fig. 6). In a recent study of human cases of CDH, we observed high levels of 6-keto-PGF_1 , a vasodilatory eicosanoid, in bronchoalveolar lavage fluid in CDH patients with PPH[27], as an attempt of the body to overcome the vasoconstrictive status of the pulmonary vasculature.

Furthermore, ET-1 is a mitogen for vascular smooth muscle cells.[12] Enhanced expression of ET-1 may also be attributed to the abnormal pulmonary arterial muscularization in CDH. In this study, there were no significant differences in the expression of ET-1 mRNA between left and right lungs of CDH rats. In human cases

of CDH, it has been reported that the pulmonary vascular abnormalities are bilateral, but the ipsilateral side is more severely changed as compared to the contralateral side.[28]

The present study was restricted to the observation at mRNA level. Further elucidation of protein expression and tissue localization are warranted in our model. Recently new trials of ET_A blockade and ET_B stimulation have been initiated in the CDH lamb model as an intervention for pulmonary vasoconstriction in CDH with promising results[29]. Our findings may support the pharmacological modulation of pulmonary vascular tone by the ET_A blockade as an alternative treatment modality for CDH associated with PPH.

4.6 Figures:

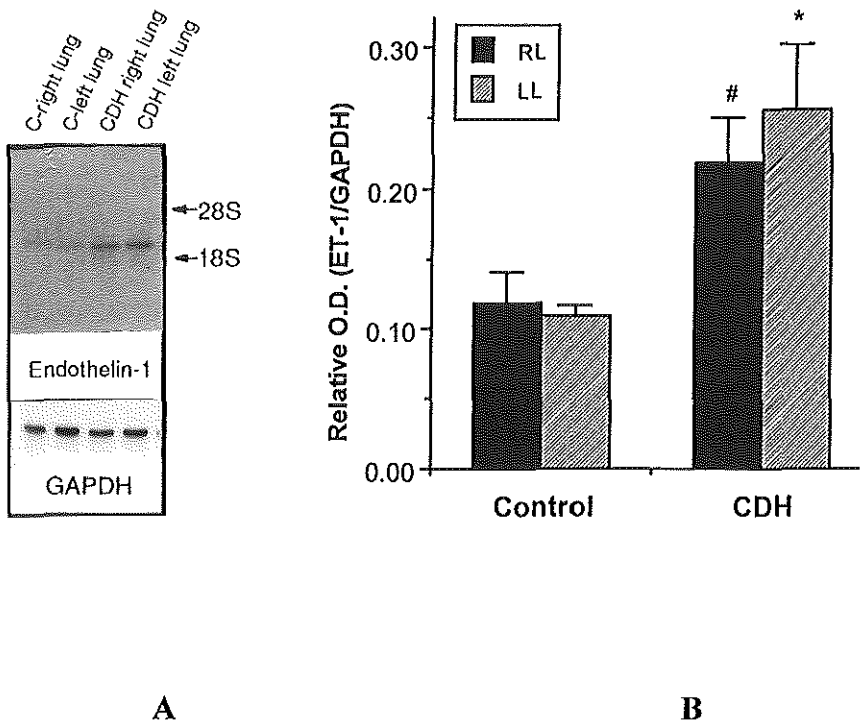


Figure 2: *Northern blot analysis of ET-1 expression in control and CDH lungs.* Panel A: Filters were hybridized with ET-1 cDNA probe (upper panel) and GAPDH (lower panel) for reference purposes. Autoradiography shows enhanced expression of ET-1 mRNA in CDH lungs. Panel B: Bar graph shows relative levels of ET-1 mRNA. #,*: significantly different from respective control lungs (both RL and LL) ($p<0.05$). RL=right lung, LL=left lung, C=control rats.

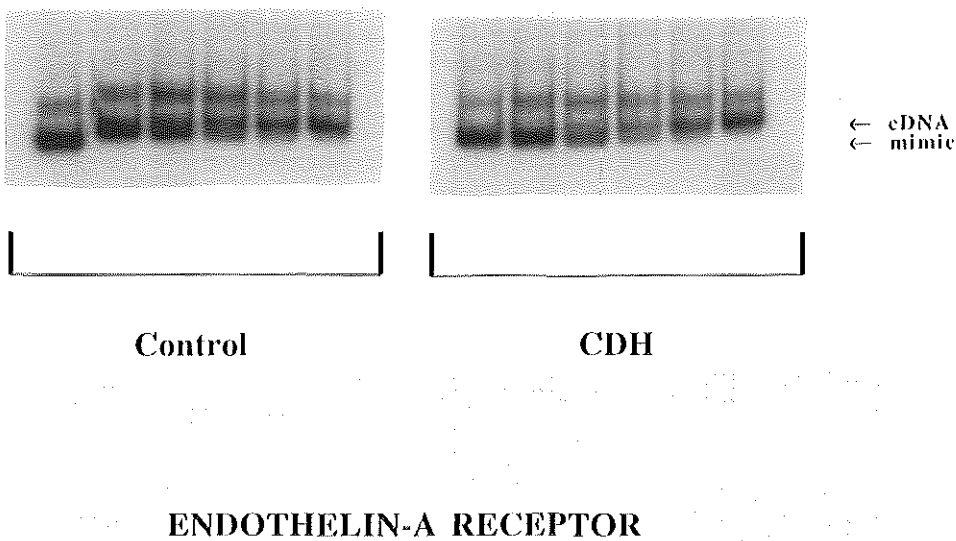


Figure 3: *Quantitative RT-PCR of ET_A receptor expression in control and CDH lungs.*
Autoradiography of ET_A receptor is shown after electrophoresis on an agarose gel

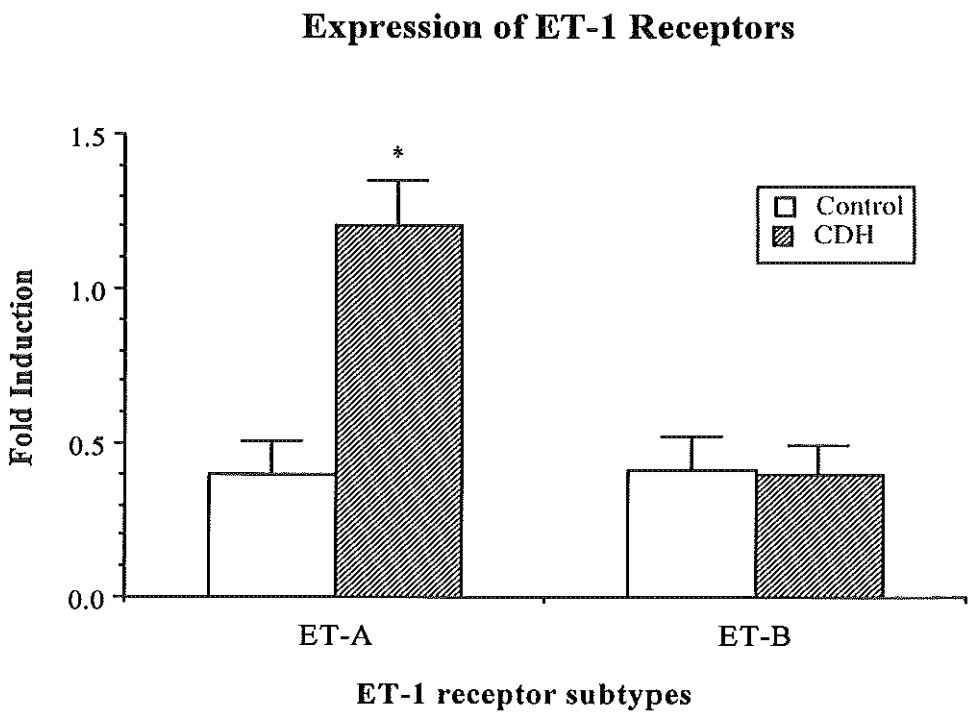


Figure 4: *Quantitative RT-PCR of ET_A and ET_B receptor expression in control and CDH rat lungs.*

Bar graph shows the values of ET_A receptor mRNA expression (left panel) as percent of control. About 3 fold increase in ET_A mRNA was observed in CDH rat lungs as compared to controls. Right hand panel shows ET_B receptor mRNA expression as percent of control. There was no significant change in ET_B mRNA expression between CDH and control lungs.

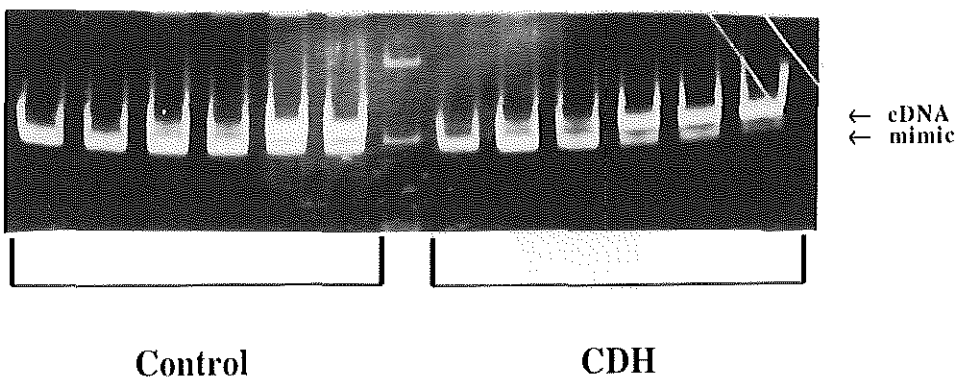


Figure 5: *Quantitative RT-PCR of ET_B receptor expression in control and CDH rat lungs.*
Ethidium bromide staining of the agarose gel for the ET_B receptor is shown.

4.7 References

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Part IV

Pulmonary Vascular Stress Responses in the Developing Rat Lungs

Chapter 5

Pulmonary Stress Response in Congenital Diaphragmatic Hernia rats after Conventional and Partial Liquid Ventilation

Based on the article:
Okazaki T, Sharma HS, Vlot J, Alshafei M, Tibboel T: Pulmonary Stress Response in Congenital Diaphragmatic Hernia rats after Conventional and Partial Liquid Ventilation – *Submitted*

5.1 Abstract

Artificial ventilation in human cases of congenital diaphragmatic hernia (CDH) may result in a progressive damage of the lung due to shear forces, activation of cytokines and oxygen toxicity. Partial liquid ventilation (PLV) might be an alternative ventilatory mode to prevent lung damage. However, it is not known whether, as a result of conventional ventilation (CV) or PLV in CDH cases, the stress imposed on lungs could result in the induction of stress genes as a sign of molecular adaptation. Using a rat model of CDH, we studied the lung mechanics and assessed the pulmonary stress imposed by two (CV and PLV) modes of ventilation by examining the expression pattern of heat shock proteins (HSPs), HSP-70 and HSP-27. To induce CDH, 100 mg of Nitrofen in olive oil was given to Sprague-Dawley female rats on day 10 of gestation while control animals received only the vehicle. Foetuses obtained by caesarean section on gestational day 22 (term) were either conventionally ventilated (freq. 40; PIP 25-17; PEEP 3; FiO2 1.0) or given perfluorocarbon (15ml/kg body weight) keeping the oxygen saturation above 96% in both groups. After 4 hours of ventilation, lung tissues were collected and processed for the measurement of lung mechanics, Northern blot analysis and (immuno)histochemistry. Following PLV, increased total lung volume in both control and CDH and improvement in opening pressure only in case of CDH group was observed as compared to respective CV groups. HSP-70 mRNA levels were enhanced in CV as compared to PLV rats in both control and CDH groups ($p<0.05$). However, the expression of HSP-27 mRNA was elevated in CV as compared to PLV rats in control group. Immunohistochemical localisation of both HSP-70 and 27 showed distinct staining in airway epithelial cells and vascular smooth muscle cells in CV and PLV lungs in both control and CDH groups. Though, the pattern of expression of HSP-70 and 27 was similar, semiquantitative analysis using an arbitrary staining scale of 0-4 showed increased expression in rats with CV as compared to PLV. Our results clearly demonstrate for the existence of endogenous defence mechanisms in hypoplastic CDH lungs and PLV appears to be less strenuous as compared to CV.

5.2 Introduction

The high mortality and morbidity rate in patients with congenital diaphragmatic hernia (CDH) is largely determined by the severity of lung hypoplasia and persistent pulmonary hypertension (PPH).[1] In survivors beyond the immediate neonatal period an increased incidence of bronchopulmonary dysplasia (BPD) has been reported in CDH because artificial ventilation with high peak inspiratory pressures and a high inspiratory oxygen fraction are often required in the neonatal period.[2,3] Moreover, the antioxidant enzyme system in the lungs of CDH cases is known to be deficient.[4] Ventilation with perfluorocarbon has been shown to improve gas exchange and lung function in clinical and experimental conditions including CDH.[5-8] Partial liquid ventilation (PLV) is a

modified technique of ventilation with perfluorocarbon, in which the lung is filled till functional residual capacity with perfluorocarbon and tidal volume ventilation is performed by additional conventional ventilation.[9] Previous studies suggested that PLV might be the solution to prevent ventilation induced chronic lung damage[10] and enhancing survival rate in patients with CDH.[11] However, it is not known whether, as a result of PLV in CDH, the stress imposed on lungs induces stress genes as a sign of molecular adaptation or alters in comparison with a regimen of conventional artificial ventilation.

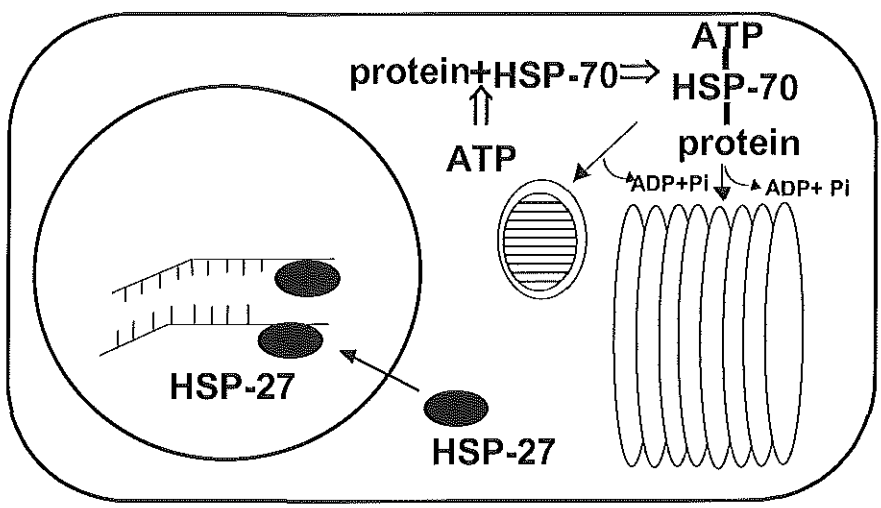

Figure 1: Schematic diagram showing the intra-cellular role of HSPs

Heat shock proteins (HSPs) are a group of highly conserved proteins that can be induced by heat shock and in a variety of pathophysiological conditions including hypoxia, oxidative and metabolic stress.[12-15] The HSP-70 family of proteins bind to ATP and are of help in post-translational import of proteins into the endoplasmic reticulum and mitochondria (Fig. 1).[15,16] HSP-70 and HSP-90 are known as regulators of glucocorticoid receptor function.[17,18] The small heat shock protein HSP-27 is expressed in developing organs as well as in cancer cells, migrates to the

nucleus upon stress, acts as a molecular chaperone, and plays an important role in signal transduction[19] Moreover, it has been reported that HSPs participate in embryonic and foetal development. Small HSPs are considered to be involved in organisation of the intracellular matrix and the preservation of cell structure.[20]

The aim of the present study was to evaluate how PLV influences the lung mechanics and to examine the mRNA expression and tissue localisation of HSP-70 and HSP-27 as stress markers in CDH and control rat lungs in a comparative study of CV and PLV.

