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Inhaled NO prevents hyperoxia-induced white matter damage in neonatal rats

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ABSTRACT

White matter damage (WMD) and bronchopulmonary dysplasia (BPD) are the two main complications occurring in very preterm infants. Inhaled nitric oxide (iNO) has been proposed to promote alveolarization in the developing lung, and we have reported that iNO promotes myelination and induces neuroprotection in neonatal rats with excitotoxic brain damage. Our hypothesis is that, in addition to its pulmonary effects, iNO may be neuroprotective in rat pups exposed to hyperoxia. To test this hypothesis, we exposed rat pups to hyperoxia, and we assessed the impact of iNO on WMD and BPD.

Rat pups were exposed to either hyperoxia (80% FiO_2) or to normoxia for 8 days. Both groups received iNO (5 ppm) or air. We assessed the neurological and pulmonary effects of iNO in hyperoxia-injured rat pups using histological, molecular and behavioral approaches.

iNO significantly attenuated the severity of hyperoxia-induced WMD induced in neonatal rats. Specifically, iNO decreased white matter inflammation, cell death, and enhanced the density of proliferating oligodendrocytes and oligodendroglial maturation. Furthermore, iNO triggered an early upregulation of P27kip1 and brain-derived growth factor (BDNF). Whereas hyperoxia disrupted early associative abilities, iNO treatment maintained learning scores to a level similar to that of control pups. In contrast to its marked neuroprotective effects, iNO induced only small and transient improvements of BPD.

These findings suggest that iNO exposure at low doses is specifically neuroprotective in an animal model combining injuries of the developing lung and brain that mimicked BPD and WMD in preterm infants.

Introduction

Bronchopulmonary dysplasia (BPD) and white matter damage (WMD) are the two most common complications of preterm birth. Their incidence reaches 20 to 40% of infants born below 28 weeks of gestation (Jobe, 2011; Volpe, 2009). Whereas advances in neonatal intensive care have resulted in a marked decrease in neonatal mortality, the incidence of both morbidities in these infants remains high. Several common risk factors, such as low gestational age, perinatal inflammation and excessive oxidative stress make more likely the devastating co-occurrence of BPD and WMD (Dammann et al., 2004). In addition, BPD increases the risk for cerebral palsy (CP), which is a common consequence of WMD in infants born prematurely. Among these common risk factors, an excessive release of free radicals induced by oxygen therapy in preterm neonates with respiratory distress was involved in the pathogenesis of WMD as well as BPD. Furthermore, recent experimental studies have supported that hyperoxia causes oxidative stress and triggers maturation-dependent cell death, maturation arrest of developing oligodendrocytes, and disruption of axon–oligodendrocyte integrity, all key features of WMD (Back et al., 2007; Gerstner et al., 2008; Ritter et al.,

Abbreviations: BPD, bronchopulmonary dysplasia; WMD, white matter damage; iNO, inhaled NO; RAC, radial alveolar count; MLI, mean linear intercept; CC3, cleaved caspase 3.

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2013; Schmitz et al., 2011; Vottier et al., 2011). For these reasons, the design of therapeutic strategies that take into account both BPD and WMD is a highly relevant, to date poorly explored challenge.

In the present study, we focused on the therapeutic use of nitric oxide (NO) in neonates. NO is widely recognized as an important messenger and effector molecule in a variety of acute and chronic inflammation systems, and also as a mediator of vascular tone and tolerance to damage (Moncada et al., 1991; Toda and Okamura, 2003; Vaucher et al., 2000). Interestingly, NO is both a physiological mediator of the central nervous system and a key factor for lung angiogenesis and alveolarization, two developmental phenomena involved in BPD pathophysiology (Jobe, 2011; Yun et al., 1996). On the other hand, we have recently reported that inhaled NO (iNO) has several critical properties in the developing brain by promoting myelination and inducing neuroprotection against excitotoxic-induced brain injury and neonatal stroke (Charriaut-Marlangue et al., 2012, 2013; Olivier et al., 2010; Pansiot et al., 2010). Interestingly, similar results were reported in preclinical model of adult stroke (Terpolilli et al., 2012, 2013). Thus, iNO appears to be a promising candidate for the prevention and/or the clinical management of WMD and BPD, but remains to be evaluated in an animal model mimicking simultaneously these two developmental diseases.

Here, we used a rat model of prolonged postnatal hyperoxia that induced BPD and WMD. We made the hypothesis that iNO, in addition to its potential effects on lung injury, may be neuroprotective in rat pups exposed to hyperoxia. The present results showed that iNO induces neuroprotection with a significant effect at both histopathological and behavioral levels, whereas it had only transient and mild effects on BPD.

