

Effect of dexamethasone on endothelial nitric oxide synthase in experimental congenital diaphragmatic hernia

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Abstract

Aims—To study the effect of prenatal glucocorticoid treatment on endothelial nitric oxide synthase (eNOS) expression in rats with congenital diaphragmatic hernia (CDH).

Methods—CDH was induced in fetal rats by the maternal administration of nitrofen on day 9.5 of gestation. Dexamethasone was administered on days 18.5 and 19.5 before delivery of the fetuses on days 20.5 and 21.5. Pulmonary eNOS protein expression was studied by western immunoblotting and immunohistochemistry.

Results—On day 20.5, eNOS expression was significantly reduced in CDH pups compared with normal control rats. Dexamethasone treated CDH pups had eNOS concentrations equivalent to those of normal animals. By day 21.5, however, there was no detectable difference in eNOS expression between the experimental groups.

Conclusions—eNOS is deficient in near term (day 20.5) CDH rats. Dexamethasone restores eNOS expression in these animals to that seen in normal rat lungs. At term, the precise role of eNOS in the pathophysiology of CDH remains uncertain.

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Keywords: congenital diaphragmatic hernia; endothelial nitric oxide synthase; glucocorticoids; nitrofen

Congenital diaphragmatic hernia (CDH) continues to have an unacceptably high mortality due to the lethal combination of pulmonary hypoplasia and pulmonary hypertension.¹ Pulmonary hypertension is characterised by persistent and severe hypoxia associated with ductal shunting from the pulmonary circuit to the systemic circulation.² In CDH a combination of factors contribute to its occurrence. A reduced and excessively muscularised pulmonary vascular bed exhibits abnormal vascular reactivity, resulting in profound vasoconstriction and refractory hypoxaemia.^{2,3} Various treatments aimed at improving pulmonary hypertension have entailed ventilatory strategies such as hyperventilation and permissive hypercapnia, vasodilators, and extracorporeal membrane oxygenation (ECMO).^{4,5}

The mechanisms responsible for abnormal vascular reactivity in CDH are not completely understood. During fetal life, the pulmonary vasculature is a high pressure bed with minimal

blood flow through the lungs. However, at birth, pulmonary vascular resistance decreases and blood flow increases as the lungs take over the function of gaseous exchange from the placenta.⁶ The pulmonary vascular endothelium is of vital importance in the perinatal regulation of pulmonary vascular tone and blood flow.⁷ It releases a host of vasodilator and vasoconstrictor substances which, acting in a paracrine manner, stimulate the contraction or relaxation of adjacent vascular smooth muscle cells.⁷ Nitric oxide (NO) is believed to have a pivotal role in the transition of the pulmonary circulation from fetal to neonatal life.⁸ A potent vasodilator, it is generated in the pulmonary vascular endothelium through the action of the enzyme, endothelial nitric oxide synthase (eNOS).^{9,10} NO diffuses into adjacent smooth muscle cells where it increases cellular concentrations of cyclic guanosine monophosphate thus producing vaso-relaxation.^{9,10} As the pulmonary circulation in some neonates with CDH fails to adapt or sustain the physiological transition to extrauterine life, it has been suggested that NO may be deficient in CDH. Inhaled NO treatment has been instituted as a specific pulmonary vasodilator in CDH patients as a means of treating pulmonary hypertension related crisis with varying clinical results.¹¹⁻¹⁷

Glucocorticoids have an important role in the regulation of normal lung development and exert profound maturational effects on the developing fetal lung.¹⁸ Antenatal corticosteroid treatment is now well established as a means of preventing respiratory distress syndrome in premature newborns.¹⁹ In experimental CDH animal models, antenatal glucocorticoids improve surfactant biochemical immaturity, increase lung compliance, and enhance lung morphology.¹⁸⁻²³ The effect of this pharmacological intervention on the prevention or treatment of pulmonary hypertension in CDH remains unknown. The aim of the present study was to evaluate the effect of antenatal glucocorticoids on eNOS expression as a marker of pulmonary vascular reactivity in the lungs of rats with congenital diaphragmatic hernia induced by nitrofen.

Methods

CREATION AND TREATMENT OF CDH

Timed pregnant Sprague-Dawley rats (Charles River UK, Ltd) (vaginal plug positive = day 0) were given 100 mg nitrofen (Zhejiang Chemicals, China) by gavage on day 9.5 of gestation (term = day 22) to induce left sided CDH in fetal rats.²⁴ Control animals received olive oil.

