REVIEW OF THE YEAR: NITRIC OXIDE (NO•) AND ORGANIC DUSTS

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Abstract

Nitric oxide synthase (NOS) is a cytosolic enzyme that produces nitric oxide (NO•). Evidence is accumulating that the constitutive form of NOS is present in various pneumocytes releasing NO• at a low basal rate to control vascular tone and act as a modulator of cellular responses. In response to inflammatory cytokines various lung cells also exhibit the upregulation of an inducible NOS (iNOS) capable of generating NO• at levels high enough to cause oxidant damage. This review summarizes evidence that endotoxin, an etiologic agent in organic dusts, can induce NO• production following in vitro or in vivo exposure in a variety of lung cell types. Induction of NO• is also demonstrated in response to inhalation of cotton dust in rats. Species variability is discussed.

Introduction

The cytosolic enzyme, NOS, converts the substrate, L-arginine, to L-citruline and nitric oxide, NO•. Nitric oxide, being a radical, is reactive. In addition, NO• can combine with superoxide anion, O^-_2, to form the highly reactive product, peroxynitrite ($OONO^-$). Peroxynitrite can combine with hydrogen ion to form peroxynitrous acid, which can generate hydroxyl radical (•OH) and nitrogen dioxide.

From the above, it can be expected that high activity of NOS in lung cells can result in a significant burden of oxidants. Indeed, several examples of oxidant injury and inflammation have been associated with high levels of NO• production. These include:

1. lipid peroxidation
2. nitrosation of tyrosine residues in proteins
3. inactivation of surfactant apoprotein A
4. mitochondrial, cell, and or DNA damage
5. increased pulmonary permeability
6. decreased production of surfactant by alveolar type II cells
7. increased production of proinflammatory prostaglandin, PGE_2
8. increased responsiveness to chemoattractants

To prevent excess oxidant injury, NO• levels are normally maintained at low levels under basal conditions. However, upon exposure of the lung to inflammatory agents, high levels of NO• can be generated which may play a significant role in disease processes. NOS exists in three isoforms which account for the production of NO• at the basal or induced rate. Two isoforms of NOS are considered constitutive and are responsible for basal release of relatively low levels of NO•. The two constitutive forms of NOS are the type 1 form, i.e., brain NOS (bNOS) or neuronal NOS (nNOS), and the type 3 form, i.e., endothelial NOS (eNOS). The constitutive forms of NOS exhibit low affinity for calmodulin, calcium dependence, and low basal release of NO•. These isoforms are involved in neurotransmission, bNOS or nNOS, or vasodilation involving eNOS. In response to an inflammatory insult, type 2 NOS, iNOS, is induced. Inducible NOS has a high affinity for calmodulin, is calcium independent, and can generate high levels of NO• in response to inflammatory exposures or cytokines.

Constitutive or inducible isoforms of NOS have been identified in various lung cell types as summarized in Table 1. Under basal conditions, eNOS has been found in alveolar macrophages, alveolar type II cells, and pulmonary endothelial cells. Type II cell eNOS appears to be more active than that found in alveolar macrophages. In contrast, nNOS is found in airway neurons but not in alveolar macrophages or type II cells. Inducible NOS has not been identified in unstimulated alveolar macrophages or type II cells. However, in response to interferon g (IFNg) or IFNg plus cytokines or lipopolysaccaride (LPS), iNOS is expressed in alveolar macrophages, interstitial macrophages, type II cells, fibroblasts, pulmonary arterial smooth muscle, and neutrophils, resulting in the production of moderate to high quantities of NO•.

Exposure to cotton or other organic dusts can result in pulmonary inflammation and airway constriction (Rylander, 1990; Rylander, 1992). Endotoxin or its biologically active component lipopolysaccaride (LPS) is considered a major etiologic agent in the pulmonary response to inhalation of organic dusts (Rylander, 1983). This review summarizes the effects of in vitro or in vivo exposure to endotoxin or of cotton dust inhalation on the upregulation of iNOS and the induction of NO• production by various lung cells.

Production of NO• by Lung Cells in Response to In Vitro LPS Exposure

A review of the literature reveals that LPS is a potent inducer of NO• production by various types of lung cells in culture. As shown in Table 2, LPS treatment of alveolar macrophages harvested by bronchoalveolar lavage of naive rats results in a dose-dependent increase in NO• production, measured by the Greiss reaction as the generation of nitrate and nitrite products in the culture medium. This elevated NO• production is accompanied by a dose-dependent increase in mRNA levels for iNOS, measured by northern blot analysis, as well as an LPS-dependent increase in iNOS protein levels, measured by western blot analysis. Gutierrez et al. (1995) have shown that LPS enhances the production of NO• by a cultured alveolar type II epithelial cell line (Table 3). LPS

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 1:177-180 (1998)
National Cotton Council, Memphis TN

plus cytokine treatment (IFNg and IL-1) causes a further induction of NO• production which is associated with an increase of message for iNOS as well of upregulation of iNOS protein levels.