Material and methods

Experimental protocol and gas exposure

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. The day before delivery, pregnant rats (Sprague–Dawley, Janvier S.A.S., Le Genest-St-Isle, France) were placed in a transparent Plexiglas chamber supplied with a gas mixture that either induced hyperoxia (FiO_2 = 80 +/− 0.5%) until postnatal day (P)7 or maintained normoxia (FiO_2 = 21 +/− 0.5%). Hyperoxia exposure of pregnant rats began the day before delivery to make sure that all rat pups were placed under hyperoxic condition immediately after birth. Adult rats were switched every 24 h between O_2 exposed and room air-exposed litters. Oxygen concentration was monitored using a Proox (Biopherix, USA). CO_2 concentration was consistently kept under 0.1% using soda lime (Intersurgical, France). To investigate the impact of exogenous NO on the developing brain and lung, iNO at low concentration (5 ppm) was introduced in chambers from embryonic day (E)21 to P7 and monitored using iNOvent system (INOTherapeutics, Clinton, NJ). NO_2 concentration was kept under 1 ppm. Low concentration of NO (5 ppm) was used according to current use in neonatal intensive care units and because a higher dose (20–40 ppm) would not be feasible for a protracted exposure.

From P7, rat pups and their mothers in all experimental groups were kept in room air. Animals were housed under controlled temperature (22 ± 1 °C) and light conditions (12 h day/night cycle) with food and water ad libitum. Neonatal mortality was checked daily.

Blood gas analysis

Blood gas was analyzed in hyperoxic and control rat pups using a clinical blood gas analyzer (ABL 80, Radiometer). Pups were decapitated and blood samples collected at various time points (1 h, 12 h, 3 days and 7 days of life) from the neck in heparinized capillary tubes, and gases measured immediately.

Ultrasound imaging

Thermoregulated rat pups (at P1 and/or P6, n = 5 per group) were subjected to ultrasound measurements under isoflurane [0.5% inhalation via a facemask in (O_2/N_2O) (1:3)] anesthesia using an echograph (Voluson i, GE Heatlthcare, Aulnay-sous-bois, France) equipped with a 12-MHz linear transducer (Bonnin et al., 2011). Heart rate and spatial-averaged-time-averaged mean blood-flow velocities (mBFV) were measured in the intracranial carotid arteries (ICA) and the basilar trunk (BT, when detectable) 1 day (P1) and 6 days (P6) after air, iNO (5 ppm), hyperoxia, and hyperoxia in combination with iNO exposure. Heart rates reflected changes in cardiac output, as the left ventricular ejection volume is quite invariable in newborns.

Tissue preparation

Pups (n = 7–9/group for each staining protocol) were sacrificed at P3, P10 or P21. The left lung was washed in physiological serum, fixed with 4% paraformaldehyde in PBS, pH 7.4, for 24 h, and paraffin-embedded. Symmetrical 5 μm-thick sections were cut from the hilum to the pleural surface.

For brain histology studies, two distinct fixation protocols were used:

(1) brains were directly removed and immersed in 4% formaldehyde and embedded in paraffin,
(2) pups were perfused transcardially with 4% paraformaldehyde in phosphate buffer (PB 0.24 M, pH 7.4). Brains were equilibrated with 10% sucrose in PB for 2–4 days, frozen in liquid nitrogen-cooled isopentane, stored at −80 °C and cut coronally into serial 10 μm-thick sections.

Lung alveolarization assessment

All sections were stained with hematoxylin and eosin, and alveolarization was assessed by performing radial alveolar counts (RAC) and median linear intercepts (MLI) as previously described (Dunnill, 1962; Emery and Mithal, 1960; Thurlbeck et al., 1970). Images of each section were captured with a magnified digital camera through a Leica microscope and were saved as PICT.jpg files. At least ten counts were performed per animal and 6–8 animals were used for each experiment.

Immunohistochemistry

Primary antibodies used in this study are listed in Supplemental Table S1. All quantification of immuno-reactive cells was carried out by investigators blind to the experimental groups. Lung angiogenesis was evaluated by measuring pulmonary vessel number and pulmonary vascular volume density (Vv), using factor VIII [von Willebrand Factor (vWF)] as an endothelial marker. Pulmonary vessel number was determined by counting microvessels (20–80 μm) stained with vWF in each high power field (100X magnification). Vv was measured by superimposing a grid of 100 points onto color photomicrographs (400× magnification) of ten random noncontiguous fields per animal. Vv was calculated as the ratio of the number of points coinciding with vWF-positive sites to the number of points on lung parenchyma (excluding large vessels and airways).

For brain immunohistochemistry, coronal sections (+1.44 to −0.48 mm from bregma) were selected and processed as previously described (Olivier et al., 2005). In each experimental group, we studied 7–8 pups in three separate experiments. Immunolabeling was visualized using the streptavidin–biotin–peroxydase method. Double-labeling was performed with secondary antibodies coupled to the green fluorescent marker Fluoroprobe S488 (Interchim, Montluçon,