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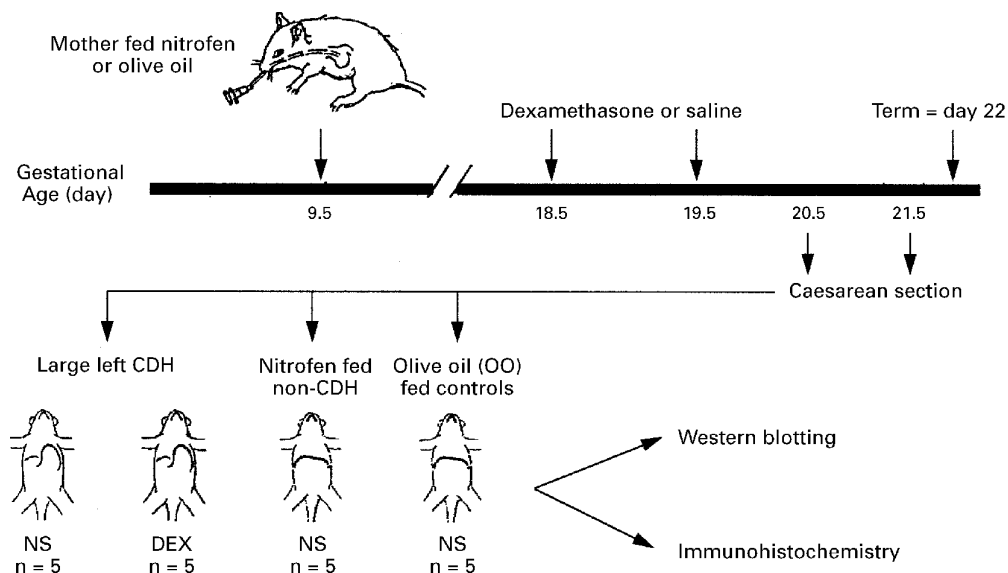


Figure 1 Experimental protocol: NS = physiological saline, Dex = dexamethasone, OO = olive oil.

Antenatal dexamethasone (David Bull Laboratories Warwick, UK) 0.25 mg/kg or an equal volume of physiological saline was administered by maternal intraperitoneal injection on days 18.5 and 19.5. Pregnant rats were terminally anaesthetised using halothane on days 20.5 and 21.5 of gestation. Fetal pups were given a lethal dose of sodium pentobarbital 100 mg/kg intraperitoneally to prevent air breathing and hypoxic stress before delivery by caesarean section. We studied the ipsilateral lungs as although the vascular abnormalities associated with CDH are known to be bilateral, they are more severe on the ipsilateral side.²⁵ Alterations in the ipsilateral lung are therefore likely to be representative of events occurring in both lungs.²⁶ Animals were divided into four experimental treatment groups (fig 1).

WESTERN IMMUNOBLOTTING

Individual lungs were stripped of all extra pulmonary tissue, flash frozen immediately in liquid nitrogen, and stored at -70°C until they were studied. The lungs were homogenised in 10 mM TRIS-HCl (pH 7.4) and 100 mM NaCl and centrifuged at $5000 \times g$ for 3 minutes at 4°C . Protein assays were performed on the supernatant fluid by the method of Lowry using bovine serum albumin to obtain standard curves.²⁷ Lung samples were then boiled in lysis buffer containing 42 mM TRIS (pH 6.8), 48 mM sodium dodecyl sulphate, 7% glycerol, and 5% β -mercapto ethanol for 3 minutes. Lung protein (100 μg) was loaded into

individual wells on a 7.5% polyacrylamide gel, separated by sodium dodecyl-polyacrylamide gel electrophoresis and then electrophoretically transferred on to a polyvinylidene difluoride membrane (ICN Pharm Ltd, Oxfordshire, UK).²⁸ The membrane was then incubated with a solution of 5% non-fat milk in 10 mM TRIS pH7.4, 100 mM NaCl, and 0.1% Tween 20 (blocking buffer) for 1 hour to prevent non-specific binding of antibody, followed by an overnight incubation with a specific murine monoclonal antibody for eNOS (Transduction Laboratories, Kentucky, USA). After 6 five minute washes a 1 hour incubation with a secondary peroxidase labelled rabbit anti-mouse antibody (Sigma Aldrich Co. Dorset, UK) was performed. eNOS protein expression was visualised using a 1 minute incubation with chemiluminescent reagents (Amersham UK, Ltd.) and exposure to photo sensitive film (Amersham UK Ltd.). Western blots were quantified by laser densitometry using Image Quant software on a Personal Densitometer (Molecular Dynamics UK). eNOS expression for each specimen was reported as mean (SEM) percentage (compared with the eNOS expression in olive oil and physiological saline treated control lungs).