5.3 Materials and Methods

Study design

Adult female Sprague-Dawley rats (Harlan Olac, England), average body weight 250 grams, rats were mated overnight. Observation of positive smears was considered as proof of pregnancy (day 0 of pregnancy). To induce CDH, 100 mg of 2,4-dichloro-phenyl-p-nitrophenylether (Nitrofen) dissolved in 1 ml of olive oil was given on day 10 of gestation as described before[4] In control animals at 10 days of gestation, the same dose of olive oil was given without Nitrofen. Water and food were supplied ad libitum during the whole period of the experiment. On gestational day 22, the fetuses were delivered by caesarean section and they were immediately anaesthetised and intubated, followed by artificial ventilation.[4] To start with, the ventilatory settings were as follows, PIP (peak inspiratory pressure) = 25 cmH_2O, PEEP (positive end expiratory pressure) = 3 cmH_2O, frequency = 40 breaths/minutes, FiO_2 (fraction of inspiratory oxygen) = 1.0. After 30 minutes of ventilation, animals were randomised in a 2:1 order to receive either 15 ml / kg of perfluorocarbon (n=81) or continuation of CV (n=42). Five minutes after perfluorocarbon administration, PIP was reduced to 17 cmH_2O and animals were ventilated for 4 hours. Oxygen saturation was kept over 96% in all animals as measured with a pulse oximetry probe (Nellcor, USA) attached to the foetal head to prevent interference with respiratory movements. The perfluorocarbon compound (RM-101) used in our experiments had the following characteristics: density 1.77 g/ml; vapor pressure 64 mmHg at 37^0C; surface tension 15 dyne/cm; O_2 solubility 52 ml/100ml; CO_2 solubility 160 ml/100ml. Following 4 hours of ventilation, foetuses were removed from the ventilator and their pressure volume curves were assessed. Lungs were dissected out and processed for the Northern blot analysis, routine histology and immunohistochemical examination.

Measurements of lung mechanics

Evaluating pressure-volume curves as described earlier lung mechanics was assessed[21]. A time on the ventilator was chosen in all our experiments because

significant metabolic derangements occur after 6 hr of ventilation this neonatal rat model of CDH. Four hrs of ventilation makes our data comparable with former studies from our laboratory[22] using the same approach. After 4 hours of artificial ventilation, at random the trachea of rat pups was exposed and cannulated with a 24 G catheter connected with a water manometer and a syringe. Subsequently pups were submerged in a saline-filled bath on an electronic balance. Air from the syringe gradually inflated the rat lungs to increase airway pressure in increments of 5 cm H_2O to a maximum pressure of 30 cm H_2O by intervals of 30 seconds for equilibration, following deflation of the lungs in 5cm H_2O decrements. Changes in lung volume were recognised as a change in weight of the system. Lung volumes at distinct airway pressure were recorded after calibrating the balance and water manometer connected with the animal submerged and serial pressure-volume curves were made. At least 5-7 curves for the different conditions were made.

Isolation of total cellular RNA and Northern blot analysis

For Northern blot analysis, both CDH and control foetuses were sacrificed immediately after caesarean section and lungs were removed from the thoracic cavity, snap frozen in liquid nitrogen and stored at -80^{0}C until analysed. Total cellular RNA was extracted from 100 mg of frozen lung tissue by using a guanidinium thiocyanate-phenol-chloroform method.[23,24] The RNA concentration was measured by spectrophotometry. For Northern hybridisation, 10 µg of total RNA was denatured at 65^{0}C and electrophoresed on 1% agarose gel containing 2.2M formaldehyde. Gels were photographed and RNA was transferred to hybond-N membrane (Amersham Nederland B.V., Den Bosch). Thereafter, filters were air-dried and UV cross-linked in a gene linker (Bio-Rad Laboratories B.V., The Netherlands). Blots were hybridised at 42^{0}C in a buffer containing 50% deionized formamide, 1.0M sodium chloride, 1% sodium dodecyl sulfate (SDS), 0.2% polyvinyl pyrrolidone, 0.2% ficoll, 0.2% bovine serum albumin, 50mM Tris-HCl (pH 7.5), 0.1% sodium pyrophosphate, 10% dextran sulfate and denatured salmon sperm DNA (100 µg/ml). cDNA probes used for hybridisation were 2.1 kb DNA fragments encoding human HSP-70 and 0.9 kb DNA fragments encoding human HSP-27. cDNA inserts were labelled employing a multiprime labelling system (Amersham Nederland B.V., Den Bosch), to a specific activity of 10^9 cpm/µg DNA using [^{32}P]-dCTP (3000 Ci/mmol, Amersham Nederland B.V. Den Bosch). After overnight hybridisation, filters were washed at room temperature for 5 min in 2x SSC (1x SSC = 0.15M NaCl, 0.015M sodium citrate) containing 0.1% SDS and at 42^{0}C in 0.1x SSC containing 0.1% SDS for 20 min. Subsequently, filters were wrapped in a household plastic wrap and exposed to Kodak X-OMAT AR films (Kodak Nederland B.V., Odijk) at –80^{0}C for 24 hours. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (1.2 kb PstI

fragment of human cDNA, procured from ATCC, USA) was used to rehybridize membranes for reference purposes. After autoradiography and densitometric measurement of signals, the optical density (OD) of the HSP signal was divided by OD of the corresponding GAPDH signal and relative mRNA levels were calculated.

Histology

For histological examination, lungs were fixed in two different ways. A 24G needle was inserted into the trachea and the lungs were fixed by injection of Davidson solution (40 vol % ethanol; 5 vol % acetic acid; 10 vol % formaldehyde; 45 vol % 0.9M NaCl; pH7.3) into the trachea at a pressure of 20 cmH_2O for at least 4 to 6 hours as previously described by Buri et. al.[25]. Consequently the lungs were expanded. Other lungs were removed together with the heart and immersed directly in Davidson solution in order to assess the aeration pattern. Additionally both CDH and control fetuses were sacrificed immediately after caesarean section and processed further for fixation and referred to as non-ventilated groups. After fixation, the lungs were embedded in paraffin. Six µm thick sections were cut and mounted on poly-L-lysin coated microscope slides and processed for the routine Hematoxylin-Eosin staining to assess the lung histology

Immunohistochemistry

Six µm thick sections from lungs following inflation fixation were processed for immunohistochemistry using a standard avidin-biotin complex (ABC) method as described earlier[26]. In brief, after deparaffinization in xylene and re-hydration through graded alcohol, the slides for HSP-70 were incubated for 20 minutes in methanol with 0.3% H_2O_2 to block endogenous peroxidase, then boiled in a citrate buffer for 15 minutes, rinsed with phosphate buffered saline (PBS) and placed in Sequenza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor, Runcorn). Non-specific binding sites were blocked by incubation in 10% normal goat serum at room temperature for 20 min. Slides were incubated at room temperature for one hr with mouse monoclonal antibody to HSP-27 (dilution 1:750) or with mouse monoclonal antibody to HSP-70 (dilution 1:25), both procured from Neo Markers, Fremont, USA. After rinsing with PBS, the slides were incubated for 30 minutes with biotinylated secondary antibody (Multilink, 1:75 dilution, Biogenex, San Ramon, MO, USA). Slides were rinsed again, incubated for 30 minutes with peroxidase conjugated streptavidin for HSP-70 and with alkaline phosphatase conjugated streptavidin for HSP-27, using a dilution of 1:50 for both (Biogenex). In case of HSP-70 slides were colored using 0.025% of 3,3-diaminobenzidine (Sigma, St Louis, MO, USA) in 0.01 mol/L PBS, containing 0.03% H_2O_2. Slides for HSP-27 staining were rinsed with 0.2 mol/L TRIS-HCL pH 8.0, incubated with levamizole in order to block the endogenous alkaline phosphatase activity, then stained with 0.3% New Fuchsin/TRIS-HCL (Sigma) and

briefly counterstained with Mayer's hematoxylin. Positive controls consisted of human breast carcinoma tissue specimens. The optimal dilutions for both HSP antibodies were identified by examining the intensity of staining obtained with a series of dilutions ranging from 1:10 to 1:1000. The dilutions chosen for this study resulted in specific and easily visible signals in paraffin sections of breast carcinoma. Negative controls consisted of omission of the primary antibody.

Semi-quantitative analysis of HSPs localisation

Expression of HSP-27 and HSP-70 was analyzed semi-quantitatively, using an arbitrary visual scale ranging from 0 to 4: grade 0 represents no staining, grade 1 represents focal staining, grades 2,3 and 4 represent diffuse weak, moderate and strong staining, respectively. This method has been widely used in earlier studies[26,27]. Prior to screening by three independent observers, sections were coded so that all three observers were unaware of the experimental groups. Sections were graded from 0-4 for the intensity of immunostaining signal for HSPs in the bronchial epithelium, as well as in the endothelium and medial smooth muscle cells (SMC) of pulmonary arteries.

Statistical analysis

Data from pressure-volume curves and densitometric analysis of Northern blots were presented as mean ± SEM and analyzed by using student's t-test. Statistical significance was accepted at p-values less than 0.05. Semi-quantitative analysis of HSPs expression at protein level was performed using visual scoring method. Immunostaining of HSPs scoring values may not be normally distributed and hence the data in addition to the student's "t" test was statistically tested using a non-parametric Mann-Whitney test where applicable. In few cases as the values were ranging from 0-3 with several values at 0, we have opted for Fisher's exact test instead of Mann-Whitney.

5.4 Results

Lung mechanics

The pressure-volume curves showed a marked increase in the lung volume at each pressure point (airway pressure between 0-30 cmH_2O with increments of 5 cmH_2O) in control rats who underwent CV as compared to those who were subjected to PLV (Fig. 2A). The mean lung volume in control rats at 30 cmH_2O increased from 373±23.1 µl after CV to 416±41.0 µl following PLV. As shown in figure 2B, a beneficial effect of perfluorocarbon on opening pressure in CDH rats was also observed (Fig. 2B). Although, CDH rats who received CV did not show any inflation at 10 cmH_20, however those CDH rats who underwent PLV demonstrated a mean lung volume of

56±25.0 µl at the same pressure. Mean lung volume in CDH rats at pressure 30 cmH_2O also increased significantly from 173±30.5 µl in CV group as compared to 242±21.7 µl in PLVgroup (Fig. 2B).

Histological examinations
In CV lungs, an alveolar aeration-pattern was observed in control rats, whereas a centro-acinar aeration pattern could be seen in CDH rats (Fig. 3A, B). Following PLV, a marked change towards improved aeration pattern was observed in both control and CDH rat lungs (Fig. 3C and D). No inflammatory cellular infiltration was observed in either group.
See colour pictures (Fig. 3) on page 126.

Effects of artificial ventilation on the expression of HSP-70 and HSP-27 mRNAs
By Northern blot analysis, we detected two mRNA species of 3.5 and 2.7 kb encoding HSP-70 in the lungs derived from both CDH and control rats (Fig. 4A). Baseline expression of HSP-70 mRNAs at birth showed no significant differences between CDH and control rats. Densitometric analysis of HSP-70 mRNAs showed significantly enhanced ($p< 0.05$) levels of HSP-70 mRNAs in rats followed CV as compared to PLV in both the control and CDH groups (Fig. 4B).

A single mRNA species of 0.9 kb hybridising to the HSP-27 probe was detected in the lungs obtained from each experimental group (Fig. 5A). Like HSP-70 expression baseline levels of HSP-27 mRNAs at birth showed no significant differences between CDH and control rats. The expression of HSP-27 mRNA was enhanced significantly in CV as compared to non-ventilated (NV) and PLV lungs in control rats (Fig. 5B). However, there were no differences in the expression pattern of HSP-27 mRNA among NV, CV and PLV in case of CDH rats.

Effects of artificial ventilation on the localisation of HSP-70 and HSP-27
Immunohistochemical localisation of HSP-70 and HSP-27 revealed that these stress proteins were expressed in conducting and terminal airway epithelial cells in the lungs of both control and CDH rats, irrespective of the mode of ventilation (Fig. 6, 7). Pattern of expression for HSP-70 and HSP-27 immunoreactivity was also observed in vascular smooth muscle cells in all above groups. Intense staining for HSP-27 in the large pulmonary arteries of both control as well as CDH groups was observed (Fig. 6A and B). The effects of mode of ventilation on the expression of HSP-27 became apparent at the level of large pulmonary arteries where the expression was less in case of PLV treated control rats (Fig. 6A and C). Endothelial staining the large pulmonary arteries of control rats kept on PLV was also observed (Fig. 7C).

Semi-quantitative analysis using an arbitrary visual scale ranging from 0 to 4 revealed that the cellular distribution patterns for both the HSPs were almost similar in the lungs derived from CDH rats irrespective of the ventilatory conditions (see also Fig. 6,7). However, pulmonary expression of HSP-27 was significantly decreased in control rats kept on partial liquid ventilation (staining score: 2.1 ± 0.16) as compared to conventionally ventilated control rats (staining score: 3.2 ± 0.24) (see Fig. 6A and C).
See colour pictures (Fig. 6 and 7) on page 128-131.

5.5 Discussion

In the present study, we demonstrated the increase in total lung volume in both control and CDH lungs and improvement of opening pressure in CDH lungs in PLV as compared to CV, indicating improved compliance by PLV. Histological examination revealed that the aeration pattern shifted from a centro-acinar to an alveolar pattern. Significantly enhanced expression of HSP-70 and HSP-27 mRNAs was observed in CV as compared to PLV in control group. In CDH rats the levels of HSP-70 mRNA were higher in the CV as compared to PLV, whereas, HSP-27 mRNA levels remained unaltered in CV as compared to PLV rats. Immunohistochemically, both HSP-27 and HSP-70 protein levels were elevated in CV rats as compared to PLV rats both in CDH as well as in control groups.