The response of pulmonary smooth muscle to in vitro treatment with LPS has been investigated by monitoring the contractility of tracheal smooth muscle or pulmonary arterial smooth muscle in response to methacholine or phenylephrine, respectively. As shown in Table 4, addition of LPS to an excised tracheal smooth muscle preparation decreases its contractile response to methacholine. This decrease in contractility is evident even in the absence of tracheal epithelial cells, indicating that a LPS-induced relaxing factor is being produced by the airway smooth muscle cells themselves. L-NAME (Nw-nitro-L-arginine methyl ester), an inhibitor of NOS, blocks this LPS-induced relaxation, suggesting that the factor involved is NO•. In contrast to airway smooth muscle, Russell et al. (1993) failed to demonstrate induction of NO• production in rabbit pulmonary arterial smooth muscle, i.e., in vitro LPS treatment does not decrease contractility in response to phenylephrine. Nakayama et al. (1992) also reported that in vitro treatment of rat pulmonary arterial smooth muscle with LPS fails to induce NO• production. However, LPS in combination with a mixture of cytokines (IL-1, TNFg, and IFNg) is a potent of NO• production by cultured pulmonary arterial smooth muscle cells. Similarily, in vitro treatment of cultured rat lung fibroblasts with LPS alone fails to stimulate NO• production, but LPS augments NO• production by fibroblasts in response to IFNg (Jorens et al., 1992).

Production of NO• by Lung Cells in Response to In Vivo LPS Exposure

LPS administered intraperitoneally (IP), intravenously (IV), by intratracheal instillation (IT), or by inhalation of aerosolized endotoxin induces NO• production in lung tissue as well as several lung cell types isolated from exposed animals. As shown in Table 5, mRNA for iNOS is dramatically elevated in lung tissue of rats exposed (IP) to LPS. Interestingly, message for constitutive eNOS is decreased by LPS. NOS activity in lung tissue is also increased after IV treatment of isolated-perfused rat lungs with LPS. This induction is inhibited by 85% with aminoguanidine, a specific inhibitor of iNOS.

Induction of NO• production measured by the Greiss reaction or by L-NAME-inhibitable chemiluminescence is substantial in alveolar phagocytes harvested from rats exposed to LPS or endotoxin (Table 6). Chemiluminescence in this study was measured in response to unopsonized zymosan. Since alveolar macrophages but not neutrophils respond to unopsonized zymosan, these data indicate that iNOS is upregulated in alveolar macrophages as well as pulmonary phagocytes in response to LPS. This increase in NO• production is associated with dramatic increases in iNOS mRNA. In addition, induction of mRNA for iNOS is observed independently of the route of LPS exposure, i.e., IT, IV or inhalation.

Wizemann et al. (1994) compared the response of alveolar macrophages, interstitial macrophages, and alveolar type II cells isolated from rat lungs following IV exposure to LPS. Message for iNOS is elevated in all three lung cell types in response to in vivo LPS, with the responsiveness of alveolar macrophages and type II cells being more substantial than that for interstitial macrophages. Ex vivo production of NO• in response to IFNg plus LPS is elevated in both alveolar and interstitial macrophages isolated from LPS-exposed rats. As with iNOS message, NO• production is greater for alveolar macrophages than interstitial macrophages.

As shown in Table 7, the contractililty of pulmonary arterial rings in response to phenylephrine is significantly decreased after IV treatment of rabbits with LPS. Similar results are also obtained after IP treatment of rats with LPS. This relaxation is partially inhibited by a NOS blocker (NLA, nitro-L-arginine) indication that NO• is the relaxation factor involved. Indeed, message for iNOS is elevated in pulmonary arterial tissue after IP treatment with LPS. In both experimental cases, pulmonary arterial smooth muscle relaxation is observed even after removal of the vascular endothelial cells, indicating NO• induction in the vascular smooth muscle cells themselves.

In contrast to the response of vascular smooth muscle, bronchial smooth muscle fails to exhibit NO•-dependent relaxation in response to IV administered LPS, i.e., no change in contractility of airway smooth muscle in response to carbachol is reported (Russell et al. 1993).

