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Effect of nitric oxide on fibroblast growth factor-10 and bone morphogenetic protein 4 expressions in the branching morphogenesis of fetal rat lung explants

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Abstract

Purpose: Nitric oxide (NO) can accelerate branching morphogenesis of fetal rat lung explants in vitro, whereas its exact mechanism remains unclear. In this study, we investigate the effect of NO on the expression of fibroblast growth factor-10 (FGF10) and bone morphogenetic protein-4 (BMP4), which plays an important role in bud formation.

Methods: Fetal rat lungs harvested on day 13.5 of gestation were cultured in serum-free medium for 72 hours with 0, 50, 100, and 200 μmol/L of an NO donor, DETA NONOate (DETA/NO) (n = 4, 3, 6, and 5). The ratio of bud increment of each cultured lung was calculated, and the FGF10 and BMP4 mRNA expression levels were analyzed by real-time reverse transcription polymerase chain reaction.

Results: Bud increment ratio was significantly increased in 50, 100, and 200 μmol/L DETA/NO (3.3 $\pm$ 0.2, 3.0 $\pm$ 0.3, and 3.5 $\pm$ 0.5) compared to controls (1.9 $\pm$ 0.3) ($P < .05$). There was a significant increase in BMP4 mRNA expression in 100 μmol/L DETA/NO (190% $\pm$ 20%) compared to controls (100% $\pm$ 30%) ($P < .05$), whereas FGF10 mRNA expression was not significantly different between each DETA/NO group and controls.

Conclusion: The NO donor not only promotes branching of fetal lung explants but also upregulates expression of BMP4, which is an important regulator of branching morphogenesis.

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Pulmonary hypoplasia, characterized by immaturity and small size, causes respiratory failure which remains the principal contributor to the high mortality and morbidity in patients with congenital diaphragmatic hernia. Control and regulation of fetal lung growth are complex and incompletely understood.

In the early stages of the lung development, lung bud initiation from the foregut is followed by relatively stereotypic branching of the buds, which results from reiterated combination of bud outgrowth, elongation, and subdivision of terminal units [1]. These morphogenetic events are regulated by coordination of paracrine peptide growth factor signaling involved in epithelial-mesenchymal interactions such as fibroblast growth factor-10 (FGF10) and bone morphogenetic protein-4 (BMP4). Fibroblast growth factor-10 is locally expressed in the mesenchyme at prospective sites of bud formation and promotes both proliferation and chemotaxis of the nearby epithelium [2].

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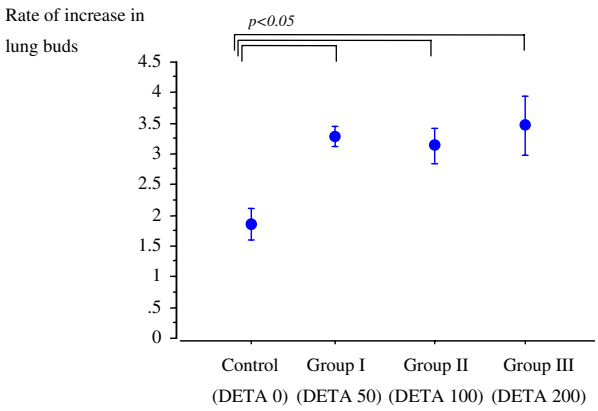

Fig. 1 Rate of increase in the bud count in the lung explant with DETA/NO 0, 50, 100, and 200 μmol/L during 72 hours of culture (mean $\pm$ SE).

Bone morphogenetic protein-4 plays a critical role in epithelial proliferation and proximal-distal cell differentiation [3]. The expression of BMP4 is localized in the distal epithelium of branching airways and dynamically coordinated with the expression of FGF10 to help reiterate stereotypic branching [4].

Recently, there is increasing evidence that oxygen-sensing pathways play an important role in the morphogenesis of respiratory structures [5]. Nitric oxide (NO) has drawn much attention in this context. Expression of NO synthase isoforms and protein S-nitrosylation has been reported in the developing lung, thus suggesting that NO may regulate some events during lung development [6,7]. Recent reports demonstrated that exogenous NO released from an NO donor affects branching morphogenesis of normal fetal lung explants as well as nitrofen-induced hypoplastic lung explants in the rat, thus suggesting an important role of NO in the lung development [8,9]. However, the mechanism of branching acceleration by NO remains unclear. Recent studies suggested that FGF10 and BMP4 expressions are regulated by transcription factors that are also involved in oxygen-sensing pathways or genomic NO sensor [5,10,11]. We designed this study to evaluate the effect of exogenous NO on the expression of FGF10 and BMP4 in fetal rat lung explant model.