HSPs can be stimulated by a variety of pathophysiological stresses.[12-15] In our experiment, the inspiratory oxygen fraction of the ventilator was 1.0 and all pups had transcutaneous saturation values of 96% or more during the 4 hours of ventilation. In control rats the expressions of HSP-70 and HSP-27 mRNA were significantly enhanced in CV group as compared to PLV. While the initial protection of the lungs from oxygen free radicals during exposure to hyperoxia and barotrauma in neonates is considered to be provided by non-enzymatic antioxidants[28], another study from our group revealed that antioxidant enzyme (AOE) activities increased gradually in normal rat pups during 5 hours of artificial ventilation but were decreased in CDH rats.[29] Our current data suggest that PLV reduces the stress of artificial ventilation with a high concentration of oxygen as indicated by decreased opening pressure, and increased total lung volume.

It has been reported that HSPs can play a role in organ development in the embryonic and fetal period. Our results showed that the same levels of HSP-70 and HSP-27 mRNA and their proteins were present in airway epithelial cells and vascular smooth muscle cells in both control and CDH rat lungs. In addition, alterations of HSP-70 and HSP-27 mRNA expressions after 4 hours of ventilation were observed in CDH lungs as well as control lungs on day 22 of gestation, indicating that lungs in CDH rats respond to ventilatory stress by induction of HSPs. In other words the hypoplastic lung is not

different from the control with regards to the baseline ability to react to hypoxic insults. These data are comparable with the response of AOE activity in this model system as shown previously.[22] It is possible that 4 hours of ventilation is too short to observe alterations at protein level as investigated by immunohistochemistry. Although rat lung development is transitional from the saccular phase to the alveolar phase on day 22 of gestation[29], previous studies from our group showed that fetal rat lungs are retarded morphologically comparable with day 20 of gestation in hypoplastic lungs in Nitrofen induced CDH rats.[30] On the other hand, significantly lower expression of surfactant protein (SP)-A mRNAs was observed in CDH rat lungs on gestational day 20, but no difference was seen between CDH and control lungs at birth. Different expression pattern of SP-B and SP-C were also observed between gestational day 15 to 18 in CDH rat lungs, whereas the levels of these mRNA were similar between CDH and control lungs on gestational day 22.[31] Thus, the relationship between morphological and functional development in CDH rat lungs is not completely clarified. Moreover, it is not known when HSPs genes are expressed for the first time and how HSPs affect the lung development and endogenous cellular defence mechanisms in CDH.

PLV has been shown to improve gas exchange and lung mechanics in animal models of CDH and in premature infants.[5-8] Recently the first positive results have become available using PLV in combination with ECMO in 7 human cases of CDH with survival in 5 of them (Jay Wilson, personal communication). Moreover, PLV combined with inhalation of nitric oxide reduces pulmonary hypertension and enhances oxygenation in CDH.[6,32] Aggressive ventilatory support is often required to maintain adequate blood gases in patients with hypoplastic lungs in CDH. The enhanced expression of HSPs mRNA in CV as compared to PLV in control rats indicates that PLV leads to a less amount of stress in ventilated lungs. We observed a different expression pattern of HSP-27 mRNA in CDH rats. As described above, functional development of lungs in CDH is still partly understood. HSP-27 is known to be a major target of phosphorylation in response to stimuli and is rapidly phosphorylated by several oxidants and oxyradical generating agents including cytokines[33], and then synthesis is enhanced. Although the duration of artificial ventilation was 4 hours and no cellular infiltration was observed, a differential expression pattern of AOE activity during artificial ventilation were observed previously in the same rat model of CDH.[22] This might result in a differential expression pattern of HSP-27 mRNA in CDH rats. Whether these different endogenous stress responses following artificial ventilation might contribute to the high incidence of chronic lung disease following artificial ventilation in high risk CDH can only be elucidated by studying human specimen following different ventilation strategies. We have recently finished a study on 24

cases of human CDH where we found a positive effect of extracorporeal membrane oxygenation (ECMO) treatment on pulmonary HSPs expression (Shehata et al. unpublished). The problem remains that in humans only autopsy cases are available representing a subset of patients with the limited diagnosis.

In conclusion, we showed the improvement of lung mechanics following PLV and enhanced expression of HSPs mRNAs in CV as compared to PLV in control rats, suggesting that PLV leads to less stress during artificial ventilation of hypoplastic lungs although a different expression pattern of HSP-27 was observed in CDH. Our study strongly supports the idea that PLV alone, or in combination with ECMO, as an additional therapy to open up the hypoplastic lungs and might be of great benefit to diminish ongoing stress of the lungs in the clinical situation.

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5.6 Figures

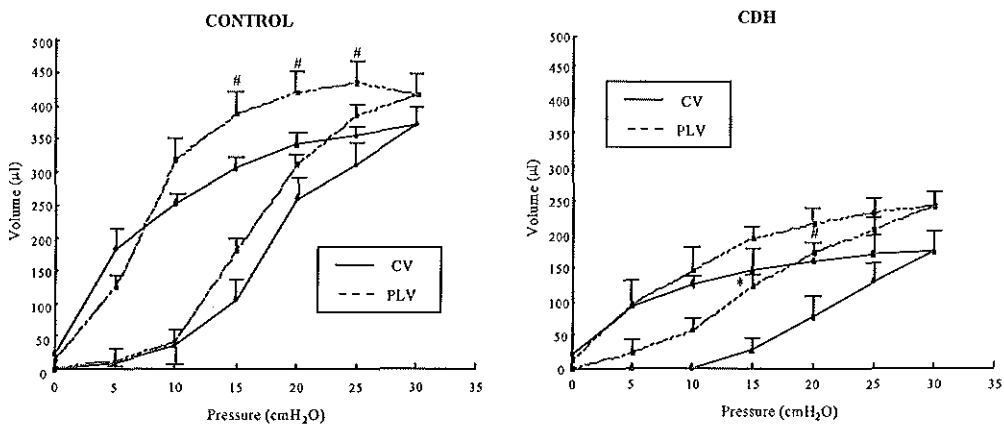

Figure 2: Pressure-volume curves in control and CDH rats after CV and PLV.

(A) Control rats subjected to CV for 4 hours (solid line) or PLV (broken line). A marked increase of the lung volume at each pressure was observed in rats given PLV as compared to CV.

(B)Pressure-volume curves in CDH rats after 4 hours of CV (solid line) or PLV (broken line). An increase of the lung volume was also found in CDH rats subjected to PLV.

#: significantly different from CV at corresponding pressure point ($p<0.05$);

*: significantly different from CV at corresponding pressure point ($p<0.01$)

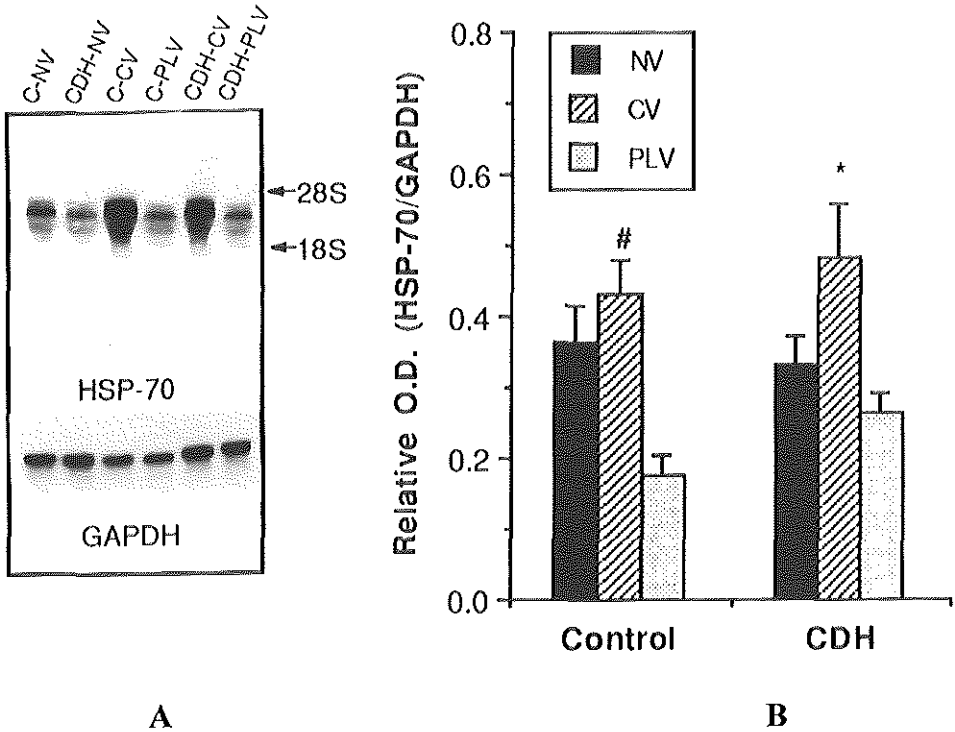

Figure 4: Northern Blot analysis of HSP-70 mRNA expression in control and CDH rats subjected to CV and PLV.

10 µg of total RNA extracted from lugs of control as well as CDH rats was denatured and electrophoresed on 1% agarose gel and blotted on a nylon membrane as described in "Materials and Methods" section. Filters were hybridized with HSP-70 cDNA probe (Panel A; upper part) and with GAPDH (Panel A; lower part) for the reference and RNA loading correction purposes. Autoradiography shows two mRNA species of 3.5 and 2.7 kb encoding HSP-70 in the lungs derived from both CDH and control rats. Panel B: Bar diagram shows relative levels of HSP-70 mRNAs in control and CDH lungs subjected to CV or PLV. Note the decreased mRNA levels of HSP-70 in PLV treated rats.

#,* depicts significantly different from respective lungs subjected to PLV ($p<0.05$).

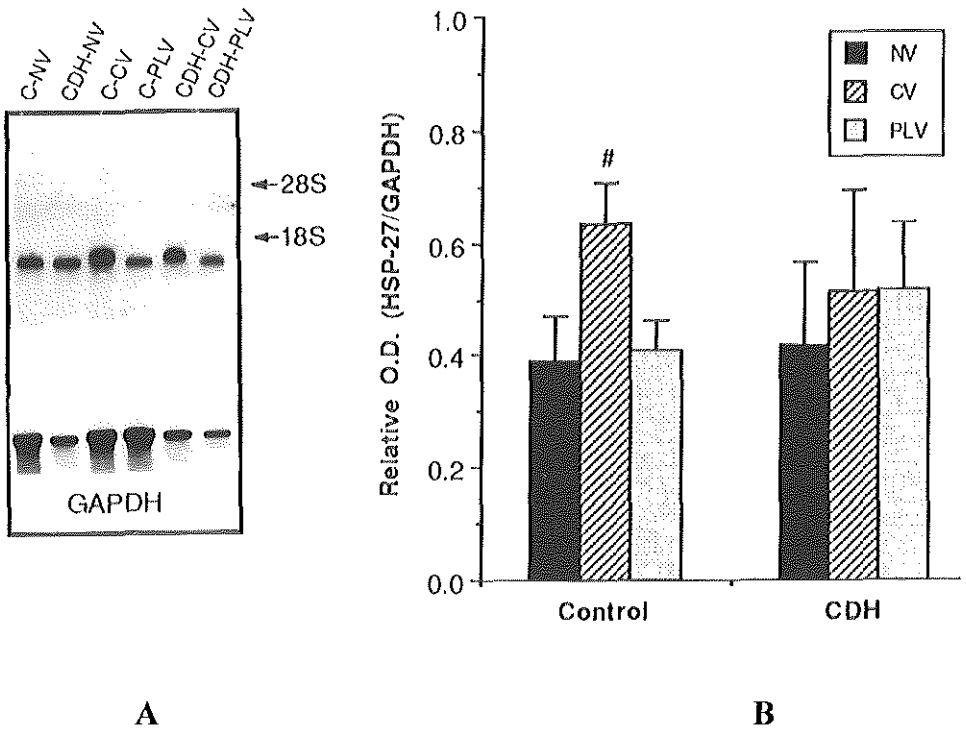

Figure 5: Northern Blot analysis of HSP-27 mRNA expression in control and CDH rats subjected to CV and PLV.

10 μg of total RNA was denatured and electrophoresed on 1% agarose gel and blotted on a nylon membrane as described in "Materials and Methods" section. Filters were hybridized with HSP-27 cDNA probe (Panel A; upper part) and with GAPDH (Panel A; lower part) for the reference and RNA loading correction purposes. Autoradiography shows a single mRNA species of 0.9 kb encoding HSP-27 in the lungs derived from both CDH and control rats. Panel B: Bar diagram shows relative levels of HSP-27 mRNA in control and CDH lungs subjected to CV or PLV. Note the decreased mRNA levels of HSP-27 only in control rats subjected to PLV.

#,* depicts significantly different from respective lungs subjected to PLV ($p<0.05$).

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Chapter 6

Enhanced Expression of Heat Shock Proteins in Lungs of New-born Rats Following Exposure to Nitrogen Dioxide

Based on the article:

T. Okazaki T, Shehata SMK, Busker R, van der Heijden B, Tibboel D and Sharma HS: NO_2-induced Airway Responses in Newborn Rats: Enhanced Expression of Heat Shock Proteins – *Submitted*

6.1 Introduction

In new-borns with respiratory insufficiency, artificial ventilation with high peak respiratory pressures, resulting in volutrauma of the pulmonary parenchyma, combined with high respiratory oxygen, levels may lead to progressive lung damage ref. [1-12]. Several reports have documented the role of a number of mediators in different tissue components as etiological factors for the development of progressive lung damage. We know that immaturity of the lung, barotrauma, and oxygen toxicity contribute to the lung injury and the pathogenesis of chronic lung disease (CLD), but the exact mechanisms by which the lung undergoes severe disruption in structure and function are not fully understood. The same holds true for mechanisms preventing lung injury. Prolonged exposure to hyperoxia and barotrauma causes acute lung injury that leads to an inflammatory reaction. Capillary permeability changes are amongst the first events to occur. The injury process is initially characterised by an influx of cells, mainly neutrophils, and an elevation of the release of various inflammatory mediators. This early inflammatory phase is followed by a subacute fibroproliferative response with fibroblast and smooth muscle proliferation, leading to interstitial and peri-alveolar fibrosis. Recent evidence suggests that the fibroproliferation response may be due to higher expression of different cytokines such as platelet derived growth factor-BB (PDGF-BB), transforming growth factor-β (TGF-β) and interleukin-1β, produced by locally accumulated alveolar macrophages and probably epithelial cells. In some cases the inflammatory and fibroproliferation responses may lead to (development of) chronic lung disease (CLD).

