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Effect of nitric oxide on the development of nitrofen-induced fetal hypoplastic lung explants

Masato Shinkai, Toko Shinkai, Martina A. Pirker, Sandra Montedonico, Prem Puri*

Children's Research Centre, Our Lady's Hospital for Sick Children and University College, Dublin 12, Ireland

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Abstract

Background/Purpose: Nitric oxide (NO) is an important cell-signaling molecule, and its generators, nitric oxide synthases, are expressed temporospatially in fetal rat lung. Recently, NO has been reported to modulate branching of the fetal rat lung lobe in vitro. We designed this study to evaluate the effect of NO on the morphogenesis of hypoplastic lung using nitrofen-induced rat lung explant model.

Methods: A hypoplastic fetal lung model and a normal control lung model were induced by feeding a pregnant rat with nitrofen (100 mg) or olive oil on day 9.5 of gestation, respectively. Fetal lungs were harvested on day 13.5 and placed in organ culture containing serum-free medium Dulbecco modified Eagle medium. An NO donor, DETA NONOate (DETA/NO), was added daily in the culture medium. The lung cultures were divided into 4 groups: group 1 (n = 8), normal controls without DETA/NO; group 2 (n = 22), normal controls with DETA/NO; group 3 (n = 13), hypoplastic lungs without DETA/NO; group 4 (n = 22), hypoplastic lungs with DETA/NO. The fetal lungs were incubated for 48 hours at 37°C with 5% CO_2. Lung bud count and area of the specimens were measured under computer-assisted digital tracings. The rate of increase in bud count and lung area was calculated as the ratio of each value at 48 hours minus each value at 0 hour, divided by the value at 0 hour.

Results: The lung bud count was significantly increased in group 2 compared with group 1 at a concentration of 50 μmol/L DETA/NO ($P < .05$). In the nitrofen group, the lung bud count was significantly increased in group 4 compared with group 3 at 100 μmol/L DETA/NO added ($P < .05$). There was no significant difference in the rate of increase in whole lung area among the 4 groups. The peak increase rates of lung area and bud count were significantly lower in group 4 compared with group 2.

Conclusions: This study demonstrates that the NO donor, DETA/NO, promotes branching of the nitrofen-induced hypoplastic fetal lung explant. These data suggest that NO may modulate the development of the nitrofen-induced hypoplastic lung.

Congenital diaphragmatic hernia (CDH) is a congenital malformation with an incidence of 1 in 2500 births. Despite significant advances in neonatal resuscitation and intensive care, newborn infants with congenital diaphragmatic hernia continue to have high mortality and morbidity. This has been attributed to pulmonary hypoplasia and persistent pulmonary hypertension. Pulmonary hypoplasia, characterized by immaturity and small size, produces respiratory failure that remains the principal contributor to high mortality and

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* Corresponding author. Tel.: +353 1 4096420; fax: +353 1 4550201.
E-mail address: prem.puri@ucd.ie (P. Puri).

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morbidity in these patients. Control and regulation of fetal lung growth are complex and incompletely understood.

Recently, there is increasing evidence that oxygen-sensing pathways play an important role in the morphogenesis of respiratory structures. Nitric oxide (NO) has drawn a lot of attention in this context. Nitric oxide is a colorless gas that can diffuse readily between and within cells as one of free radical molecules and exhibits various important roles in the homeostatic regulation of the immune, cardiovascular, and nervous systems [1]. Many of its physiological effects are exerted by increase in cyclic guanosine monophosphate levels as a result of NO molecule binding to Fe^{2+} heme groups in the enzyme soluble guanylyl cyclase.

Nitric oxide is produced by several nitric oxide synthases (NOS) through sequential oxidation of a terminal nitrogen of L-arginine. At least 3 NOS isoforms are recognized. NOS I (nNOS) was initially isolated in association with neuronal and epithelial tissues. NOS II (iNOS) was first identified in macrophages and liver cells treated with endotoxin or cytokines. NOS III (eNOS) was originally found as an endothelium-derived relaxing factor that relaxes blood vessels. NOS I and NOS III are constitutively expressed and calcium responsive and usually produce nanomolar concentrations of NO. NOS II is inducible, calcium independent, and capable of producing high concentrations of NO.

