Human pulmonary nitric oxide synthases (NOS) and nitrotyrosine: Findings during neonatal development and in chronic lung disease (CLD)

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NO is a key mediator of a complex physiologic process in the transition from intra-uterine to extra-uterine life. Studies in the past 15 years have broadened NO's proposed role as an important biological messenger from pulmonary vasodilation, hypertensive shock and neurotransmission to smooth muscle cell inhibition and modulation of inflammation. Recently several well designed clinical trials in neonatology have reported differing results regarding inhaled NO in preterm infants.

NO plays a key role in many biochemical interactions

NO's biochemical interactions are multiple and complex. It is synthesised by NOS when L-arginine is oxidised to L-citrilline. There are three isoforms of NOS: neuronal or nNOS (NOS1), inducible or iNOS (NOS2) and endothelial or eNOS (NOS3). The major interactions involving NO include:

- NO activation of the guanylate cyclase pathway leading to increased GCMP causing vasorelaxation
- rapid reaction with oxyhaemoglobin leading to NO inactivation by formation of methemoglobin
- radical-radical interaction of NO and superoxide to form the reactive nitrogen species peroxynitrite which can then nitrate phenolyic compounds like tyrosine.

Nitrotyrosine is a stable and measurable end-product and can serve as a footprint for NO production. Sheffield et al. conducted a case-controlled, retrospective study with the aim of assessing the relative and prospective density of the three NOS isoforms in the neonatal lung at specific levels of the airways and vascular trees (unpublished data).

NOS isoforms are present in the lung, even in very premature neonates

Lung tissue samples from 56 neonates with varying degrees of prematurity were assessed utilising immunohistochemistry to evaluate different airway levels (bronchial, proximal broncholic etc.) and two vascular tree levels with regard to normal ontogeny and the effects of CLD. The primary hypothesis was that there would be no relationship between the presence of any of the NOS isoforms or nitrotyrosine and the gestational or the postnatal age nor a correlation between NOS or nitrotyrosine and the degree of CLD.
Post mortem lung tissues samples from neonates with a post-conceptual gestational age of 22 to 42 weeks, who had not received any ions or inhaled NOS, were compared with control samples from infants who died from nonpulmonary causes or who died less than 48 hours before significant pulmonary rearchitecturing could occur. Infants with a ROM >48 hours, multiple congenital abnormalities, lungs that could not be inflated or those with extensive haemorrhage were excluded.
The control group was compared with the neonates stratified according to severity of lung disease and gestational age. Lung disease was defined using the respiratory SCORE to characterise samples as mild, moderate or severe. Five airway and two vascular levels were evaluated for staining intensity by an observer blinded to medical history on a semiquantitive scale of 0 = no stain to 3 = intense/heavy staining.
Stains for each of the three isoforms were found at all gestational ages from youngest (22 weeks) to oldest (42 weeks). In the control and CLD groups NOS isoforms were found in the airway epithelium from the bronchus to the most distal levels. The only significant difference was at the respiratory bronchial level; eNOS (NOS3) was markedly reduced in neonates with severe CLD.

These results confirmed the developmental expression and intra-pulmonary location of NOS enzymes and the enzymatic potential for NO production, even in the youngest neonates. However, the question remained as to what evidence is available for NO activity. To answer this question the nitrotyrosine end-product was measured.

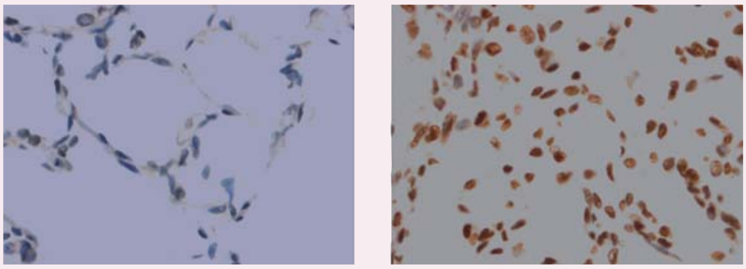
Nitrotyrosine is a fingerprint for the production of NO

Nitrotyrosine can serve as a fingerprint for the production of the reactive nitrogen species formed from the radical-radical interaction of superoxide and NO to form peroxynitrite. Evidence indicates that there are increased levels of nitrotyrosine in the lungs of adults dying from RDS,[1] and there is a correlation between plasma levels and the severity of BPD.[2]

On a randomly selected group of tissues computerised quantitative image analysis was performed to determine the mean area of staining intensity of nitrotyrosine *(Figure 1)*.

Figure 1. Nitrotyrosine staining for control versus severe CLD lung tissue.

a b



Nitrotyrosine Staining: a: Control lung showing - little staining. b: Severe chronic lung disease demonstrating heavy nitrotyrosine staining. 400x

Nitrotyrosine staining was found in the pulmonary tissues of all gestational ages tested (23 to 40 weeks). Staining was found mainly in the nuclear/perinuclear areas of airway epithelium. Compared with control and mild CLD, where nitrotyrosine staining was minimal, neonates with severe CLD had a >4 fold increase in staining ($p<0.001$). All three NOS isoforms were detectable by immunohistochemistry at even the earliest lung development stages. NOS ontogeny shows no significant changes in abundance or distribution with advancing gestational age and only eNOS was altered in infants dying with CLD. Nitrotyrosine, however, is significantly increased in those with severe CLD.

The current study is the most complete human study for the developmental expression of NOS isoforms. It also demonstrated that there is a 4-fold increase in the intensity of staining which is correlated with the severity of CLD and this may have implications for clinical use of NO. The investigators acknowledge that this study poses several limitations, however the study was designed to minimise any potential sources of error. While NO staining shows only expression, this study also analysed the nitrotyrosine end-product in an effort to predict function. The risk of post mortem changes was minimised by exclusion of samples with autolysis, and a single individual prepared and fixed all lung tissue samples. Given that the arbitrary scoring system of stain intensity was semiquantitive and had the potential for subjectivity, this was addressed by utilising computer imaging analysis. Furthermore, immunohistochemistry has innate, specific limitations including it is only semiquantitive and in this study staining was performed for an enzyme rather than a structural protein.

Finally, the results of this study suggest that in infants with very severe CLD it may not always be useful to administer exogenous NO, although recent data indicates a possible benefit in less critical infants. Evidence of activation of any of the NOS isoenzymes with subsequent production of NO may serve as a relative contraindication to use of exogenous NO. It is possible to speculate that exhaled NO measurements may still provide a tool for more specific and precise use of inhaled NO to those who are most likely to benefit.

References

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2. Lorch SA, Banks SA, Christie J, et al. Plasma 3-nitrotyrosine and outcome in neonates with severe bronchopulmonary dysplasia after inhaled nitric oxide. Free Radic Biol Med 2003; 34: 1146-1152.