IMMUNOHISTOCHEMISTRY

Cryostat lung sections (8 μm thick) were cut and the sections post-fixed in 1% formaldehyde for 1 minute, and acetone for 5 minutes. Sections were incubated with 2% bovine serum

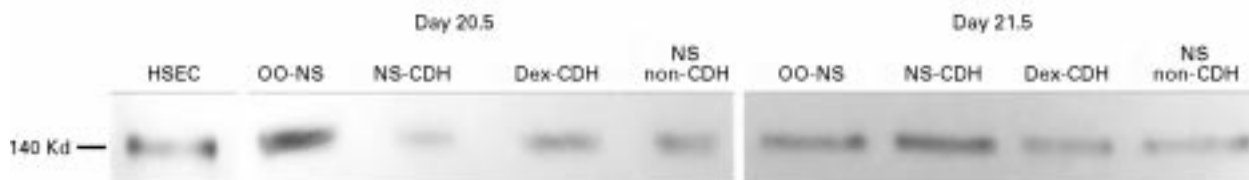


Figure 2 Western blots representative of five independent experiments showing pulmonary eNOS expression on days 20.5 and 21.5 of gestation: HSEC human suspended endothelial cells (positive control for eNOS).

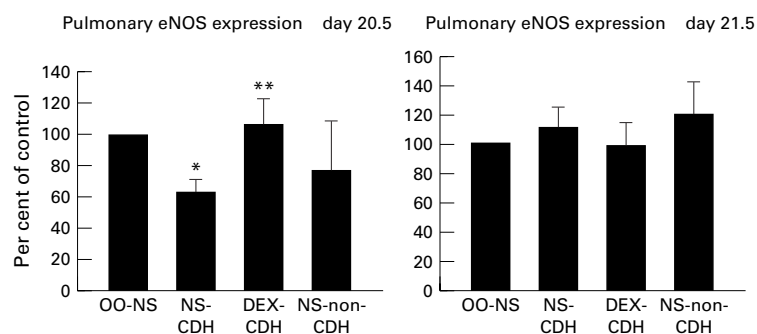


Figure 3 Results of five independent western blot experiments showing pulmonary eNOS expression on days 20.5 and 21.5 of gestation. Results are expressed as mean percentage (SEM) compared with eNOS expression in normal controls given olive oil and physiological saline. On day 20.5 of gestation, CDH rats given physiological saline showed reduced eNOS expression compared with normal controls (OO-NS) (* $p=0.03$). CDH rats treated with dexamethasone had significantly higher eNOS concentrations than CDH fetuses given physiological saline (** $p=0.02$) and equivalent to that seen in the lungs of normal controls ($p=0.8$).

albumin in TRIS buffer (pH 7.6) to reduce non-specific binding of antibody and then with a specific murine monoclonal anti-eNOS antibody (Transduction Laboratories, Kentucky, USA) for 1 hour followed by two washes in TRIS buffer (pH 7.6). This was followed by incubation with a secondary monoclonal biotin labelled rabbit anti mouse "link" immunoglobulin (Sigma Aldrich Co. Dorset, UK) and a streptavidin/horseradish peroxidase complex (DAKO Ltd. Bucks, UK). Staining for eNOS was visualised using a cobalt enhanced 3,3' diaminobenzidine chromogen (Sigma Aldrich Co. Dorset, UK). To confirm antibody specificity, control sections were subjected to all the above steps with omission of the primary anti eNOS antibody.

The unpaired *t* test was used to determine statistical difference between the groups. A *p* value of < 0.05 was considered significant.

Results

WESTERN IMMUNOBLOTTING

The results of five independent experiments and a representative western blot are shown in figs 2 and 3 for day 20.5. CDH lungs showed a significant reduction in eNOS protein expression compared with controls ($p=0.03$). In contrast, the lungs of CDH rats treated with dexamethasone showed eNOS expression equivalent to that seen in the lungs of normal animals ($p=0.02$ vs CDH treated with physiological saline). Nitrofen physiological saline-non CDH pups had intermediate eNOS expression which were lower than but not statistically different from olive oil physiological saline normal controls ($p=0.07$).

The results of five independent experiments and a representative western blot are shown in figs 2 and 3 for day 21.5. There were no differences in eNOS protein expression between any of the experimental groups studied.

IMMUNOHISTOCHEMISTRY

eNOS immunoreactivity was localised to the endothelium of pulmonary arteries of varying size in all the lungs studied (fig 4). Large hilar vessels showed the greatest intensity of staining compared with the smaller peripheral arterioles. There were no obvious differences in the

intensity of staining between the groups. eNOS immunoreactivity was also detected in the bronchial epithelium, but no differences were observed between the experimental groups. Detailed morphometric analysis was not performed.