To investigate the pathogenesis of lung damage in the new-born, numerous articles have focused on the role of hyperoxia in this process, suggesting a complicated interplay between mediators and cells [10, 11, 13-17]. Besides hyperoxia models, another approach resulting in progressive tissue damage and remodelling is exposure of adult rats to a combination of ozone and nitrogen dioxide (NO2) [18-22]. The effects are well documented, both at the biochemical level and morphologically. The consequences of exposure of adult animals to either NO2 or Ozone have been published as well [23-30]. As other models for neonatal lung damage have proven to be a highly resistant to changes, such as hyperoxia, or are extremely expensive due to the complicated infrastructure needed, for example in the premature baboon model, a neonatal rat lung model was used.

Inducing airway inflammation by damaging the airway epithelium air pollutants are thought to provoke asthmatic symptoms[31-33]. Nitrogen dioxide (NO_2), is one of the major components of atmospheric air pollution that has been reported to alter lung morphology and to affect pulmonary function [34,35]. Morphological changes after NO_2 exposure include damage to Clara, ciliated, and type-I epithelial cells in the

bronchioalveolar region [36,37], interstitial fibrosis [38], and increase in medial thickness of pulmonary arteries [39], indicating that NO_2 can cause lung remodeling. Furthermore, these morphological changes are closely correlated with NO_2 concentration and exposure time [39]. Although many reports have documented that nitrogen dioxide (NO_2) can cause lung injury and airway remodeling in adults [36-39], virtually nothing is known regarding the effects of NO_2 exposure on the neonatal developing lung.

A number of genes are expressed immediately when cells are subjected to stress, stretch, and in case of acute and chronic lung injury [40,41]. Among these are the heat shock proteins (HSPs), a group of highly conserved proteins that can be induced by exposure to heat, as well as by a variety of pathophysiological conditions including hypoxia, oxidative and metabolic stresses [42,43]. The HSP-70 family of proteins binds to adenosine tri-phosphate (ATP) and plays a role in the transport of proteins into the endoplasmic reticulum and mitochondria [44,45]. The small heat shock protein HSP-27 is a 27 KDa stress related protein, considered a molecular chaperone expressed in developing organs. From cellular cytoplasm it migrates to the nucleus upon stress and plays a role in signal transduction and drug resistance [45,46]. HSPs have been reported to participate in embryonic and fetal development [47]. To our knowledge, few studies have examined HSPs expression in NO_2 induced lung injury and remodeling, particularly in the developing lung.

In order to establish the nature and time course of NO_2-induced lung damage in early life, we investigated the expression of mRNAs and tissue localization of HSP-27 and HSP-70 in a model of lung injury induced by chronic NO_2 exposure in weanling rat pups.

6.2 Materials and Methods

Experimental model

Specific pathogen-free, 3-days-old Wistar strain rat pups were used for this study. A total of 36 animals were divided into exposed and non-exposed (control) groups. Exposed pups were chronically exposed to NO_2 at 7 parts per million (ppm) for 8 hours daily for 7 days. Nitogen dioxide (NO_2) was purchased from Hoekloos (Schiedam, the Netherlands) in a 500 g lecture bottle. The bottle was supplied with a T-connector with a septum. A series of two impingers was connected to the lecture bottle, with an end pressure of 4 cm water. After the cylinder was opened, it was heated using an air heater until all air in the system was replaced by (brown) NO_2 fume. Then 70 ml off NO_2 vapor was sampled, under heating, into a 100 ml gas tight syringe, by repeated movement of the plunger. The animal exposure chamber (77x77x125 cm +741 l) consisted of glass walls, an entrance, a gas inlet and sampling outlets. At the start of the

exposure, the desired amount of NO_2 was injected into the chamber (see below). To maintain the desired concentration while applying the necessary amount of fresh air into the chamber, the inlet (of the chamber) was connected to a Gilson personal sampling pump, which delivered fresh air at 1.2 l/min. Using a T-connector, a Braun infusion pump was connected to the same inlet. Using this pump the gas tight syringe containing 100 ml NO_2 was emptied with the desired flow (see below). The temperature of the infusion pump was kept at 25°C with a re lamp.

For 20 ppm of NO_2 : 20 x741/1000=15 ml of NO_2 was needed as a loading dose. With an air refresh rate of 1.2 ml/min, an additional amount of NO_2 was supplied. For this purpose, 20 ml of NO_2 per l, i.e. 24 ml per min=1.44 ml/h, was theoretically needed. However, empirically it appeared that 4 ml/h was necessary. This is due to loss of NO_2 to the walls, tubings, water vapour, animals etc.

The NO_2 concentration in the chamber was determined using Drager indicator tubes: nitrous fumes 20/a 20-500 ppm. Sampling was performed at 5 min after start of the experiment, and each hour thereafter. When necessary the NO_2 flow was adjusted to maintain the desired concentration. Using this system the NO_2 levels were well controlled:

If aimed at 7 ppm: 7.0 ± 0.2 ppm
If aimed at 20 ppm: 17.4 ± 1.3 ppm

After exposure pups were allowed to recover for either one week (group I, 12 pups) or two weeks (group III, 12 pups). Following the recovery period the animals were sacrificed. Age-matched controls were exposed to room air alone in the same way, forming two groups of 6 pups each (groups II and IV respectively). During exposure (periods), animals in each group were kept in a cage, which was placed in a gas-tight chamber equipped with an inlet for a mixture of NO_2 and compressed air. The NO_2 concentration was measured and kept to 7 ppm. After exposure, all rat littermates were returned to room air for recovery. They were kept in conventional cages with their mothers and fed *ad libitum* until tissue preparation. The Erasmus University committee for research and animal studies approved all the experimental protocols.

Tissue preparation
At day 7 (group I +II) and 14 days (group III + IV) after exposure, the pups in each group were anaesthetized by intraperitoneal injection of 1 ml sodium pentobarbital. Through a midsternal thoracotomy, extended to the cervical portion, both lungs and the trachea were exposed. For isolation of total cellular RNA, the two lungs were removed from the thoracic cavity, frozen in liquid nitrogen and stored at -80 °C until analysis. For histological and immunohistochemical examinations, a 22G needle was inserted

into the trachea and the lungs were fixed by injection of Davidson solution (40 vol % ethanol; 5 vol % acetic acid; 10 vol % formaldehyde; 45 vol % saline; pH7.3) at a pressure of 20 cmH_2O [49]. Following lung expansion, both lungs and heart were removed as one preparation and immersed in Davidson solution for at least 24 hours and processed for the histology.

Isolation of total cellular RNA and Northern blot analysis

Total cellular RNA was extracted from 100 mg frozen lung tissue by the guanidinium thiocyanate-phenolchloroform method of Chomzynski and Sacchi [48]. The RNA concentration was measured by spectro-photometry. For Northern hybridization, 10 µg of total RNA was denatured at 65°C in buffer containing formamide and ethidium bromide, and electrophoresed on 1% agarose gel containing 2.2M formaldehyde. The gels were photographed and RNA was transferred to hybond-N membrane (Amersham Nederland B.V., Den Bosch). Thereafter, filters were air-dried and UV cross-linked in a gene linker (Bio-Rad Laboratories B.V., The Netherlands). Blots were hybridized at 42°C in a buffer containing 50% deionized formamide, 1.0 M sodium chloride, 1% sodium dodecyl sulfate (SDS), 0.2% polyvinyl pyrolidine, 0.2% ficoll, 0.2% bovine serum albumin, 50mM Tris-HCl (pH 7.5), 0.1% sodium pyrophosphate, 10% dextran sulfate and denatured salmon sperm DNA (100 µg/ml). cDNA probes used for hybridization were a 0.9 kb DNA fragment encoding human HSP-27, and a 2.1 kb DNA fragment encoding human HSP-70. cDNA inserts were labeled employing a multiprime labeling system (Amersham Nederland B.V., s'Hetogenbosch), to specific activity of 10^9 cpm/µg DNA using [^{32}P]-dCTP (3000 Ci/mmol, Amersham Nederland B.V. Den Bosch). After overnight hybridization, filters were washed at room temperature for 5 min in 2x SSC and 0.1% SDS and at 42°C in 0.1x SSC and 0.1% SDS for 20 min. Subsequently, filters were wrapped in household plastic wrap and exposed to Kodak X-OMAT AR films (Kodak Nederland B.V., Odijk) at -80°C for 24 hours. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (1.2 kb PstI fragment of human cDNA, procured from ATCC, USA) was used to rehybridize membranes for reference purposes. After performing autoradiography and densitometric measurement of signals, fold induction of gene expression was calculated and compared between different study groups.

Immunohistochemistry

After Davidson fixation, the lungs were embedded in paraffin. Sections were cut at 6 µm thickness and mounted on 3-amino-propyl-trioxysilane coated slides (Sigma, St Louis, MO, USA), followed by immunohistochemistry using the avidin-biotin complex (ABC) method. The sections were deparaffinized and endogenous peroxidase was quenched by 2 % hydrogen peroxide in methanol for 20 min. Non-specific binding sites were blocked by pre-incubation with 10% normal goat serum for 15 min.

Monoclonal mouse anti-human antibodies against HSP-27 {diluted 1:250} and HSP-70 {diluted 1: 100}(NeoMarkers, Fremont, USA) were used as primary antiserum. The sections were incubated with primary antiserum at room temperature for 30 min. Negative controls were performed by omission of the primary antiserum. After washing in 0.5% Tween in PBS solution, the slides were incubated for 30 min with mouse biotinylated anti-rabbit IgG (Multilink, 1:75) and with Peroxidase conjugated streptavidin in a dilution of 1:50, both supplied by Biogenex (San Ramon, MO, USA). Slides were visualized after developing the color using DAB (0,025% 3,3'-diaminobenzine tetra hydrochloride dihydrate, Sigma, St. Louis, Mo, USA). Sections were counter stained with Mayer's hematoxylin.

Semiquantitative analysis HSPs localization

Expression of HSP-27 and HSP-70 was analyzed semi-quantitatively and scored by two independent observers, using an arbitrary visual scale ranging from 0 to 4: grade 0 represents no staining, grade 1 represents focal staining, grades 2,3 and 4 represent diffuse weak, moderate, and strong staining, respectively [50].

Statistical analysis

Results were calculated as mean ± SEM. Statistical analysis was based on the unpaired Student's *t* test, and significance was accepted at $P < 0.05$.

6.3 Results

Expression of mRNAs of HSPs

By Northern blot analysis we detected mRNAs encoding both HSP-27 and HSP-70 in control as well as in NO_2 exposed rat tissues at 7 days after exposure (Fig. 1 and 2). In control lungs, the levels of expression of HSPs mRNA decreased gradually. However, significantly enhanced expression levels ($P \leq 0.05$) of HSPs mRNA were detected at 7 days after exposure (group I) and these levels remained elevated until 14 days after exposure to NO_2 (group III) as compared to their respective controls (groups II and IV). Densitometric analysis of the blots revealed that expression levels of both HSPs were significantly elevated at 7 days after NO_2 exposure as compared to 14 days after exposure to NO_2 (Fig. 1B and 2B). Although, the expression levels were decreased in the pups at 14 days, their expression values were not statistically different in controls and NO_2 treated groups.

Histology

Hematoxylene and eosine staining assessed pulmonary tissue histology in relation to NO2 exposure. Routine histological examination showed minimal differences between controls and NO_2 exposed groups (Fig. 3). However, NO2 exposure resulted in partial

epithelial shedding in the tracheal airways of pups derived from group I and III (Fig 3A).
See color pictures on page 132.

Tissue localization of HSPs
Immunoreactivity for both HSPs was mainly localized in bronchial and bronchiolar epithelial cells in both experimental and control groups (Fig. 4 and 5). NO_2 exposure resulted in intense pulmonary staining for HSPs as compared to the control group at 7 days after exposure. Intense staining of HSP-27 was detected in bronchiolar epithelial cells, and also in the adjacent alveolar type II epithelial cells of lungs in NO_2 exposed pups (Fig. 4). On the other hand, vascular smooth muscle cells showed immunoreactivity of HSPs, which did not change drastically after NO_2 exposure (Fig 4). Semi-quantitative analysis using a visual score revealed that the expression levels of HSP-27 and HSP-70 were reduced in rats at 14 days of exposure as compared to those after 7 days but still the values were higher than those of their respective controls.
See color pictures on page 134-137.

6.4 Discussion
In the present study, we demonstrated enhanced expression of HSP 27 and HSP 70 at both mRNA and protein levels in NO_2 exposed lung tissues of neonatal rats. Histological evaluation revealed only minimal differences with controls. NO_2 is one of the most important air pollutants, that are known to cause lung injury and airway remodeling. NO_2 could be produced following oxidation of NO in the lung. In clinical circumstances, NO inhalation therapy has been used in neonatal patients with acute respiratory distress syndrome or persistent pulmonary hypertension because of its vasodilator property [51]. Although levels of NO_2 produced by NO therapy have been estimated to be as low as 2 ppm [52], they might induce injury, and exposing the lung to a stress condition.

The effects of NO_2 on the capillary alveolar septal network and the phospholipid pool in the postnatal lung remodeling have been described previously [53]. In addition, our present results point at to an important role of NO_2-induced airway inflammatory damage at a molecular level with the subsequent early pathophysiologic stages that may eventually account for the childhood asthma [31]. These findings are supported in part by our findings of increased vascular endothelial growth factor (VEGF) at both mRNA and protein levels in newborn pups following chronic exposure to NO_2 [Sharma et al. Submitted].

Heat shock proteins are one group of the lung's defensive mechanisms, beside others such as the oxygen scavenger system including the antioxidant enzyme system [53,54]. These mechanisms are of specific importance in cytoprotection and prevention of

parenchymal lung damage after exposure to certain injurious stimuli [55,56]. Our findings of increased expression levels of HSPs, which are known molecular markers of pulmonary cellular stress [38,46,55], may indicate a possible role of HSPs in postnatal lung development and remodeling. Lung development in rats defines the formation of the main structural lung tissue, including the capillary network in alveolar septa, which occurs within three weeks after birth [57]. Enhanced expression of HSPs in NO_2-exposed lungs might indicate its involvement in tissue protection and recovery processes following NO_2-induced injury in the developing lung. The expression levels of HSPs mRNA, which are maximal following the exposure and decreased by time, remained in this study even significantly higher than in the respective controls, in correspondence to the protective role of HSPs in response to degree and time-peak of NO_2-induced injury.

Immunoreactivity for both HSPs was mainly cytoplasmic, with minor degree of nuclear positivity, as has been reported previously [46]. Although previous studies showed histologic alterations in adult lungs exposed to NO_2 [36-39], our data could not support these findings as no remarkable changes in lung tissue at routine histological examination of neonatal lungs were seen. It remains unclear if the protective mechanisms against NO_2 exposure are more efficient in neonatal rats than in adults rats.

