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

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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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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