1. Materials and methods

1.1. Fetal lung explant model

Timed-pregnant Sprague-Dawley rats were sacrificed on day 13.5 of gestation (noon on the day of vaginal plug represented day 0.5 of gestation) by cervical dislocation under deep anesthesia with isoflurane inhalation, and the fetuses were removed from the amniotic sacs. Whole fetal lungs were dissected free in Hank's 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's modified Eagle's 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 72 hours without changing the medium.

1.2. Morphometry

The cultured lungs were photographed daily on an inverted microscope, and the digitized lung images were analyzed for evaluating lung bud counts of the specimens. The increase in bud count was calculated as the ratio of each value at 72 hours minus each value at 0 hour, divided by the value at 0 hour.

1.3. NO donor

Following the previous method [9,10], we adopted DETA NONOate (DETA/NO) (Alexis Biochemicals, 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: control (n = 4) without DETA/NO; group I (n = 3) with 50 μmol/L DETA/NO; group II (n = 6) with 100 μmol/L DETA/NO; group III (n = 5) with 200 μmol/L DETA/NO.

1.4. RNA extraction

At 72 hours of culture, each lung was detached carefully from the culture dish insert filter with a scalpel under a microscope, resuspended in RNA*later* solution (Ambion, UK), and stored at −70°C. The total RNA of each lung was

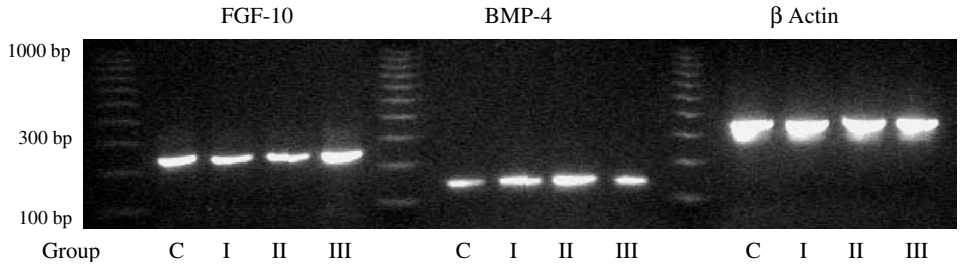

Fig. 2 Agarose electrophoresis of RT-PCR products. C indicates control.

extracted using guanidine-isothiocyanate lysis and silica-gel membrane affinity chromatography (RNeasy Micro Kit, Qiagen, UK). DNA contamination was removed by a DNase treatment.

1.5. Real-time RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) was performed using OneStep RT-PCR Kit (Qiagen) according to the manufacturer's protocol. The primer sequences for FGF10 and BMP4 were published previously [12]. Reverse transcription was performed at 50°C for 30 minutes, followed by an initial PCR activation at 95°C for 15 minutes, and 40 cycles of the amplification (denaturation at 95°C for 1 minute, annealing at 60°C-61°C for 1 minute, extension at 72°C for 1 minute) with final extension at 72°C for 10 minutes. The PCR products with and without RT were separated by 2% agarose gel electrophoresis and stained with ethidium bromide to confirm the specificity of the product and rule out DNA contamination.

Real-time RT-PCR was carried out to semiquantify the amounts of mRNA expression of FGF10 and BMP4 in the cultured lungs. Relative levels of gene expression were measured by real-time PCR (iCycler iQ Multicolor Real-Time PCR Detection System, Bio-Rad Laboratories, CA). RT-PCR reaction was performed using QuantiTect SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer's instruction. After RT at 50°C for 30 minutes, 40 cycles of amplification were carried out (denaturation at 95°C for 15 seconds, annealing at 60°C-61°C for 30 seconds, and extension at 72°C for 30 seconds). After the amplification program, melt curve analyses were performed to confirm that a single specific amplified product was produced. Serial dilution of one sample RNA was prepared to create a standard curve for the relative quantification of mRNA in the samples. The relative changes in levels of specific FGF10 and BMP4 mRNA were expressed in percent of the control values that were set equal to 100%, after the normalization by the level of β-actin expression in each sample. Product specificities were assured by 2% agarose electrophoresis.

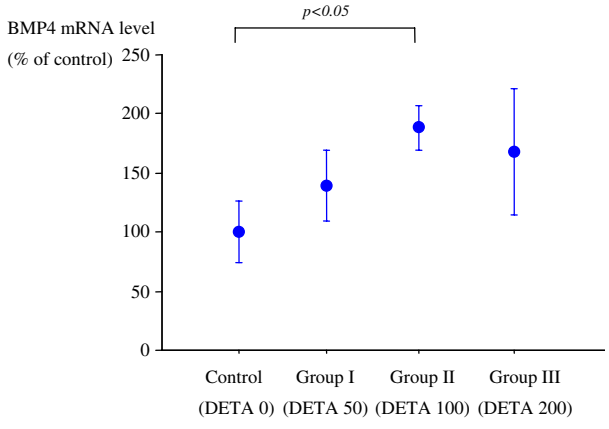

Fig. 3 Relative levels of BMP4 mRNA expression in the lung explant with DETA/NO 0, 50, 100, and 200 μmol/L (mean ± SE).