Production of NO• by Alveolar Phagocytes Following Inhalation of Cotton Dust

Recently, Huffman et al. (1997) reported the induction of NO• generation following inhalation of cotton dust and compared these responses to those following inhalation of an aerosol of endotoxin. In this experiment, rats were exposed for 3 hours to 41 mg/m^3 cotton dust and alveolar phagocytes, obtained by bronchoalveolar lavage, monitored 18 hours post-exposure. NO• in the acellular lavage fluid was monitored by the Greiss reaction and is elevated by 27% above control. In addition, NO• production by alveolar phagocytes, measured by the Greiss reaction, or by alveolar macrophages, measured as NO•-dependent chemiluminescence, is increased dramatically by cotton dust inhalation. Message levels for iNOS in bronchoalveolar lavage cells also demonstrate induction in response to cotton dust exposure. These data are summarized in Table 8. In general, the induction of NO• in alveolar phagocytes was similar after inhalation of either cotton dust or endotoxin.

Species Variability of LPS-induced NO• Responses

Upon review of the literature, it becomes apparent that most laboratories including my own have been unsuccessful in demonstrating LPS or cotton dust-induced NO• production in

guinea pigs. These data are summarized in Table 9. For example, in vivo treatment with LPS induces NO•-dependent relaxation of pulmonary arterial smooth muscle in the rat and rabbit, but not in the guinea pig. Likewise, LPS exposure induces NO•-dependent relaxation of airway smooth muscle and induction of iNOS in lung tissue of rats, but not guinea pigs. Lastly, inhalation of endotoxin or cotton dust aerosols induces NO•-dependent chemiluminescence from alveolar macrophages of rats, but not guinea pigs.

It is not clear why investigators are unsuccessful in observing induction of NOS in the guinea pig. However, the lack of induction leads one to question the role of NO• in the development of pulmonary reactions to cotton or other organic dusts, since the guinea pig appears to be an excellent model for the acute responses to these organic dusts (Castravova et al., 1996). Indeed, humans and guinea pigs exhibit the following similarities in acute organic dust reactions:

1) febrile response
2) increased breathing rate
3) neutrophil recruitment
4) activation of alveolar macrophages
5) increased airway reactivity
6) time course of response
7) Monday accentuation
8) dependent on endotoxin

Therefore, either unknown technical difficulties obscure the induction of NOS in guinea pigs or NO• production is a response to organic dust-induced inflammation rather than a cause of the inflammatory reaction.

Summary

Upregulation of iNOS and NO• production is demonstrated in response to in vitro exposure in alveolar macrophages, alveolar type II cells, and tracheal smooth muscle. In vivo exposure to LPS by an IV, IP, IT, or inhalatory route increases iNOS and NO• production in lung tissue, alveolar macrophages, interstitial macrophages, type II cells, and pulmonary arterial smooth muscle. Following inhalation of cotton dust, induction of mRNA for iNOS and NO• production in alveolar macrophages and pulmonary phagocytes is observed. Although the guinea pig is an excellent animal model for acute reactions to organic dust and endotoxin exposure, LPS or cotton dust stimulated induction of NOS is difficult to document in this species. Therefore, the role of enhanced NO• production as a cause of the inflammatory pulmonary reaction to endotoxin or organic dusts may be questioned and requires further investigations.

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Table 1. Types of nos found in lung cells.

Lung Cell	NOS isoform	Condition	Presence	Reference
Alveolar Macrophages	eNOS	Basal	Low	Miles et al, in press
	nNOS	Basal	No	ibid
	iNOS	Basal	No	ibid
	iNOS	IFNg	High	Huffman et al, 1998
Interstitial Macrophages	iNOS	IFNg/LPS	High	Wizemann et al, 1994
Type II Cells	eNOS	Basal	Moderate	Miles et al, 1997
	nNOS	Basal	No	ibid
	iNOS	IFNg or IL-1	Moderate	Gutierrez et al, 1995
Fibroblasts	iNOS	IFNg	High	Jorens et al, 1992
Pulmonary Arterial Smooth Muscle Cells	iNOS	cytomix (IL-1b, TNFa, INFg, LPS)	High	Nakayama et al, 1992
Pulmonary Endothelial Cells	eNOS	Basal	Low	Dinh-Xuan et al, 1991
Airway Neurons	nNOs	Basal	Low	Belvisi et al, 1992
Neutrophils	iNOS	silica stimulated	High	Blackford et al, 1994

Table 2. Response of rat alveolar macrophages to in vitro exposure to lps.

Treatment	$NO^{\bullet}$ Production (% Control)	iNOS Protein (Western)	iNOS mRNA (Northern)
Control	100	no	no
LPS (10ng/ml)	3621 ± 297*	___	yes
LPS (100ng/ml)	4697 ± 539*	yes	yes

Values for $NO^{\bullet}$ production are given as % of control levels (means ± SEM of 3 experiments). Message and protein for iNOS is designated as detectable (yes) or undectable (no). Data modified from Huffman et al, 1998 or Ruetten and Thiemerman, 1996. *indicates a significant increase from control.