Discussion

Pulmonary hypertension in combination with pulmonary hypoplasia continues to present a major obstacle to the successful management of CDH. Despite advances in neonatal intensive care, the current mortality associated with this condition remains as high as 50%.^{5 29 30} An improved understanding of the natural history and pathophysiology of CDH together with the availability of antenatal diagnosis has led to fetal intervention in an effort to rescue lung growth and maturation. Recently, antenatal glucocorticoid treatment has been shown to improve biochemical immaturity, increase lung compliance, and enhance lung morphology in experimental CDH animals.²⁰⁻²³ The effect of this treatment on the pulmonary vascular bed in CDH has not been studied.

In this study, we used the nitrofen induced CDH rat model which reproduces many of the pathologic hallmarks of CDH seen in people. The diaphragmatic defect is induced early in gestation during the embryonal phase of lung development producing lungs which are markedly hypoplastic, surfactant deficient, poorly compliant and display hypermuscularisation of the pulmonary vasculature.^{20-24 31 32}

Nitric oxide is believed to have a pivotal role during the transition of the fetal pulmonary circulation to extrauterine life.⁸ Experimentally, inhibiting NO production before birth can prevent the normal postnatal increase in pulmonary blood flow.^{8 33} Clinically, inhaled NO has been administered as a specific pulmonary vasodilator to CDH patients with varying clinical response.¹¹⁻¹⁷ An eNOS deficiency in CDH has been investigated by previous authors.^{20 26 34} eNOS activity is normal in CDH lambs, suggesting that a deficiency is not implicated in this late gestational animal model.³⁴ In contrast, studies in CDH rats have yielded conflicting results.^{20 26 35} In our study, we showed that eNOS was deficient in CDH rats at 20.5 days of gestation. This agrees with the findings of a previous study.²⁶

We noted that dexamethasone treated CDH rats had eNOS concentrations restored to those seen in the lungs of normal olive oil controls. This suggests that antenatal steroids may enhance endothelium dependent vasorelaxation in fetal CDH rat lungs before term. However, at term (day 21.5), we were unable to show an absolute deficiency of eNOS, and dexamethasone had no additive effects on its expression. Supporting these findings, immunohistochemistry revealed no qualitative differences in eNOS immunoreactivity in the large and small pulmonary arteries between experimental groups. These findings therefore agree with those of another study which showed normal eNOS mRNA expression at 21.5 days in CDH rats.²⁰ A fetal eNOS deficiency near term (day 20.5) may reflect an inadequate priming

