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Early inhaled nitric oxide at high dose enhances rat lung development after birth

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ABSTRACT

Rational: Inhaled nitric oxide (NO) is frequently administered to full term and preterm newborns in various clinical settings in order to alleviate pulmonary hypertension whilst improving oxygenation. However, the physiological effect of NO on early postnatal lung development has not yet been clearly described. We therefore investigated whether NO administered by inhalation affects lung development at early postnatal life.

Methods: Pregnant rats were placed in a chamber containing 5 ppm (iNO-5 ppm group) and 20 ppm NO (iNO-20 ppm group), started from the last day of their pregnancy in order to keep rat pups under ambient NO from birth to 7 days postnatal. Control animals were kept at room air and all rat pups were sacrificed at postnatal *day 7* and *day 14*.

Results: Lung-to-body weight and wet-to-dry lung weight ratios did not significantly differ among 3 groups at postnatal *day 7* and *day 14*. Vascular volume densities (Vv) in both NO groups (5 and 20 ppm) were higher than controls ($P < 0.05$; $P < 0.001$). Pulmonary vessel number was significantly increased in iNO-20 ppm group. Radial alveolar counts (RAC) and mean linear intercepts (MLI) markedly increased (consistent with increased alveolarization) in iNO-20 ppm group. This was associated with upregulation of VEGF/VEGFR-2, MT1-MMP/MMP2 and HO-1 protein expression in iNO-20 ppm group.

Conclusions: We concluded that inhaled NO at 20 ppm enhanced lung development possibly through increased expression of HO-1, VEGF/VEGFR-2, and MMP2 at early stage of postnatal rat life.

Introduction

Inhaled nitric oxide (NO) is a potential therapy in very ill preterm and term newborns. Recent studies showed that early use of inhaled NO in preterm newborn might decrease the risk of brain injury [1], improve neurological development outcome, and prevent incidence of bronchopulmonary dysplasia [2]. It is known that preterm newborns with severe respiratory failure are at high risk of mortality due to intracranial hemorrhage and lung injury. Inhaled NO is an important adjunctive treatment to current therapy of persistent pulmonary hypertension (PPH) in term newborns with hypoxemic respiratory failure. As inhaled NO is a selective pulmonary vasodilator [3]; it can selectively decrease pulmonary vascular resistance and ventilation – perfusion mismatching, leading to oxygenation improvement [4]. Further, in severe respiratory failure newborns, inhaled NO also reduces the requirement for extracorporeal membrane oxygenation and improves short term pulmonary outcomes [5]. Although the rational of dose and duration of inhaled NO in very ill preterm and term newborn has been demonstrated by previous studies [6,7], the effects of inhaled NO on normal lung development have not been completely understood.

It is recognized that NO has a potential role in airway branching morphogenesis and vascular development. NO is produced endogenously from the amino acid L-arginine by enzymatic action of nitric oxide synthases (NOS) which is expressed in endothelial and epithelial cells [8]. A number of studies have shown that eNOS-deficient mice exhibit severe abnormalities in lung development with a high mortality due to fatal respiratory distress [9,10]. However, the precise mechanism by which pulmonary vascular bed develops during fetal and early postnatal life under effects of inhaled NO has not been completely understood. Recently, the role of endogenous NO on

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lung vascular growth has been suggested by its effect on upregulation of vascular endothelial growth factor (VEGF), a key factor for angiogenesis during embryonic and fetal development [11]. NO modulates a number of VEGF-regulating factors such as hypoxia-inducible factors-1alpha (HIF-1α) and heme oxygenase-1 (HO-1) which induce VEGF synthesis during lung development [12].

VEGF binds to its receptor VEGFR-1 (flt-1) and VEGFR-2 (KDR in human and flk-1 in mice), which then interact and modify the biological effects of VEGF [13]. While VEGFR-1 acts as a silent receptor for VEGF due to its relatively weak kinase activity, VEGFR-2 plays an important role for lung development via phosphatidylinositol 3-kinase/Akt pathway [14]. Beside the angiogenesis and vasculogenesis mediated by VEGF/VEGFR-2 pathway, lung development before and after birth is marked by an alveolarization. In early postnatal life, the factors controlling alveolar stage are abundant and very complex. Among of these, the matrix metalloproteinase (MMPs), especially matrix metalloproteinase 2 (MMP2 or gelatinase A) and its activator MT1-MMP, play an important role in alveolarization [15]. The activity of MMPs is mediated by hypoxia or hyperoxia status and oxidative stress [16].

However, until now, the effect of exogenous NO (inhaled NO) on normal lung development has not been clearly demonstrated. Therefore, we hypothesize that inhaled NO at early postnatal life affects lung growth via VEGF/VEGFR-2 pathway. Then, we postulate that the effect of inhaled NO on postnatal lung growth will depend on the dose and the duration of inhalation. To test this hypothesis, we first determined whether inhaled NO during an early postnatal life changes alveolar and vascular growth in normal neonatal rat lungs. Then, we described the effect of inhaled NO at low and high dose on VEGF/VEGFR-2 pathway and its regulating factors in animal model.