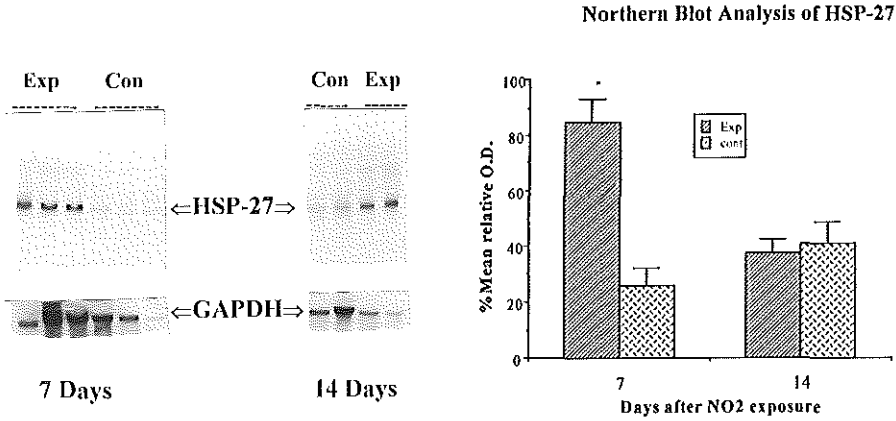
Our data show that NO_2 exposure induces a significant increase in the expression levels of HSPs genes, which had been reported to participate in cellular defense mechanisms and perhaps to enable the lungs to recover from stressful conditions [50]. Furthermore, the enhanced expression of HSPs we found, represents a state of cellular injury. HSPs are molecular markers of pulmonary stress, possibly triggering cytoprotective mechanisms against further pulmonary parenchymal damage, as demonstrated in previous reports on the cytoprotective role of HSPs in both experimental and human tissues [58-60]. The enhanced expression of HSPs possible reflects a neonatal attempt to establish a protective mechanism against stress as shown earlier for antioxidant enzyme activity in a congenital diaphragmatic hernia rat model [60]. Interestingly, our results demonstrate that the bronchiolar epithelial cells exhibit the most intense expression of HSPs in the developing lung in response to NO_2 exposure, indicating cellular injury, which is more pronounced in the distal airway passages. This is in accordance to a literature report documenting significantly raised expression levels of HSP-70 in inflammed airways in, for instance, asthmatic patients [61]. These findings, in view of the fact that in inflammatory airway diseases the distal bronchioli are the more affected ones, could explain that recurrent exposure to air pollutants in infancy heightens susceptibility to airway inflammation at later life. A recent report demonstrates nasal mucosal inflammation following chronic exposure to low dose NO_2 and ozone in humans in vitro [62]. One of the mechanisms underlying this notion could be that the distal air passages become hyper-responding or less protected against

inhalation injury. These findings could be useful in evaluating age-dependent changes and susceptibility of the pulmonary system to environmental pollutants.[63].

From the results of the current study and previous reports concerning the understanding of the pathogenesis of primary airway inflammatory disease, we hypothesize that neonatal rat lungs might have different cellular or functionally more efficient -already existing- defense mechanisms as compared to the adult rat lung.

Acknowledgments
Authors thank Mrs Emine Yilmaz for her technical assistance.

6.5 Figures



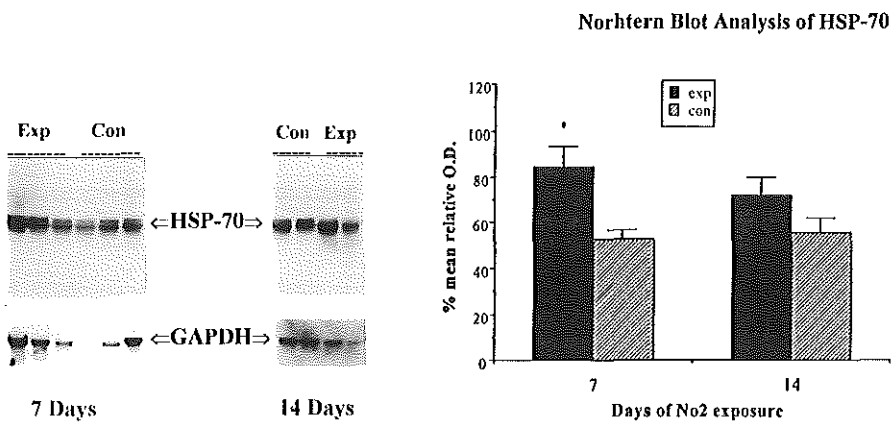
(A) (B)

Figure 1: Northern Blot analysis of HSP-27 mRNA in control and NO_2 exposed rat pups

Total RNA isolated from pooled lungs from 3-4 pups from control as well as from NO_2 exposed groups was subjected to gel electrophoresis and subsequently for blotting and hybridization as described in "Materials and Methods". Filters were hybridized with HSP-27 cDNA probe (***Panel A:*** upper part) and with GAPDH (***Panel A:*** lower part). Autoradiography showed a single mRNA species of 0.9 kb encoding HSP-27 in the lungs of all pups.

Panel B: Bar diagram depicting relative mRNA levels of HSP-27 in control as well as in NO_2 exposed pups at 7 and 14 days of exposure. Note the induced expression of HSP-27 mRNA at 7 days of NO_2 exposure as compared to respective controls. Values are mean ± SEM from 3-4 different blots. P values of <0.05 were accepted as significant.

*depicts significantly increased levels of HSP-27 mRNA as compared to respective controls.



(A) (B)

Figure 2: Northern Blot analysis of HSP-70 mRNAs in control and NO_2 exposed rat pups

Total RNA isolated from pooled lungs from 3-4 rat pups from control as well as from NO_2 exposed groups was subjected to gel electrophoresis and subsequently for blotting and hybridization as described in "Materials and Methods". Filters were hybridized with a HSP-70 specific cDNA probe (***Panel A:*** upper part) and with GAPDH (***Panel A:*** lower part) used for reference purposes. Autoradiography showed two mRNA species of 3.5 and 2.7 kb encoding HSP-70 in the lungs of all pups.

Panel B: Bar diagram depicting relative mRNA levels of HSP-70 in control as well as in NO_2 exposed rat pups at 7 and 14 days of exposure. Note the induced expression of HSP-70 mRNAs at 7 days of NO_2 exposure as compared to respective controls. Values are mean ± SEM from 3-4 different blots. P values of <0.05 were accepted as significant.

*depicts significantly increased levels of HSP-70 mRNAs as compared to respective controls.

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Part V

General Discussion and Summary

Chapter 7

General Discussion and Concluding Remarks

7.1 Introduction

Congenital diaphragmatic hernia (CDH) is a congenital anomaly, which manifests itself in 1 out of 2000-3000 newborns [1-3]. Patients with CDH still experience a high mortality despite intensive medical and surgical treatment. This mortality is caused by a combination of pulmonary hypoplasia and persistent pulmonary hypertension [4,5]. Histopathologically, abnormalities in the pulmonary vasculature in CDH are well documented [6,7]. However, the exact causes attributing to the morphological changes and abnormal pulmonary vascular reactivity [8] are not fully understood in neonates with pulmonary hypertension. In the clinical setting, most of the patients with CDH require artificial ventilation with high inspiratory pressure and high percentages of oxygen to provide adequate oxygenation in the immediate neonatal period, which is believed to be a main cause of pulmonary sequel. It is not known whether and to what extent artificial ventilation and other environmental stress, not only in CDH but also in normal developing neonatal lungs, affect their developmental pattern.

Air pollutants are thought to provoke airway inflammation by damaging the airway epithelium [9-11]. Nitrogen dioxide (NO_2), is one of the major components of atmospheric air pollution that has been reported to alter lung morphology and affect pulmonary function [12,13]. Morphological changes after NO_2 exposure include damage to Clara, ciliated, and type-I epithelial cells in the bronchioalveolar region [14,15], interstitial fibrosis [16], and increase in medial thickness of pulmonary arteries [17], indicating that NO_2 can cause lung remodeling. Furthermore, these morphological changes are closely correlated with NO_2 concentration and exposure time [17]. Although many reports had documented that nitrogen dioxide (NO_2) can cause lung injury and airway remodeling in adults [36-39], virtually nothing is known regarding the effects of NO_2 exposure on the neonatal developing lung. Therefore the aim of the studies presented in this thesis were as follows:

1. to investigate differences in pulmonary vascular development between healthy and CDH lungs in humans and a rat model;
2. to investigate the expression patterns of vasoactive mediators in CDH lungs in an animal model of CDH;
3. to evaluate the effect of insults such as different modalities of artificial ventilation and exogenous stimuli on stress response and vasoactive mediators in neonatal healthy and CDH lungs;

4. to examine the respons of neonatal rat lung parenchyma and the pulmonary vascular system during NO2 exposure in an attempt to develop a model resembling the early changes in bronchopulmonary dysplasia.

7.2 Interpretation and implications of the studies

7.2.1 Morphological and functional pulmonary vascular development

In cases with CDH, a decreased number of pulmonary vessels, increased muscularity of the peripheral arteries and thickened adventitia have been reported in the hypoplastic lungs. Such histopathological findings in man have also been confirmed in experimental rat and lamb models of CDH [18,19]. Morin and Stenmark reviewed the normal structural development of the pulmonary vasculature with special attention towards the role of altered smooth muscle cell differentiation in the development of abnormal pulmonary tone [20]. We have to take into account that the models used are the neonatal hypoxic calf model or rats exposed to hypoxia or hyperoxia after birth. No detailed information is available on smooth muscle cell differentiation during abnormal pulmonary vascular development such as in case of CDH. For this reason we examined the expression of myosin heavy chain isoforms in pulmonary vascular smooth muscle in the rat model of CDH to evaluate the smooth muscle cell differentiation. The lungs of controls and those of rats with CDH showed similar patterns in the expression of SMemb, SM1, SM2 and alpha-actin, indicating that differences in smooth muscle cell differentiation do not account for the vascular abnormalities in CDH. Consequently a hampered differentiation of pulmonary SMC resembling on earlier phase of vascular development, in which high pulmonary vascular tone is a normal feature, can not be used as an argument.

A number of different growth factors such as fibroblast growth factor, transforming growth factor-beta, platelet derived growth factor, insulin like growth factors I and II, and vascular endothelial growth factor (VEGF) have been shown by different techniques such as immunohistochemistry, in situ hybridisation during pulmonary vasculogenesis and angiogenesis [19-22]. VEGF is an endothelial mitogen, which is regulated at the receptor level. Fms-like tyrosine kinase (Flt-1) and Flk-1 are receptors for VEGF and are expressed during early vascular development in human embryos. VEGF has been shown to play a role in fetal angiogenesis, its expression increases at mid gestation to enhance angiogenesis and formation of vascular beds and decreases towards term. Many studies have been performed in different types of PH, where the expression of VEGF was found to be up regulated in persistent pulmonary hypertension of neonates (PPHN) [22-24].
In our rat model of CDH, VEGF was detected in the vessels at the hilum and in parenchyma from gestational day 20 onward in control lungs, but it was absent in lungs

from CDH rats from day 16 to day 22. This finding suggests that decreased VEGF expression in Nitrofen induced CDH rat lungs accounts for altered endothelial cell growth. Recently the group of Shannon investigated expression of VEGF and its receptors in the Nitrofen rat model during prenatal development. These authors were not able to find differences between CDH and control animals (Shannon, personal communication). We reported the enhanced expression of VEGF in small pulmonary vessels of CDH cases complicated with pulmonary hypertension as compared to their age-matched controls at the level of the SMC and endothelium. Our data indicate that there is persistence of the stunted lung vessel growth in CDH cases as VEGF was reported to be increased at mid-gestation in human fetuses. This is in accordance to the findings of Wigglesworth and Desai who reported that infants with lung hypoplasia in CDH born at 34-39 gestational weeks have a lung cell population comparable to that of a normal fetus at 20-22 weeks. In our study, the increased expression possibly represents a fetal attempt to stimulate angiogenesis of the stunted bed in cases of CDH.

We do not have a clear answer for this difference. Possible explanations may be that CDH rat pups were not exposed to hypoxic conditions during the experiment, or phases of lung development in utero are various among different species. It is also possible that the pathogenesis of the vascular changes induced by Nitrofen is very different from the "natural" course in the human fetus with CDH.

7.2.2 Regulation of pulmonary vascular tone

In utero, the pulmonary blood flow is low and the pulmonary vascular resistance is high. Several factors modulating pulmonary vascular resistence in utero and during transition from intrauterine to extrauterine life have been reported [25-27]. Two factors, nitric oxide (NO) and endothelin-1 (ET-1), have been put forward as essential vasoactive mediators in the perinatal pulmonary circulation [25,28]. In lambs with CDH, a quantitative analysis showed no differences in the presence of endothelial nitric oxide synthase (eNOS) in the main pulmonary artery trunks and controls [29]. However, quantitative studies of the lung parenchyma in rats with CDH showed that eNOS activity and the mRNA expression were lower than in control lungs [30,31]. On the other hand, normal eNOS mRNA levels were reported in lungs of CDH rat pups which were not altered by prenatal treatment with dexamethasone [33]. Moreover, clinical observations show the reactions to a variety of vasoactive agents, including inhaled NO, to be highly unpredictable [8]. These findings leads us to investigate the expression of ET-1 and its receptors in CDH rat pups. Significantly enhanced levels of ET-1 mRNA were observed in CDH rats compared with controls. In addition, a two- to four fold increase in ET-A receptor mRNA were observed in CDH as compared with controls in contrast to equal levels of ET-B receptor mRNA. The upregulated expression of ET-1 and ET-A receptor mRNA may contribute to the pulmonary

vascular dysbalance in CDH. Furthermore, new trials of ET-A receptor blockade and ET-B receptor stimulation have been initiated in experimental CDH models as an intervention for pulmonary vasoconstriction in CDH with promising results (Mercier JC, personal communication).

Our findings support the pharmacological modulation of pulmonary vascular tone by ET-1 and ET-A blockade as an alternative treatment modality for CDH associated with persistent pulmonary hypertension. It is not known whether a dysbalance between the different isoforms of NOS and ET-1 and its receptors is already present before birth. It is possible that the state of vasoconstriction before birth in an environment of low oxygen tension is mediated by a profound ET-1 effect. Developmental studies in humans and rat models are essential in this respect. Evaluation of pulmonary vascular tone and respons on pharmacological agents in a standardised manner may provide us the necessary answers for future guidance of the proper vasoactive drugs [33]. In addition, other experiments showed that the prevention of hypoxia by artificial ventilation including partial liquid ventilation decreased the expression of ET-1 mRNA in control and CDH rat pups (unpublished observations). It will be necessary to search for the most effective combinations of pharmacological modulation and ventilatory strategy in the future.