Expression of NOS isoforms has been reported in the developing lung, thus, suggesting that NO may regulate some events during lung development. In the fetal rat, NOS I expression is localized to the bronchiolar epithelium, detectable between 13 and 16 days of gestation, and increased to maximal levels at 20 days of gestation. NOS III expression in the endothelium is found between 14 and 16 days of gestation, remarkably increased during vasculogenesis and angiogenesis [2,3]. Protein S-nitrosylation, which provides a significant route for conveying NO-derived bioactivity, is developmentally expressed in human lungs [4]. Elevated NO production in the late gestation may play a role in decreasing lung liquid production and inhibitory regulation of bronchomotor and vasomotor tone during the late fetal and early postnatal life [5,6]. However, little is known about the role of NO expression in the early gestation. Recently, Young et al [7] used compounds that release NO, an NO donor, in culture medium and showed that an altered concentration of exogenous NO affects branching morphogenesis of the lung explants, thus, suggesting an important role of NO in the lung development. We designed this study to evaluate the effect of NO on the morphogenesis of hypoplastic lung using nitrofen-induced rat lung explant model.

1. Materials and methods

1.1. Normal and hypoplastic lung model

A hypoplastic fetal lung model was induced by gavage-feeding timed-pregnant Sprague-Dawley rats with 100 mg nitrofen (Wako Chemicals, Osaka, Japan) dissolved in 1 mL olive oil on day 9.5 of gestation under brief anesthetics as described earlier [8]. The day that the vaginal plug was positive represented day 0.5 of gestation. A normal fetal lung model was also attained using the same procedures but feeding without nitrofen.

1.2. Culture system

On day 13.5 of gestation, the dams were deeply anesthetized with isoflurane inhalation, and the fetuses were removed from the uterus. Whole fetal lungs were dissected free in Hanks buffered saline under a dissecting microscope. The lungs were placed on a polycarbonate culture dish insert filter (3-μm pore size, 12-mm diameter, Transwell, Costar, UK) and cultured at the air-culture medium interface in serum-free Dulbecco modified Eagle medium (Gibco BRL-Life Technologies, UK) containing 100 U/mL penicillin and 100 μg/mL streptomycin. The lungs were placed in humidified incubators at 37°C with an atmosphere of air plus 5% CO_2 and cultured for at least 48 hours without changing the medium.

1.3. Morphometry

The explant lungs were photographed daily on an inverted microscope, and the digitized lung images were analyzed for lung bud count and area of the specimens using Image J 1.29 software (National Institutes of Health, Bethesda, MD). The increase in bud count and lung area was calculated as the ratio of each value at 48 hour minus each value at 0 hour, divided by the value at 0 hour.

1.4. NO donor

Following the method by Young et al [7], we adopted DETA NONOate (DETA/NO, Alexis Biochemicals, Nottingham, UK) as an NO donor, and it was added daily to the medium to reach final concentrations described below. The lung cultures were divided into 4 groups: group 1 (n = 8), normal controls without DETA/NO; group 2 (n = 22), normal controls with DETA/NO; group 3 (n = 13), hypoplastic lungs without DETA/NO; group 4 (n = 22), hypoplastic lungs with DETA/NO. In groups 2 and 4, DETA/NO was added in the medium at the final concentration of 50, 100, and 200 μmol/L, respectively (n = 4, 8, and 10 in group 2, and n = 9, 8, and 5 in group 4).

1.5. Statistical analysis

Analysis was carried out using the StatView 5.0 program (SAS Institute, Inc, North Carolina). Data were expressed as means ± SE, and statistical comparisons were performed by 2-way analysis of variance and post hoc test (Bonferroni-Dunn test).

All protocols were carried out in compliance with current European Union regulations for animal investigations

(B100/3439) and with prior ethical approval under license from the Department of Health, Ireland.