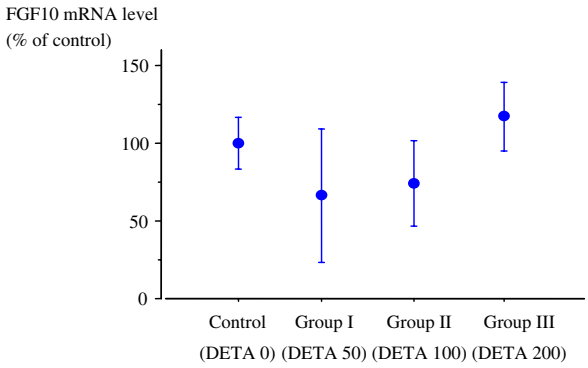

Fig. 4 Relative levels of FGF10 mRNA expression in the lung explant with DETA/NO 0, 50, 100, and 200 μmol/L (mean ± SE).

1.6. Statistical analysis

Data were analyzed using the StatView 5.0 program (SAS Institute Inc, Cary, NC). Values were expressed as mean ± SE, and statistical comparisons were performed by 2-way ANOVA and post hoc test (Bonferroni-Dunn test). Statistical significance was defined as $P < .05$.

All experiments 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

Lung bud counts were significantly increased in group I (3.3 ± 0.2), group II (3.0 ± 0.3), and group III (3.5 ± 0.5) compared to controls (1.9 ± 0.3) ($P < .05$) (Fig. 1). The length of the RT-PCR products corresponded well with the predicted molecular weights of FGF10, BMP4, and β-actin (Fig. 2). The levels of BMP4 mRNA expression in group II were significantly increased (190% ± 20%) compared to controls (100% ± 30%) ($P < .05$). Although there was relative increase in its levels in group I and III compared to controls (140% ± 30%, 170% ± 50%, respectively), this was not statistically significant (Fig. 3). No significant difference was found in the levels of FGF10 mRNA expression between each group and control (Fig. 4).

3. Discussion

Nitric oxide is a colorless gas which 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. Nitric oxide exerts its influence on a variety of protein functions through its reaction with cysteine thiol,

S-nitrosylation, and transition metal centers [13]. In a previous report, we demonstrated the potential for NO to influence lung branching morphogenesis not only in normal fetal lungs but also nitrofen-induced hypoplastic lungs [9]. However, the mechanism and role of NO in modulating lung branching morphogenesis remain obscure.

Fibroblast growth factor-10 and BMP4 stimulate branching morphogenesis not only on their own but also by their temporospatial interactions [4,13]. Recent reports suggested that the FGF10 promoter contained a consensus Sp1 binding site, which is known to be negatively controlled by transforming growth factor-β [10], and Nkx2.1, which is a transcriptional regulator for BMP4 [11]. Interestingly, transcription factors such as Sp1 family and Nkx2.1 act as dimerization partners for hepatocyte nuclear factor family, which is involved in oxygen-sensing pathways [5]. Moreover, Sp1 family is reported to function as a genomic NO sensor for various gene transcription [14]. Conceptually, these data suggest that NO may modulate the expression of FGF10 and BMP4, which lie under downstream regulation of Sp1 or Nkx2.1. Based on these data, we tested the possibility that NO might accelerate branching by modulating these paracrine peptide expressions and analyzed the levels of mRNA expression of FGF10 and BMP4 using the fetal rat lung explant model cultured with or without exogenous NO. In this experiment, we found not only significant acceleration of branching but also significant increase in mRNA expression of BMP4 in the lung explants cultured with exogenous NO compared to the ones without NO. On the other hand, we did not find any difference in mRNA expression of FGF10. These results suggest the possibility that exogenous NO may accelerate branching morphogenesis by upregulating the expression of BMP4. In our study, we found only one concentration of the NO donor that exerts a significant effect on BMP4 mRNA expression, although other concentrations of the NO donor also showed their effects in the same direction. We suppose a few reasons for this result. First, we evaluated the expression of the growth factors in the whole lung at one particular time of culture. Because both FGF10 and BMP4 are reported to exhibit their functions in a temporospatially dynamic fashion in the developing lung, these peptides may show different degrees of expression in different portions of the lung during different times of culture [15]. Second, lung branching results from the interaction of various paracrine peptides other than FGF10 and BMP4, which include transforming growth factor-β1, insulin-like growth factor, and vascular endothelial growth factor. Further studies are required to confirm the positive effects of NO on the expression of those paracrine factors, which include the evaluation of paracrine gene expression and proliferation on cellular basis at the distal airways during different periods of culture.

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