7.2.3 The effect of "insults" in neonatal lungs

7.2.3.1 Artificial ventilation including "Partial Liquid Ventilation"

The use of ECMO has been advocated to prevent the lungs from being further damaged by mechanical ventilation and to correct PPHN [5]. The mean survival rate of CDH patients treated with ECMO has not improved markedly as compared with other ventilatory strategies, for instance high frequently oscillatory ventilation (HFOV) with NO [34,35], although some other centers using ECMO reported even higher survival rates [36,37]. Partial liquid ventilation (PLV) with perfluorocarbon has only recently been tried in humans, and the first results in adults, pediatric patients, premature babies with respiratory distress syndrome, and CDH patients are encouraging [38-40]. Previous reports showed that lung compliance and gas exchange in infants and lambs with CDH improved significantly during PLV [40-42]. Especially the group of Lachmann (Rotterdam, the Netherlands) contributed significantly in the understanding of the positive effects of PLV on lung mechanisms; oxygenation and the combined use of PLV, NO and surfactant (reviewed in 43-45). However, the effect of stress at cellular and molecular levels, as a result of PLV in CDH, has not been evaluated.

We examined the changes in lung mechanics and the expression pattern of heat shock protein (HSP) - 70 and 27 as stress markers in control and CDH rats which underwent

conventional ventilation (CV) and PLV in a randomized manner. Following PLV, increased total lung volume and improvement in opening pressure in CHD rats were observed. The expression of HSP mRNAs was enhanced after CV as compared with PLV. Our results clearly demonstrated for the existence of endogenous defense mechanisms in hypoplastic CDH lungs and PLV appears to be less strenuous as compared to CV. These findings indicate that PLV may become a useful ventilatory modality for CDH patients and it may be essential to define new ventilatory strategies, for instance the combination of ECMO and PLV (J.M.Wilson, personal communication). In this respect a thorough evaluation of the different therapeutic modalities available for the treatment of CDH patient is warranted. Besides the documented release of vasoactive mediators such as PGF1 and TBX2 in rat CDH pups; cell counts and a number of inflammatory mediators in human CDH, almost no information is available [46,47]. The same holds true for the use of ECMO: lung rest is only a synonyme to hide our lack of knowledge.

7.2.3.2 Stress responses in developing lungs

The high incidence of chronic lung disease in surviving patients with CDH together with the well known clinical observations of the fast progression of altered lung morphology, resulting in adequate gas exchange in a number of patients were considered as arguments to perform these experiments. In fact two well-documented models are available in the literature: the premature baboon and the adult rat exposed to NO2 and Ozone (O3). In the premature baboon model bronchopulmonary dysplasia develops following artificial ventilation. Especially the group of Coalson has contributed extensively to this model (reviewed in Bancalari) [48]. Due to the high costs of the animals, laboratory facilities and need for an animal intensive care one centre of research is at present active in the United States.

For the rat O_3 + NO2 model special equipment is needed and facilities to control gas flow and environmental spread of noxious gas mixtures. Thus far only in adult animals the combined use results in progressive lung fibrosis [49-52]. As described above, we investigated the expression of stress genes during artificial ventilation in CDH lungs. We extended our research to examine the expression of stress genes and the angiogenic growth promoter VEGF in developing lungs under stressful condition, i.e. nitrogen dioxide (NO2) exposure. NO2, is one of the major air components of atmospheric air pollution, was reported to alter lung morphology and affect pulmonary function. Previous studies on adults rats revealed that morphological changes in response to NO2 exposure include damage to Clara, ciliated, and type-I epithelial cells in the bronchoalveolar region, intestinal fibrosis, and increased medial thickness of pulmonary arteries, indicating that NO2 can lead to lung remodelling.

We demonstrated that the expression of HSP-27 and 70 both at mRNA and protein levels were enhanced in chronic NO2 exposed lung tissues than in control lungs of rat pups in spite of the observed minimal histological changes between both NO2-exposed and control rats. This is indicated that the enhanced expression of HSPs also reflects a neonatal attempt to establish a protective mechanisms against stress as shown earlier for antioxidant enzyme activity in the CDH rat model [53]. Regarding the expression of VEGF under NO2 exposure enhanced expression of VEGF in vascular smooth muscle, bronchial and bronchiolar epithelial cells in NO2 exposed lungs as compared to controls was observed. These findings suggested that inducing VEGF expression under stress might contribute to vascular remodelling or initiate certain structural change at the stage of alveolar formation during lung development. However, our data are preliminary and further investigation will be required to evaluate stress response in developing lungs, particularly in hypoplastic CDH lungs. It will not be a simple choice to identify a key molecule in the complicated interaction of mediators. These mediators, locally produced or transferred from neighbouring cells, result in triggering the ongoing lung damage leading in a long lasting sequel and eventually fixed pulmonary damage. In this respect predetermined evaluation of stress markers in routine performed bronchoalveolar lavages might be helpful in this respect in the clinical situation. The question remains whether information obtained from BAL-fluid is a true representative of the changes occurring in the lung parenchyma.

7.3 Concluding remarks

Although many aspects of lung hypoplasia and persistent pulmonary hypertension in CDH have been studied, many issues remain to be clarified. The use of different animal models and patient selection of human cases (autopsy material) with CDH has revealed confusing and sometimes contradictory data. However, we need to elucidate these results and to implicate these in our clinical daily practice. A complete understanding of the pathogenesis of lung development of CDH and of those factors that contribute to lung injury eventually determine the prenatal, prenatal and postnatal management of patients with CDH. Especially the latest achievements in treatment modalities such as prenatal tracheal plugging and the use of corticosteroid after prenatal diagnosis (CDH study group) warrants careful, well prepared and documented evaluation [54-56]. In this thesis, we proposed some therapeutic strategies such as endothelin blockade and PLV. In addition, recently beneficial effects of prenatal hormonal modulation in CDH have been reported in the rat model of CDH [57,58]. However exact mechanisms of these effects still remain unknown. The large number of suggested interventions illustrate that we still have not found the ultimate solution how to manage the fragile hypoplastic lung and lethal persistent pulmonary hypertension in CDH.

7. 4 References

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Chapter 8

8.1 Summary

Neonates with congenital diaphragmatic hernia (CDH) experience a high mortality due to pulmonary hypoplasia and persistent pulmonary hypertension (PPHN) despite intensive medical and surgical treatment. To clarify the exact mechanisms of pulmonary hypoplasia and PPHN is essential to achieve advancements in the management of neonates with CDH. This thesis was undertaken in order to investigate the mechanisms of abnormalities of the pulmonary vasculature and the abnormal pulmonary vascular reactivity. Furthermore, we included our studies on the pulmonary stress responses in developing normal and hypoplastic CDH lungs.

This thesis consists of five parts: Part I (*Chapter 1*) is the general introduction including the objectives of the thesis. Part II (*Chapter 2 and 3*) describes studies focusing on pulmonary vascular development and Part III (*Chapter 4*) is a study about control of pulmonary vascular tone. Part IV (*Chapter 5, 6*) consists of studies focusing on pulmonary stress responses in developing lungs in two different rat models of CDH and CLD. Lastly, Part V (*Chapter 7 and 8*) includes the general discussion and concluding remarks as well as the summary of the entire work included in this thesis.

Chapter 1 gives a review of the literature concerning CDH research including recently applied aspects of molecular biological techniques and introduces the subjects of this thesis.

In the next two chapters, we investigated pulmonary vascular development in CDH and control lungs. In chapter 2, we examined the expression of a potent angiogenic growth promoter, vascular endothelial growth factor (VEGF), and myosin isoforms in a Nitrofen induced rat model of CDH. Myosin heavy chain (MHC) isoforms such as SMemb, SM1 and SM2 are important molecular markers used to study vascular smooth muscle cell differentiation. Differences in expression pattern of MHC isoforms between CDH and control rat lungs during gestational day 16 to 22 (term) were not observed, VEGF was positive in pulmonary vessel walls from gestational day 20 in control rat lungs in contrast to absent expression of VEGF during gestational day 16 to 22 in CDH rat lungs. These findings suggest that pulmonary vascular development differ between CDH and control rat lungs. Altered endothelial cell growth might be related to the different pulmonary vascular reactivity in CDH. In chapter 3, we examined the expression of VEGF in human cases with CDH and controls. In contrast to the findings from the Nitrofen induced rat model of CDH, the VEGF expression in the pulmonary vasculature of infants who had died from CDH was increased, especially in the small, pressure-regulating pulmonary arteries, as compared with that in lungs of control patients. Although these two chapters suggest that VEGF plays an important role in angiogenesis and in vascular remodelling in cases with CDH, further investigation at gene regulation level is warranted to explain these differences.

Chapter 4 is a description of one aspect of pulmonary vascular tone in CDH. The background consists of previous reports of different expression patterns of nitric oxide synthase (NOS) in experimentally induced CDH lungs and unpredictable responses against inhaled nitric oxide (NO) in patients with CDH. For this reason we examined a potent vasoconstrictor and a mitogen for vascular smooth muscle cells, Endothelin-1 (ET-1), and its receptors using a rat model of CDH. Significantly enhanced levels of ET-1 mRNA were observed in CDH rats compared with control rats. In contrast to equal levels of ET-B mRNA, a two to fourfold increase in ET-A mRNA levels was observed in CDH compared with control rats. The upregulation of ET-1 and ET-A receptor mRNA in CDH rat lungs at term indicates that this peptide plays an important role in pulmonary vasoconstriction and altered pulmonary vascular muscularization in CDH. We speculate that the use of endothelin blockade for the management of PPHN should be considered against the background of the unpredictable and variable response to inhaled NO in patients with CDH.

In chapter 5, we report the effects of partial liquid ventilation (PLV) in CDH and control rats with regards to lung mechanics and cellular and molecular adaptation using heat shock protein (HSP)-70 and 27 as stress markers during ventilation. Following 4 hours of PLV increased total lung volume in both control and CDH rats and improvement in opening pressure in CDH rats were observed as compared to rats with conventional ventilation (CV). Both mRNA and proteins of HSP-70 and 27 were detected in control and CDH rat lungs on gestational day 22 (term). Furthermore, significantly enhanced levels of HSP-70 mRNA were observed in CV rats as compared to PLV rats in both control and CDH lungs. However, the expression of HSP-27 mRNA was enhanced in CV rats as compared to PLV rats only in control group. We concluded that endogenous defence mechanisms in hypoplastic CDH lungs are not developmentally retarded and that PLV appears to be less strenuous as compared to CV.

To evaluate pulmonary vascular stress responses in developing lungs, we investigated the expression of HSP-70 and -27 and VEGF in newborn rat lungs after acute or chronic nitrogen dioxide (NO2) exposure. Chapter 6 describes the expression of HSPs in lungs after chronic exposure to NO2. After 7 days exposure to 7 parts per million (ppm) NO2 to 3 days old rat pups showed significantly enhanced levels of HSPs mRNA up till 14 days after exposure as compared to non-exposed control rat lungs. Histologically, although no apparent differences between NO2 exposed and control lungs were seen at routine histological examination level, intense HSPs immunoreactivity was observed in NO2 exposed rat lungs as compared to controls. These findings suggest that HSPs reflect a neonatal attempt to establish a protective mechanism against stress in developing lungs.

In conclusion, congenital diaphragmatic hernia is one of the major challenges for paediatric surgeons and neonatologists. Many questions still remain to be answered concerning the exact mechanisms of lung hypoplasia and PPHN in relation to various

unpredictable responses against medical and surgical management to the prevention of ongoing lung injury. The information in this thesis may guide new strategies of modulating pulmonary vascular tone and prevention of ongoing pulmonary damage.

8.2 Samenvatting

Pasgeborenen met congenitale hernia diafragmatica (CHD) hebben een hoge sterftekans door het optreden van pulmonale hypoplasie en persisterende pulmonale hypertensie (PPHN), ook bij intensieve medische en chirurgische behandeling. Dit proefschrift beschrijft onderzoek naar mogelijk onderliggende afwijkingen in het pulmonale vaatstelsel en in de vasculaire reactiviteit in de longen, alsmede onderzoek naar de reactie op stress in zich ontwikkelende normale en hypoplastische longen bij CHD.

Hoofdstuk 1 geeft een overzicht van de literatuur betreffende onderzoek naar CHD, met inbegrip van recent toegepaste molecuiair-biologische technieken, en introduceert de onderwerpen die in dit proefschrift behandeld worden.

De volgende twee hoofdstukken gaan in op de vaatontwikkeling in CHD- en controlelongen. Hoofdstuk 2 behandelt de expressie van VEGF, een belangrijke vasculaire endotheel groeifactor, en van *myosin heavy chain (MHC) isoforms* in ratten met CHD opgewekt door Nitrofen. De expressie van *MHC isoforms* tijdens dag 16 tot 22 (term) van de ontwikkeling voor de geboorte verschilde niet tussen CHD- en controlelongen, terwijl VEGF positief was in pulmonale vaatwanden vanaf dag 20 in controlelongen, maar niet in CHD-longen gedurende dag 16 tot 22. Dit doet vermoeden dat de vaatontwikkeling in CHD-longen verschilt van die in normale longen. De afwijkende endotheelcelgroei zou verband kunnen hebben met de afwijkende pulmonale vasculaire reactiviteit bij CHD. Hoofdstuk 3 behandelt de expressie van VEGF in kinderen met CHD en in controlepatiënten. In tegenstelling tot wat we bij ratten hadden gevonden, was hier sprake van verhoogde expressie – in vergelijking met controlepatiënten – van VEGF in het pulmonale vaatstelsel van kinderen die tengevolge van CHD waren overleden, vooral in de kleine drukregulerende longslagaders. Ofschoon de bevindingen uit deze twee hoofdstukken doen vermoeden dat VEGF een grote rol speelt in de angiogenese of de vasculaire hermodellering in geval bij CHD, dient nader onderzoek te worden verricht om de verschillen te kunnen verklaren.

Hoofdstuk 4 beschrijft een aspect van de longvaattonus bij CHD. Eerder beschreven afwijkende expressie van stikstofoxide synthase in de longen van proefdieren, en onvoorspelbare reacties op geïnhaleerde stikstofoxide bij kinderen met CHD vormden een aanleiding om de rol van Endothelin-1 (ET-1) en Endothelin-receptoren te bestuderen in ratten met CHD. Significant hogere concentraties ET-1 mRNA werden waargenomen in CHD ratten in vergelijking met controleratten. De concentraties ET-B mRNA waren gelijk, maar in CDH ratten waren de ET-A mRNA concentraties twee tot vier maal zo hoog als bij controleratten. De bevindingen duiden op een grote rol van dit peptide in de vasoconstrictie en in de vaatspierontwikkeling in CDH-longen. Voor de behandeling van PPHN kan, gezien de onvoorspelbare en variabele reacties op geïnhaleerd stikstofoxide bij patiënten met CHD, een endothelineblokkade worden overwogen.