2. Results

2.1. Normal lung

The bud count was significantly increased in group 2 at 50 μmol/L DETA/NO compared with group 1 (2.7 $\pm$ 0.3, 1.7 $\pm$ 0.1, P = .012). Higher doses of the NO donor did not improve the branching rate of the explant (Fig. 1). No significant difference in the rate of increase in the area of the lungs was found between groups 2 and 1 (P = .15) (Fig. 2).

2.2. Nitrofen-induced hypoplastic lung

The bud count was significantly elevated in group 4 at 100 μmol/L DETA/NO compared with group 3 (2.2 $\pm$ 0.3, 1.6 $\pm$ 0.1, P = .03) (Fig. 1). There was no significant difference in the rate of increase in the area of the lungs in group 4 compared with group 3 at any concentration of DETA/NO (Fig. 2). High doses (200 μmol/L) of DETA/NO suppressed the increase in lung area compared with lower doses and possibly caused deterioration of the growth of the explants. Moreover, the peak increase rates of whole lung area and bud count were both significantly lower in group 4 compared with group 2. Representative photographs of the hypoplastic lungs, which accelerated branching with the NO donor, are shown in Fig. 3.

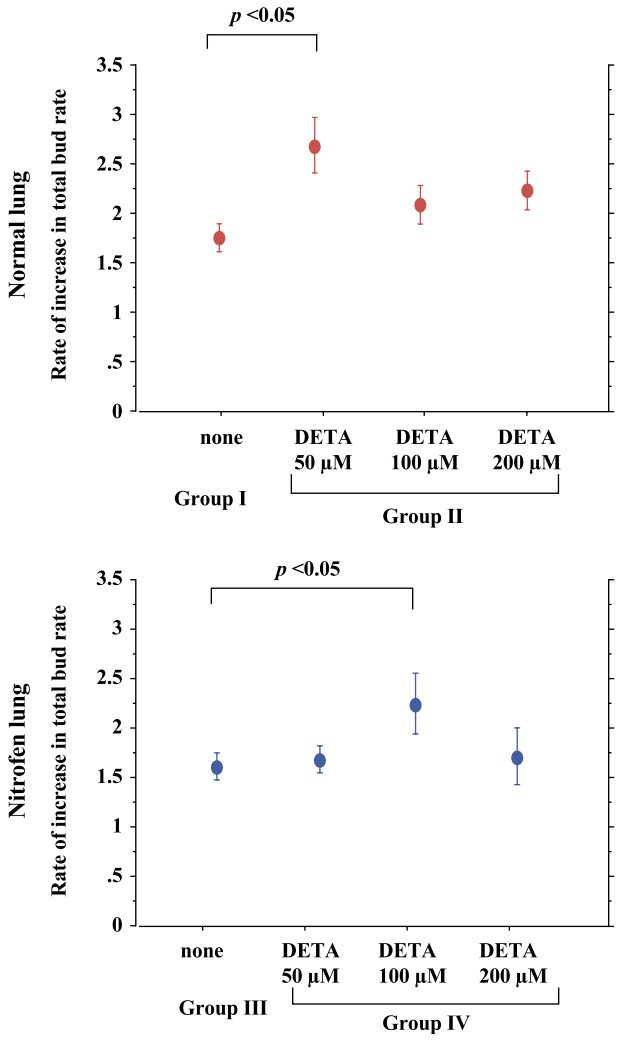

Fig. 1 Rate of increase in the bud count in the normal lung and nitrofen-induced hypoplastic lung with or without DETA/NO (mean $\pm$ SE).