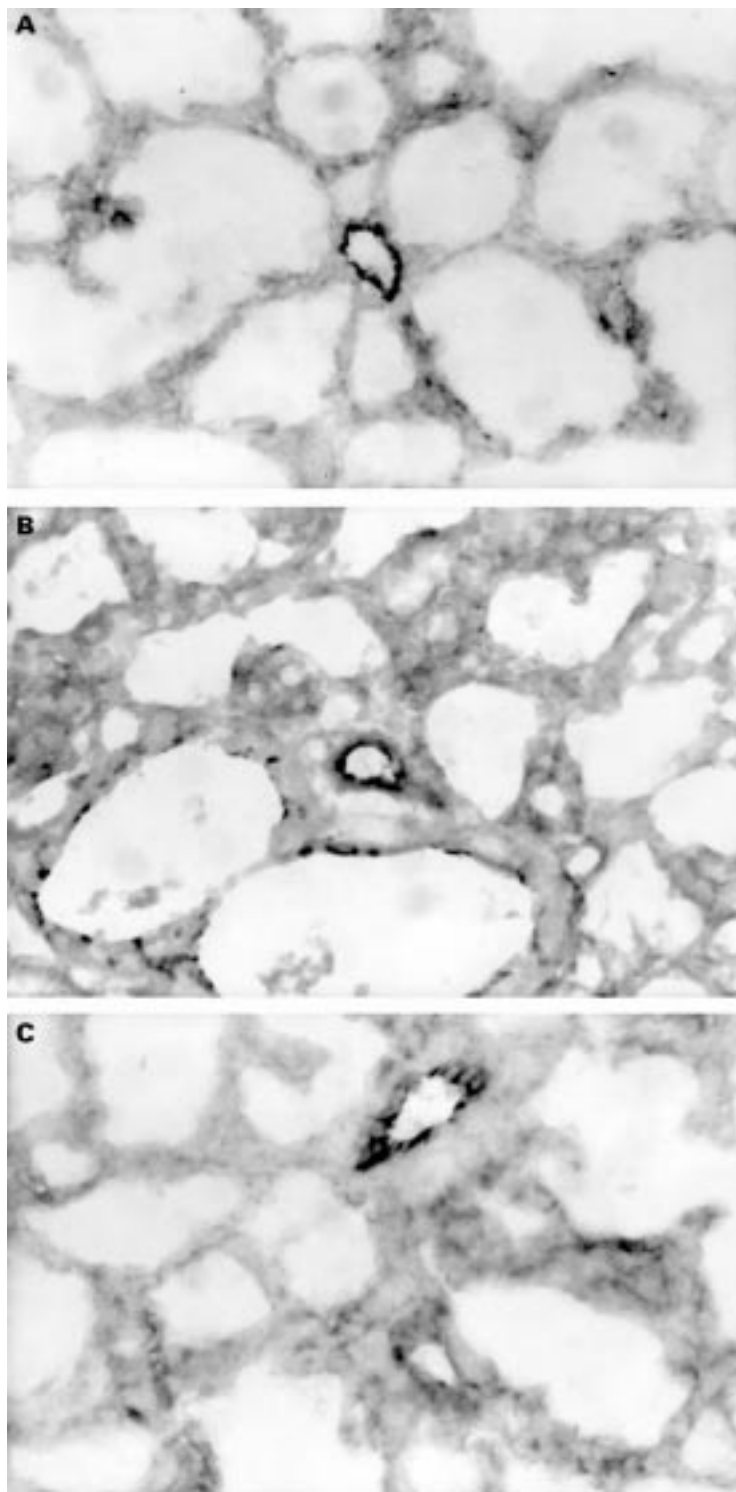


Figure 4 Photomicrographs showing eNOS immunoreactivity (black stain) in the endothelium of small pulmonary arteries in the experimental groups studied. eNOS immunoreactivity is also observed in the bronchial epithelium (B): small peripheral arteries of OO-NS (A); NS-CDH (B); and dexamethasone-CDH (C).

of the fetal pulmonary vascular bed for extrauterine life, as NO is known to have an important role in the regulation of fetal pulmonary vasoreactivity.⁷ By restoring eNOS expression at this time point, steroid treatment may improve the haemodynamic effects of fetal eNOS deficiency. However, at term the precise role of eNOS deficiency in CDH rats remains uncertain and this partly may explain the

varied clinical response to exogenous NO treatment in people with CDH.¹¹⁻¹⁷ An imbalance or deficiency in eNOS may perhaps exist postnatally due to overwhelming vasoconstrictor influences and parenchymal CDH abnormalities. Antenatal glucocorticoids may address this imbalance by modulating vasoregulatory pathways and enhancing lung maturation.^{20-23 31 36}

Notably, we also showed eNOS immunoreactivity in the bronchial epithelium. This supports the findings of an earlier study that noted immunolocalisation within the ciliated bronchial epithelium of newborn rats.³⁷ Thus NO may have an important role in modulating bronchial smooth muscle tone as well as vascular reactivity during the transition to neonatal life.

Glucocorticoids have profound maturational effects on the developing fetal lung. These effects include enhanced alveolar differentiation, thinning of alveolar septae and capillary walls, and upregulation of surfactant production.^{18 38} The mechanisms through which steroids produce these effects remain incompletely understood. They may occur as a result of the direct activation of glucocorticoid receptors or through the downstream paracrine or autocrine effects of growth factors.³⁹⁻⁴²

The precise regulation of eNOS expression and activity has yet to be determined. eNOS gene expression can be upregulated by shear stress and hypoxia.⁴³ Oestrogen may also upregulate eNOS expression through the activation of oestrogen receptors.⁴⁴ Basic fibroblast growth factor and transforming growth factor β 1 can increase eNOS mRNA and protein expression.^{45 46} Both growth factors can be susceptible to glucocorticoid modulation.^{47 48} It is conceivable, therefore, that corticosteroids influence eNOS expression in the developing fetal lung which involves complex epithelial mesenchymal interactions.⁴⁹ This process may be more complicated in pathological conditions such as CDH related lung hypoplasia where the mechanisms causing abnormal pulmonary growth and differentiation are incompletely understood.

In summary, antenatal glucocorticoid treatment can enhance the expression of eNOS in the lungs of fetal CDH rats near term. This could have implications for the therapeutic manipulation of fetal pulmonary vascular reactivity and may reduce the risk of pulmonary hypertension developing in the lungs of neonates with antenatally diagnosed CDH. Further studies are required to understand the regulatory mechanisms governing eNOS expression and NO activity in congenital diaphragmatic hernia.

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