Materials and methods

Animals and experimental protocols

This study was approved by the National Institute of Health and Medical Research and complied with the instructions of the Institutional Animal Care and Use Committees INSERM 676-Paris. Pregnant Sprague–Dawley rats (SDR) were purchased from Charles River Laboratory (L'Arbresle, France). Animals were maintained at 20–24 °C in autoclaved cages and exposed to alternative day–night cycles every 12 h (lights on at 8:00 am) throughout the study period with free access to standard food and water. Rat pups were delivered naturally at termed gestation.

Pregnant SDR were randomized into three groups at the last day of their gestation: SDR exposed to room air (control group), SDR exposed to low-dose NO at 5 ppm (iNO-5 ppm group), and SDR exposed to high-dose NO at 20 ppm (iNO-20 ppm group). Twenty-four hours before giving birth, pregnant SDR from inhaled NO groups were placed in a transparent Plexiglas chambers, connected to NO source (BioSpherix, Redfield-IL, USA) in order that neonatal rats could expose to inhaled NO since their first inspiration. NO and NO_2 levels were continuously monitor with specific apparatus (Datex-Ohmeda, Inc., Madison, WI53707-7550, USA). The concentration of NO_2 was kept less than 1 ppm. Inhaled NO was performed only for 7 days. Rat pups were killed at postnatal *day 7* and *day 14* to obtain lung tissue for histology, morphometric, and protein analysis. All postnatal *day 14* rat pups were maintained at room air condition from *day 7*. At least 6 animals were used for each group unless otherwise noted.

Chemical reagents and antibodies

All chemical reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise noted. For all washing steps in immunohistochemical (IHC) staining and Western blot, phosphate buffered saline (PBS, pH 7.4) was used with Tween 20 (0.05%), noted as PBS-T. Primary antibodies for CD31 (PECAM-1), VEGF and VEGFR-2 (Flk-1), 3-nitrotyrosine, β-actin, and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Incorporation. Vectastain Elite ABC Kit for secondary biotinylated antibodies for IHC and haematoxylin was purchased from Vector Laboratories Inc. (Burlingame, CA, USA). Quantikine VEGF Immunoassay kit was supplied by R&D Systems Europe Ltd (Abingdon, UK). Rat hemeoxygenase-1 (HO-1) ELISA Kit was purchased from Stressgen (Ann Arbor, MI, USA). Antibodies used in Western blot were diluted in non-fat milk (5%), PBS-T, and those for immunohistochemistry in PBS.

Lung tissue preparations

Rat lungs were first flushed *in situ* with PBS via right ventricular puncture and pulmonary artery to get rid of blood from pulmonary circulation. Rat lungs were removed from thoracic cavity. Right lungs were dissected, snap-frozen in liquid nitrogen, and stored at −80 °C until use for Western blot or ELISA. Left lungs were washed in physiological solution (NaCl 0.9%, pH 7.4), infused with 4% paraformaldehyde (PFA in PBS, pH 7.4) at 20 cm water pressure via an intratracheal catheter for 1 h [15]. Main left bronchus was ligated under pressure, and left lungs immersed into the same fixative solution for 24 h at room temperature. Lung tissues were then dehydrated and embedded in paraffin by automatic procedure (Shandon Citadel 2000 Tissue Processor, Rankin Biomedical Corp., MI, USA).

Wet-to-dry lung weight

Other groups of newborn rats ($n = 4$ in each group) were used for determining wet-to-dry lung weight. Rat lungs were removed from thoracic cavity. After trachea and stem bronchi removal, the whole lung was weighed to obtain wet weight. Lungs were then dried to constant weight in a microwave oven with a low power of 200 W for 60 min as previously described with a few modifications [17].

Lung alveolarization assessment

Lung blocks were cut into 5-μm sections and serially mounted onto SuperFrost Plus slides (Braunschweig, Germany). All lungs were sectioned by the same manner, with symmetrical slices from hilum to pleural surface. Haematoxylin and eosin staining was carried out on all sections to evaluate the alveolarization. Alveolarization was assessed by performing radial alveolar counts (RAC) and median linear intercepts (MLI). Images of each section were captured with a magnified digital camera through a Leica microscope (Leica Microsystems, Wetzlar, Germany).

The RAC, described by Emery and Mithal [18], represents the alveolar number across terminal respiratory units. To assess the RAC, respiratory bronchioles were identified as bronchioles lined by epithelium in one part of the wall. Then, a perpendicular line was drawn from the centre of respiratory bronchiole to the outer edge of acinus, as defined by a connective tissue septum or pleura. The number of septa intersected by this line was counted.

For MLI assessment, the technique had been previously described by Dunnill and Thurlbeck [19,20]. The MLI represents the average size of alveoli or distance between airspace walls. The same images as above mentioned were used. To measure the intercepts, a transparent sheet with 10 horizontal and 11 vertical lines was laid over the images. The intercepts of alveolar walls with these lines were counted. Intercepts of bronchioli, blood vessels or septa were counted for one half since they are more or less part