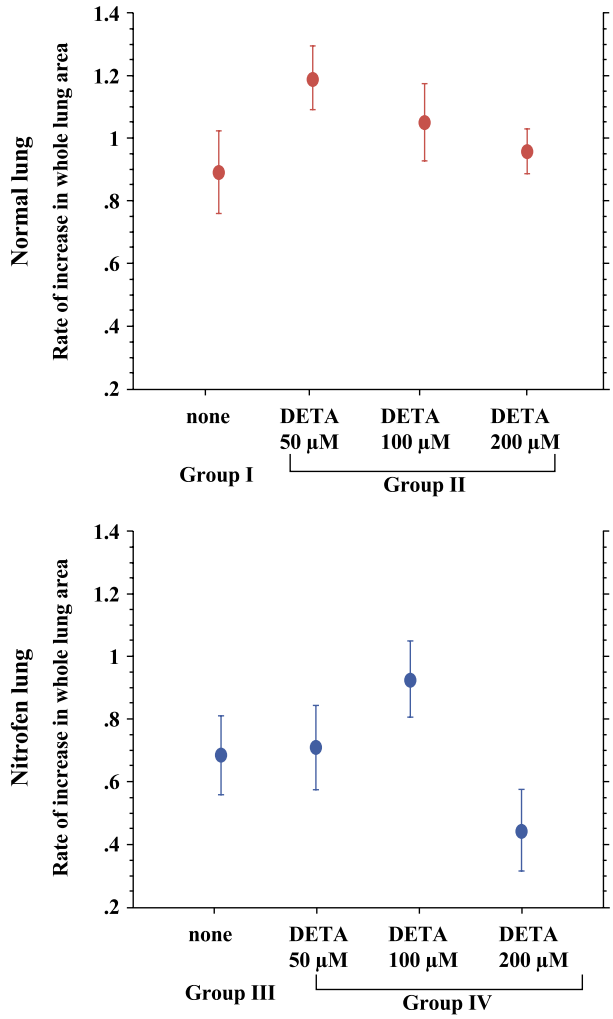

Fig. 2 Rate of increase in the whole lung area in the normal lung and nitrofen-induced hypoplastic lung with or without DETA/NO (mean $\pm$ SE).

3. Discussion

The intracellular oxidation-reduction (redox) state is delicately maintained by the balance between oxidant production including various reactive oxygen species (ROS) and antioxidant capacity of the cell. Reactive oxygen species have been regarded as toxic by-products of metabolism with the potential to cause damage to lipids, proteins, and DNA. However, recent studies revealed that lower amount of