Table 3. Response of human type ii cells to in vitro exposure to lps.

Treatment	NO• Production (% Control)	iNOS Protein (Western)	iNOS mRNA (Northern)
Control	100	no	no
LPS (10 mg/ml)	250*	_____	_____
LPS + cytokines	800*	yes	yes

Values for NO •production are given as % of control release. Protein and message for iNOS is designated as detectable (yes) or undetectable (no). Data modified from Gutierrez et al. (1995). *indicates a significant increase from control.

Table 4. Response of guinea pig trachea to in vitro exposure to lps.

	Contractility to Methacholine		
Treatment	Intact Epithelium (% Control)	Denuded Epithelium (% Control)	Effect of L-NAME
Control	100	100	None
LPS (10 mg/ml)	27*	68*	Decrease in EC_{50}

Values for contraction in response to methacholine are given as % of control. L-NAME is an inhibitor of NOS. Data modified from Fedan et al. (1995). *indicates a significant decrease from control.

Table 5. Response of rat lung tissue to in vivo exposure to lps.

Treatment	NOS Activity (L-citruline production - % Control)	eNOS mRNA (Northern, % Control)	iNOS mRNA (Northern, % Control)
Control	100	100	100 (very low)
LPS	600*	33*	900*

Values are given as % of control. Data modified from Griffiths et al. (1997) or Liu et al. (1996). *indicates a significant difference from control.

Table 6. Response of alveolar phagocytes to in vivo exposure to lps.

Treatment	NO• Production (% Control)	NO •-dep. CL (% Control)	iNOS mRNA (Northern, % Control)
Control	100	100	100
LPS			
1. IT (0.25mg/100g, 18 hr post)	_____	1800 ± 400*	2333 ± 267*
2. Inhalation (2.2 EU/m^3 for 3 hr, 18 hr post)	195 ± 49*	5700 ± 2300*	1314 ± 450*
3. IV (5mg/kg, 24 hr post)	_____	_____	Increase

Values are given as % of control (mean ± SEM of 3 experiments). *indicates a significant increase from control. Data modified from Blackford et al. (1994), Huffman et al. (1997), or Wizemann et al. (1994).

Table 7. Response of pulmonary arterial smooth muscle to in vivo exposure to lps.

	Contractility to Phenylephrine (% Control)		
Treatment	Intact Endothelium (% Control)	Denuded Endothelium (% Control)	iNOS mRNA (Northern, % Control)
Control	100	100	100
LPS (IV, 200 mg/kg; 4 hr post)	50*	38*	___
LPS + NLA	70+	61+	___
LPS (IP, 20 mg/kg; 4 hr post)	50*	45*	1200*

Values are given as % of control. *indicates a significant decrease from control. + indicates a significant inhibition of LPS-induced relaxation in the presence of a NOS inhibitor. Data modified from Russell et al. (1993) or Griffiths et al. (1995).

Table 8. No• production following Inhalation of cotton dust by rats.

Treatment	NO • in Lavage Fluid (% Control)	NO• Production by BALC (% Control)	iNOS mRNA (Northern, % Control)	NO•-dep CL (% Control)
Control	100	100	100	100
Cotton Dust (40 mg/m^3 for 3 hr, 18 hr post)	127 ± 5*	271 ± 45*	2200 ± 143*	350 ± 100*

Values are given as % of control (means ± SEM of 3-6 experiments). *indicates a significant increase from control. Data modified from Huffman et al. (1997).

Table 9. Species variability of lps-induced no• responses.

Treatment	Species	Decreased Pulmonary Artery Contractility	Decreased Airway Contractility	NO • -dep CL from AM	Lung Tissue iNOS	References
LPS (IP)	rat	yes	___	___	___	Griffiths et al, 1995
	guinea pig		no			Fedan et al, 1995
Endoxtin (IV)	rabbit	yes	no	___	___	Russell et al, 1993
	guinea pig	no	no	no		Russell et al, 1994
LPS (IV)	rat	___	___	___	yes	Salter et al, 1991
	guinea pig				no	
Endotoxin (Inhalation)	rat	___	yes	yes	___	Huffman et al, 1997; Pauwels et al, 1990 Russell et al, 1994 Folkerts et al, 1988
	guinea pig	no	no	no		
Cotton Dust (Inhalation)	rat	___	___	yes	___	Huffman et al, 1997; Castranova (unpublished)
	guinea pig			no		