Hoofdstuk 5 beschrijft de uitwerking van *partial liquid ventilation* (PLV) op longmechanismen en cellulaire en moleculaire adaptatie in ratten met en zonder CHD. Na 4 uur PLV was in beide het totale longvolume toegenomen, en was de openingsdruk in ratten met CDH verbeterd ten opzichte van ratten die conventioneel waren beademd. Bepaalde eiwitten die een rol spelen bij de afweerreactie, heat shock proteins genaamd, werden waargenomen in de longen van CHD- en controleratten a terme. Bij ratten die conventioneel beademd waren, werden significant hogere waarden van een van de beide stressmarkers waargenomen. De bevindingen van dit onderzoek geven aan dat de endogene defensiemechanismen in hypoplastische CHD-longen niet een vertraging in de ontwikkeling hebben opgelopen, en dat PLV minder belastend is dan conventionele beademing.

In hoofdstuk 6 wordt een onderzoek beschreven naar de vasculaire stress reacties in de zich ontwikkelende longen van pasgeboren ratten na acute of chronische blootstelling aan stikstofdioxide (NO2). De longen van ratten van drie dagen oud werden blootgesteld aan 7 per miljoen (ppm) delen NO2 vertoonden significant hogere concentraties van de markers tot 14 dagen daarna, in vergelijking tot de longen van ratten die niet aan NO2 waren blootgesteld. Histologisch werd in de longen van aan NO2 blootgestelde ratten sterke immunoreactiviteit van de markers vastgesteld. Deze bevindingen doen vermoeden dat er in de zich ontwikkelende longen een beschermend mechanisme tegen stress wordt gevormd.

Tenslotte, congenitale hernia diafragmatica blijft een grote uitdaging voor kinderchirurgen en neonatologen. Er zijn nog vele vragen over de precieze mechanismen die een rol spelen bij longhypoplasie en PPHN, over de uiteenlopende en onvoorspelbare reacties op medische en chirurgische behandelingen, en over het voorkómen van blijvende longschade. De gegevens uit dit proefschrift kunnen wellicht als richtsnoer dienen bij nieuwe strategieën voor het moduleren van de longvaattonus en het voorkómen van blijvende longschade.

Abbreviations

ABC	avidin-biotin complex method
ATCC	American type culture collection
α-SMA	alpha smooth muscle actin
CDH	congenital diaphragmatic hernia
cDNA	complementary deoxyribonucleic acid
CV	conventional ventilation
DAB	diaminobenzidine
DH/TL	diaphragmatic hernia + tracheal ligation
ECMO	extracorporeal membrane oxygenation
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
ET-1	endothelin 1
ET_A	endothelin receptor subtype A
ET_B	endothelin receptor subtype B
EvG	elastic von Gieson stain
FiO_2	fraction of inspired oxygen
GAPDH	glyceraldehyde-3-phosphate dehydregenase
HE	hematoxylin eosin stain
HSP	heat shock protein
I(L)GF	insulin (like) growth factor
iNOS	inducible nitric oxide synthase
LH	lung hypoplasia
LW/BW	lung weight/ body weight ratio
MHC	myosin heavy chain
mRNA	messenger ribonucleic acid
MTμ	medial thickness in microns
nNOS	neuronal nitric oxide synthase
NO	nitric oxide

NO_2	nitrogen dioxide
NOS	nitric oxide synthase
PAP	peroxidase-anti-peroxidase
ppm	parts per million
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PEEP	peak end expiratory pressure
PIP	peak inspiratory pressure
PH	pulmonary hypertension
PLV	partial liquid ventilation
PPH	persistent pulmonary hypertension
PPHN	persistent pulmonary hypertension of the neonate
PVR	pulmonary vascular resistance
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
SMC	smooth muscle cell
TGF-β_1	transforming growth factor-β_1
TL	tracheal ligation
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell

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Curriculum Vitae of T. Okazaki

7 November 1962:	Born at Mito, Japan.
March 1981:	Diploma, Mito First High School, Mito, Japan
March 1988:	M.D. Juntendo University School of Medicine, Tokyo, Japan.
Jun 1988-May 1990:	Surgical Resident in Juntendo University Hospital, Tokyo, Japan
Jun 1990-Jul 1995:	Pediatric Surgical Resident in Department of Pediatric Surgery Juntendo University Hospital
Aug 1995-Jul 1997:	Research Fellow to work on "the experimental rat models of CDH and CLD" in collaboration with Prof. Dr. D. Tibboel (Department of Pediatric Surgery) and Dr. H.S. Sharma (Department of Pharmacology) of Erasmus University.Medical Center Rotterdam, The Netherlands
Aug 1997-present:	Pediatric Surgical Resident in Department of Pediatric Surgery Juntendo University Hospital, Tokyo, Japan

List of Publications

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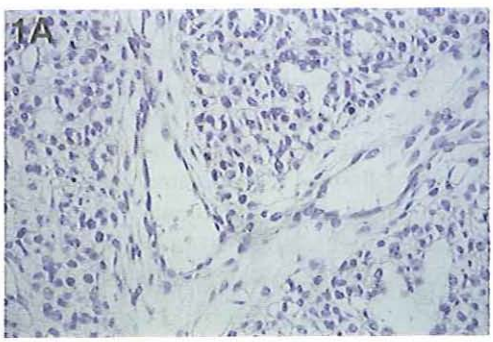
Colour Pictures

Figure 1. (A) Expression of VEGF is not seen in the vessel walls in a CDH rat at day 20 immunostained for Ab VEGF and counterstained with haematoxilin (original magnification x 400)
(B) Moderate to strong expression of VEGF (arrows) in the vessel walls in a control rat at day 20. Immunostaining as in Fig 1A (original magnification x400)

Figure 2 (A) Strong expression of SMemb (arrow) in smooth muscle cells in the vessel in a CDH rat at day 16 immunostained for Ab SMemb and counterstained with haematoxylin (original magnification x100)
(B) Expression of SMemb is not seen in the vessel walls in a CDH rat at day 22 immunostained for AB SM2 and counterstained with haematoxylin (original magnification x400). No differences in a control lung were observed.

Figure 3 (A) Strong expression of alpha-actin in the smooth muscle cells in the vessel walls and in the airways in a CDH rat at day 18 immunostained for Ab alpha-actin and counterstained with haematoxylin (original magnification x400).
(B) Strong expression of alpha-actin in the smooth muscle cells in the vessel walls and in the airways in a control rat at day 18. Immunostaining as in Fig 3A (original magnification x400) A,airway; V, vessel.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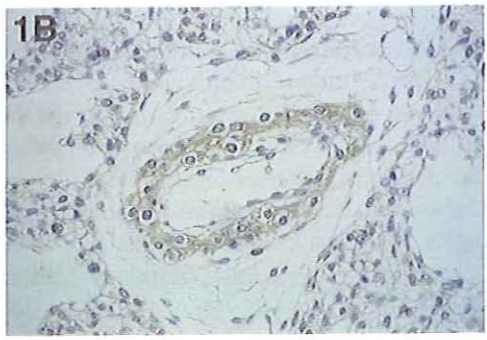
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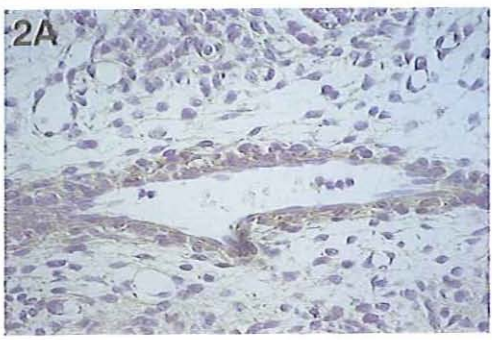
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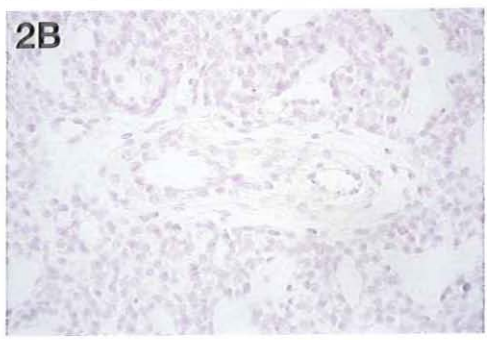
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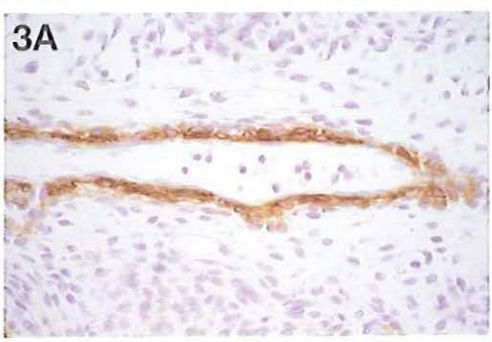
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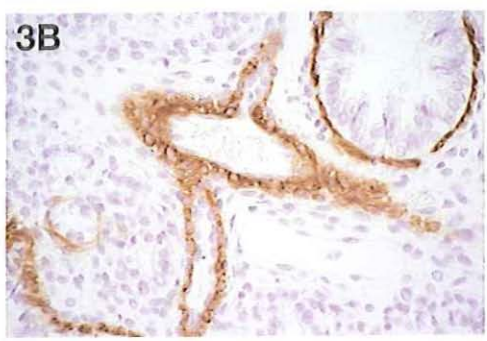
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Page 35:

Figure. 1: Immunohistochemical localization of VEGF in the human lung tissue.
Where: A; intense expression of VEGF in the medial smooth muscle cells of small diameter pulmonary arteries and in the bronchial epithelium of CDH lung tissue, B; expression of VEGF in the bronchial epithelium and faintly in the smooth muscle cells of the pulmonary arteries of control non-hypoplastic lung tissue, C; expression of VEGF in the arterial endothelium (arrow) and medial smooth muscle cells of pulmonary arteries in CDH hypoplastic lung tissue, and D; arterial endothelium identified (arrow) by CD31 staining using peroxidase technique.[Calibration bars = 50 μm in A and B and 25 μm in C and D].

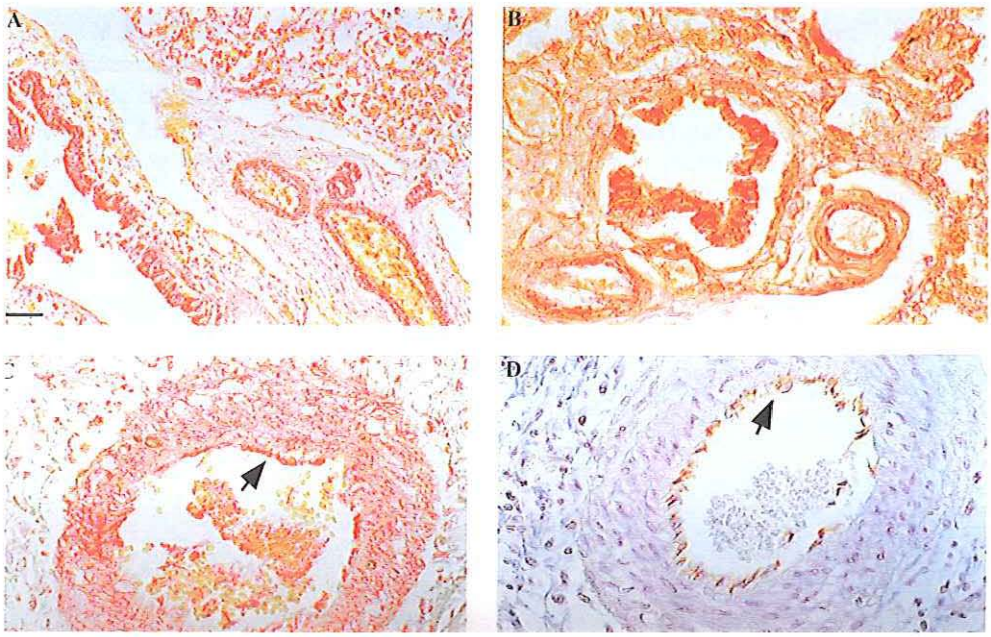
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Figure 3: Pulmonary histology in control and CDH rats after CV and PLV
For histological examination, lungs were fixed, embedded in paraffin and processed for the routine Hematoxylin-Eosin staining to assess the lung histology. Note an alveolar aeration-pattern in control rats (Panel A) and a centro-acinar aeration pattern in CDH rats (Panel B). An improved aeration pattern could be seen after PLV in both control and CDH rat lungs (Panel C and D).

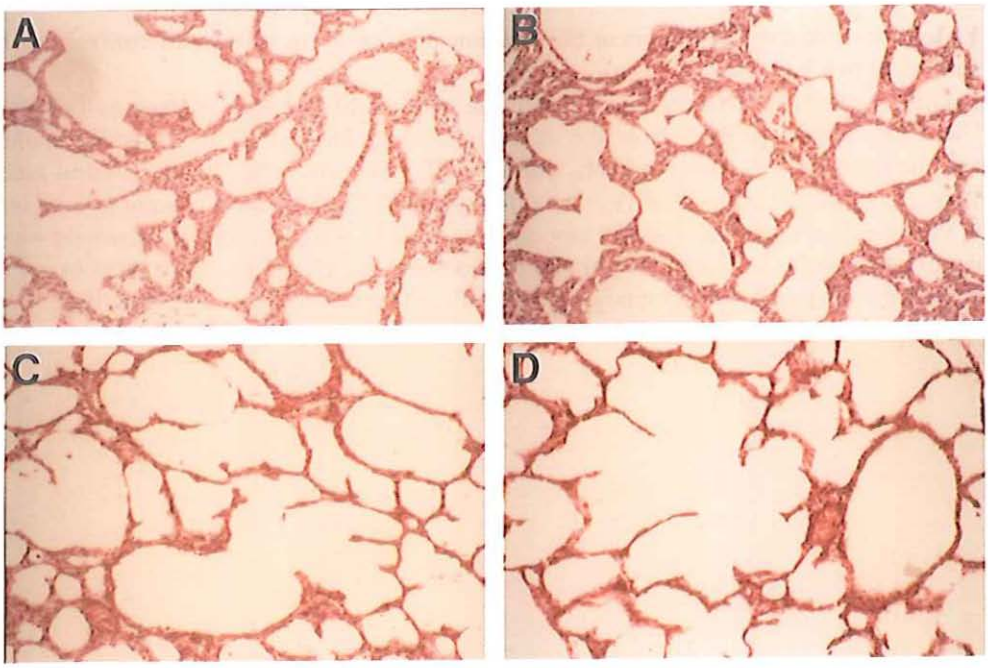
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Figure. 6: Immunohistochemical localization of HSP-27 in CDH and control rat lungs subjected to CV or PLV.