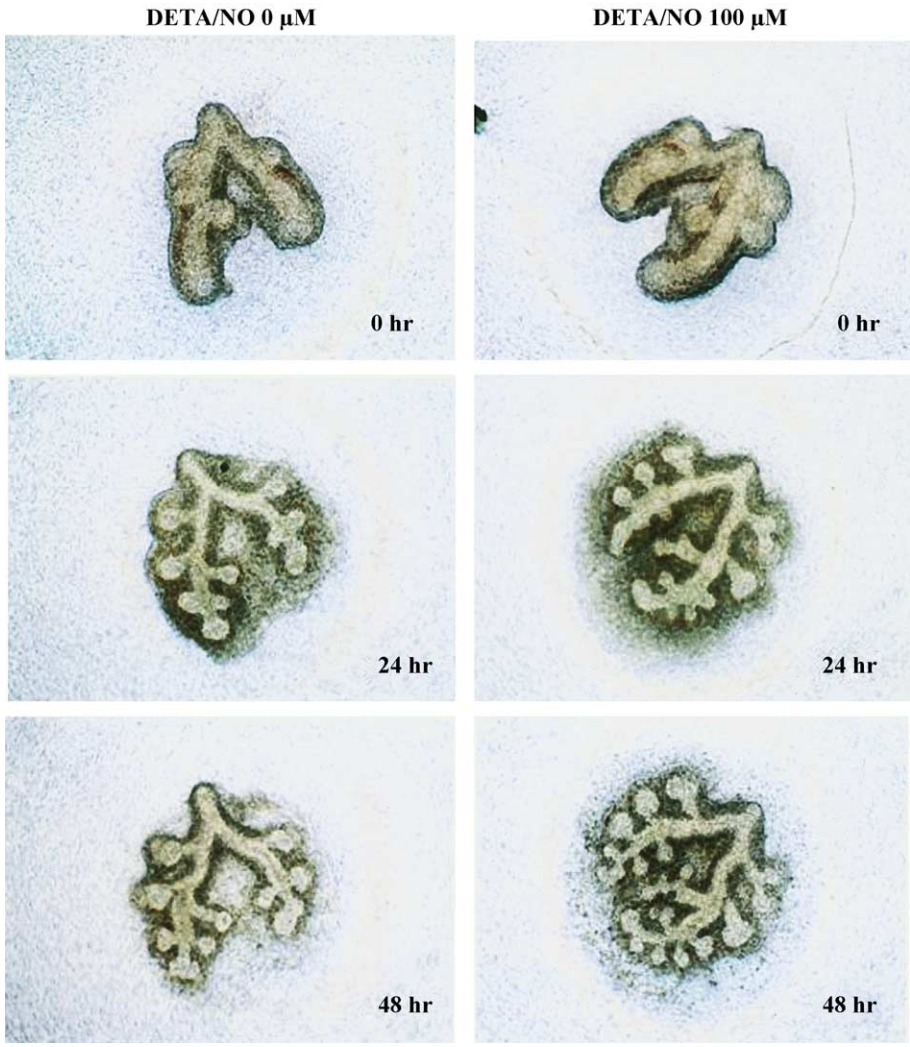

Fig. 3 Representative photographs of the nitrofen-induced hypoplastic lungs cultured with or without DETA/NO.

ROS plays an essential role in cell signaling and regulation, and many growth factors and cytokines exhibit normal physiological bioactivities by inducing ROS generation [9]. This is analogous to the effects of NO, which exhibits both regulatory functions and cytotoxic effects depending on the NOS isoform and the amount of its production.

In mammals, embryonal and fetal development occurs in an extremely low-oxygen tension environment, and a lot of data suggest that the ability to detect and respond to changes in oxygen availability is an integral part of fetal development [10]. Nitric oxide is reported to play an important role in this mechanism. Hypoxia promotes important transcription factors such as hypoxia-inducible factor, hepatocyte nuclear factor family, and SP-1 family and is also known to increase intracellular production of NO, which is suppressed by transforming growth factor β, a promoter of mesenchymal proliferation and inhibitor of airway branching [10,11]. Moreover, hypoxia-induced increase in mitochondrial NO regulates mitochondrial respiration as well as its proliferation.

Although NOS III knockout mice developed abnormally increased pulmonary vasoconstriction in response to hypoxia, no lung abnormality has been noted in NOS I and NOS II knockout mice [12]. However, ubiquitous and integrated distribution of NOS isoforms in the respiratory tract might substitute the knocked-out isoforms, and the role of NO cannot be underestimated. A recent report [7] showed that an altered concentration of exogenous NO affects branching morphogenesis of the lung explants and suggested the important role of NO in the lung development. Furthermore, Fisher et al [13] reported that the growth of lung explant was promoted by ascorbic acid and antioxidants, which are also known to promote NO release from *S*-nitrosothiol.

In our experiment, we compared the effect of various doses of the NO donor on both the normal lung and nitrofen-induced hypoplastic lung explant in the organ culture using serum-free medium. We confirmed a significant increase over control branching at 50 μmol/L DETA/NO in the normal lungs, which is consistent with the previous report of Young et al [7]. This effect of NO on

lung branching was also observed in nitrofen-induced hypoplastic lungs at 100 μmol/L DETA/NO, although the extent of branching enhancement in hypoplastic lungs was significantly lower than that of the normal lungs and higher doses of the NO donor did not exert a positive effect. These findings suggest that development of nitrofen-induced hypoplastic lungs is modulated by NO as that of the normal lungs is, and the effect of NO is reduced in nitrofen-induced hypoplastic lungs compared with the normal lungs.

This report shows that exogenous NO accelerates the growth of the nitrofen-induced hypoplastic fetal lung explant and demonstrates the potential for NO to influence lung development at the branching morphogenesis stage. The mechanism of branching acceleration by NO remains unclear. One expected effect of NO would be to inhibit apoptosis. A second mechanism by which NO could modulate branching would be through stimulating cell proliferation.

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