Paraffin sections of 6 μm were stained using alkaline phosphatase method as described in "Materials and Methods". Note the immunoreactivity for HSP-27 in Bronchial epithelium and medial SMC of (A) control rats with CV, (B) CDH rats with CV, (C) control rats with PLV and (D) CDH rats with PLV. Panel E depicts the localization of α-smooth muscle actin in the bronchial as well as vascular smooth muscle cells, whereas, panel F shows the negative control prepared by omitting the primary antibodies in staining protocols in order to verify the specificity and background staining for our HSPs antibodies. (Magnification X 400).

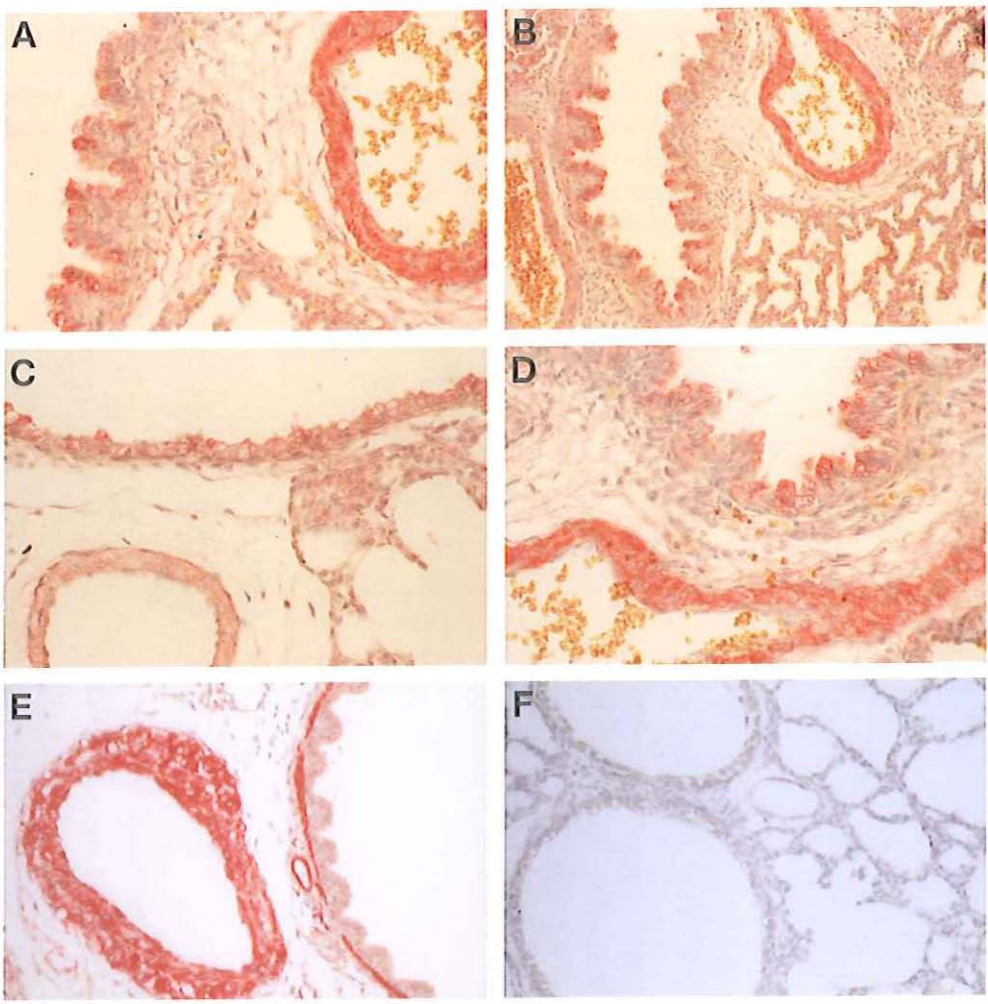
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Figure. 7: Immunohistochemical localization of HSP-70 in CDH and control rat lungs subjected to CV or PLV.

6 μm thick paraffin embedded tissue sections were stained using peroxidase method as described in "Materials and Methods". Note the Immunoreactivity for HSP-70 was seen in bronchial epithelium and medial smooth muscle cells of (A) control rats with CV, (B) CDH rats with CV, (C) control rats with kept on PLV and (D) CDH rats subjected to PLV. Microphotographs were taken at the magnification of 400 X.

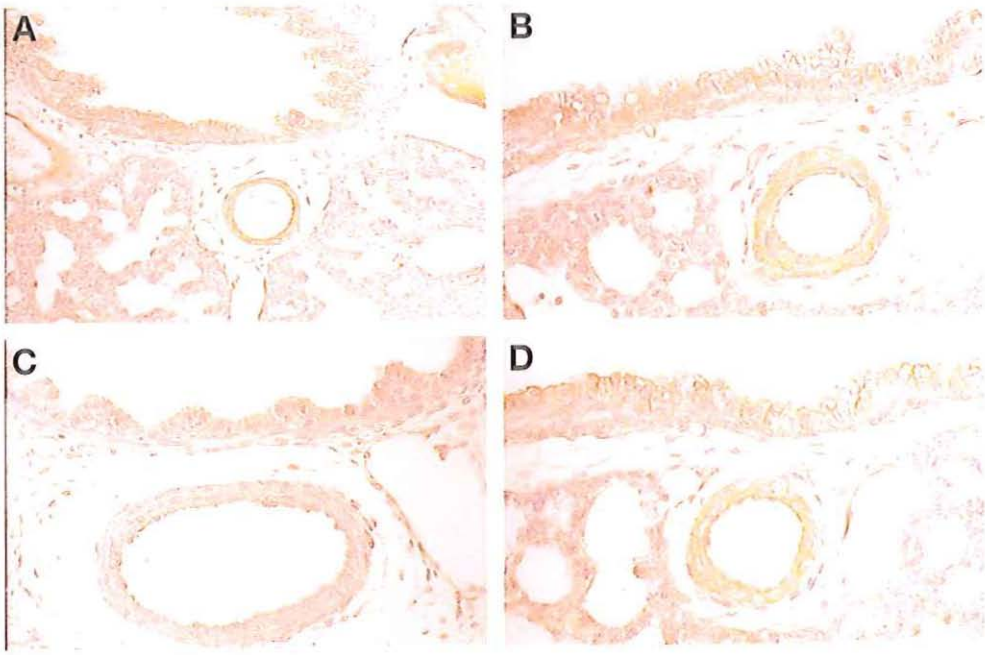
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Figure 3: Histological examination of tracheal rings obtained from control and No_2 exposed rat pups

Tracheal rings obtained from control (panel A) as well as from No_2 exposed rat pups (panel B) were fixed in formalin and embedded in paraffin. 5 μ tissue sections were stained with hemotaxylin-eosin and visualized under light microscope. Note the partial epithelial shedding in the No_2 exposed rat pups. Magnification X 200.

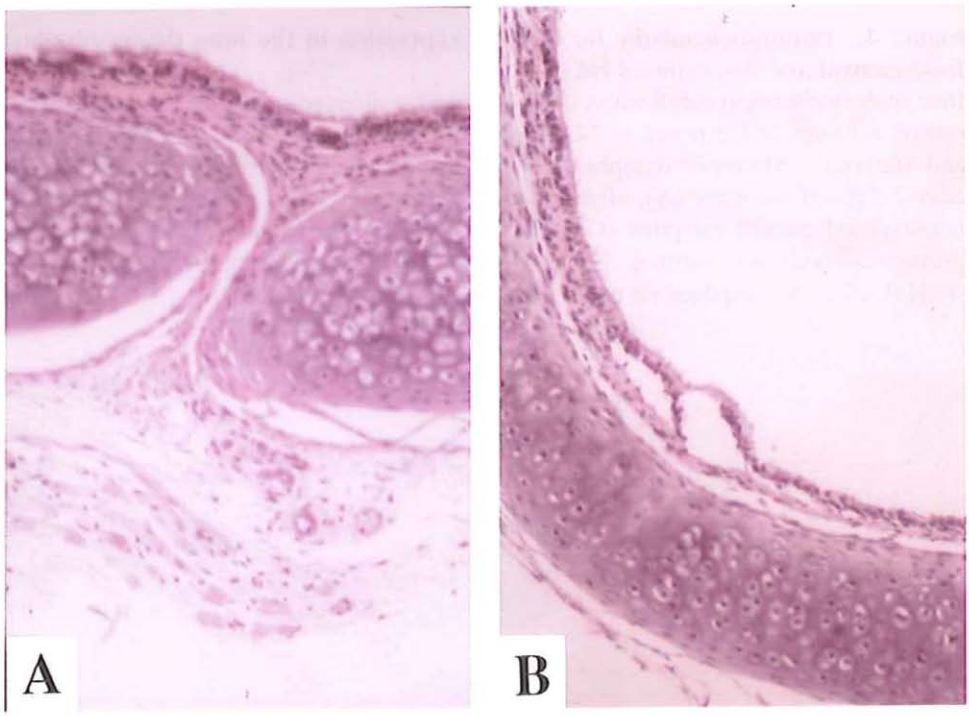
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Figure 4: Immunochemistry for HSP-27 expression in the lung tissues obtained from control and No_2 exposed rat pups

Immunohistochemical localization of HSP-27 in the developing rat lungs obtained from control rat pups and exposed to No_2 gas was performed as described in the "Materials and Methods". Microphotographs show pulmonary specimens of No_2 exposed rat pups after 7 days of recovery (A); after 14 days of recovery (B); from 7 days age matched non-exposed control rat pups (C). Panel D depicts the negative control where the primary antibody was omitted. Note the epithelial and vascular smooth muscle staining for HSP-27 in No_2 exposed rat pups. Magnification was set at X 200.

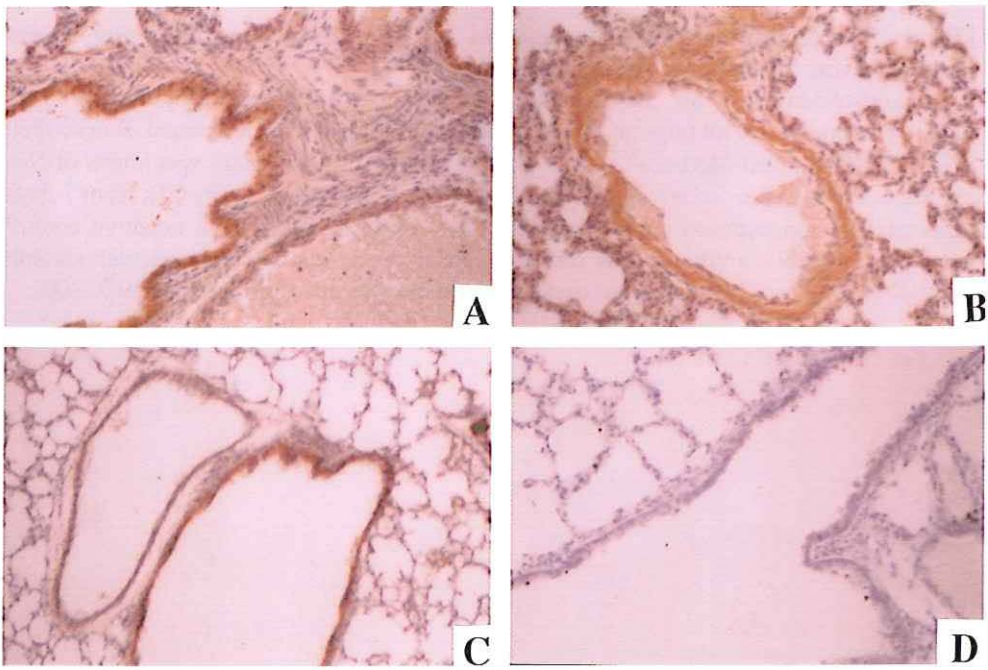
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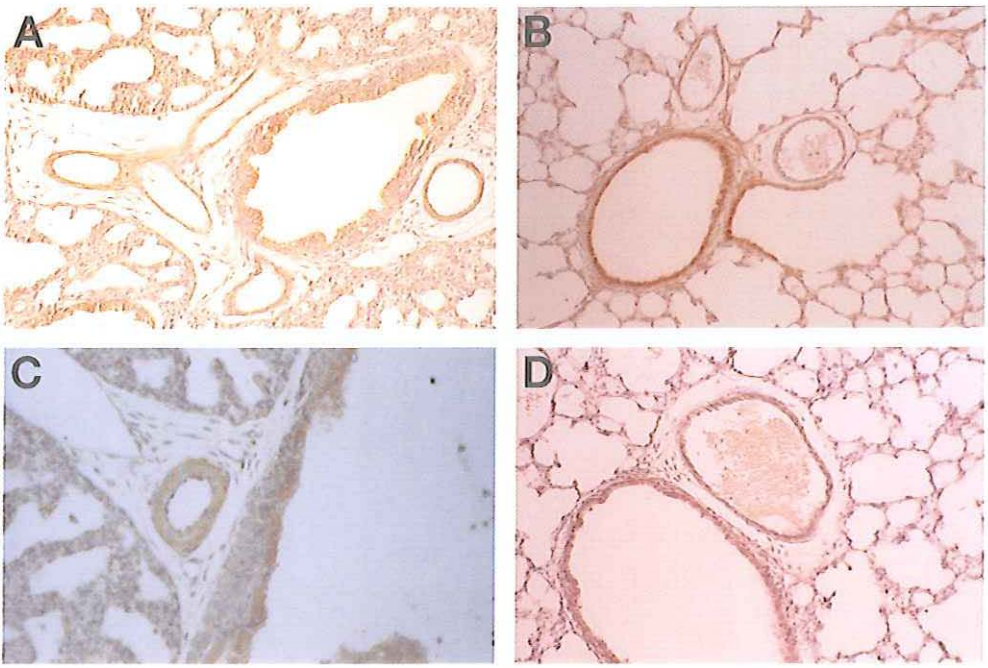
Figure 5: Immunochemical localization of HSP-70 in the developing lungs obtained from control and No_2 exposed rat pups

Immunohistochemistry for the expression of HSP-70 in the developing rat lungs obtained from control rat pups and those exposed to No_2 gas was performed as described in the "Materials and Methods". Microphotographs show pulmonary specimens of No_2 exposed rat pups after 7 days of recovery (A); after 14 days of recovery (B); from 7 days age matched non-exposed control rat pups (C). Panel D depicts the negative control where the primary antibody was omitted. Note the epithelial and vascular smooth muscle staining for HSP-27 in No_2 exposed rat pups. Magnification was set at X 200.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