Cytokines Stimulate Lung Epithelial Cells to Release Nitric Oxide

Richard A. Robbins* and O Jung Kwon**

Research Service, Omaha VA Medical Center, Omaha, NE 68105
*Pulmonary and Critical Care Medicine, Department of Internal Medicine,
University of Nebraska Medical Center, Omaha, NE 68198-5300
**Division of Pulmonology, Department of Medicine, Samsung Medical Center, Seoul, Korea

INTRODUCTION

Nitric oxide (NO) is an important mediator of vaso-regulation. Many recent reports reflect an increasing awareness that NO also plays important roles in a number of physiologic and pathophysiologic processes. Among these proposed roles for NO are modulation of edema formation, inflammation, and regulation of non-vascular smooth muscle tone. Many of these would implicate NO in basic allergic mechanisms and disorders.

NO is formed when the guanido group of the essential amino acid L-arginine is cleaved forming NO and L-citrulline. The reaction is catalyzed by nitric oxide synthase (NOS) and several cofactors. NOS exists in several forms. A constitutive NOS (cNOS) has been identified in endothelial and a variety of non-endothelial cells, which is a constitutively expressed, calcium-dependent enzyme accounting for the baseline production of small, picomolar amounts of NO. An inducible form of NOS (iNOS) has also been detected in a variety of tissues and organs. iNOS is not usually expressed in most tissues, but is induced by lipopolysaccharide (LPS) or cytokines resulting in the formation of larger, nanomolar amounts of NO.

NO is a highly reactive gas with a half life of seconds in biological tissues. NO reacts rapidly with O2· to form peroxynitrite (ONOO−) through several intermediate reactions. Nitrite is stable for several hours in water and plasma but is rapidly converted to nitrate in whole blood.

A mechanism with potential relevance in asthma has been the observation that macrophages from asthmatics spontaneously release cytokines such as TNF and IL-1. These macrophage-derived cytokines may in turn interact with airway epithelium resulting in a number of proinflammatory events including secondary release of cytokines such as interleukin-8 and RANTES. Airway blood vessel dilatation and edema have been proposed to play a major role in the airway obstruction in asthma. Paré and colleagues, through the use of mathematical modeling, have demonstrated that small amounts of airway edema can result in significant airway narrowing, resulting in airway obstruction. NO might contribute to edema formation and narrowing. In support of this concept, NO inhibitors reduce microvascular permeability of guinea pig airways in response to inhalation.
of histamine, platelet activating factor, substance P, A23187, or ovalbumin in sensitized animals\textsuperscript{22} and expression of iNOS has been demonstrated in bronchial epithelial cells in patients with asthma\textsuperscript{23}.

In the above context we made the hypothesis that cytokines released from stimulated alveolar macrophages in disorders such as asthma might interact with airway epithelial cells resulting in induction of iNOS, increased NO formation, and resultant edema and airway narrowing(Fig. 1).

**ALVEOLAR MACРОPHAGE STIMULATION OF NO PRODUCTION IN LUNG EPITHELIAL CELLS**

In order to determine the capacity of alveolar macrophages to stimulate iNOS in lung epithelial cells, bronchoalveolar lavage was performed in 7 normal, nonsmoking adults\textsuperscript{24}. The resultant cell population consisted of >85% alveolar macrophages(AM) with occasional ciliated cell, neutrophil, eosinophil, or other cell type identified. The AMs were suspended at $1 \times 10^6$ cells/ml in RPMI-1640 with 10% fetal calf serum and cultured for 24 hours in the presence of opsonized zymosan\textsuperscript{25}. After centrifuging to remove the zymosan, 100 µl of the AM supernatant was added to confluent cultures of the human lung epithelial cell line, A549, or the murine lung epithelial cell line, LA-4, with 900 µl of Ham’s F-12 medium. After 24 hours, the supernatant fluids were harvested and evaluated for nitrite and nitrate.

Nitrite was measured by a sensitive(<1 nM) method employing conversion of nitrite to NO in glacial acetic acid with 1% KI under a nitrogen stream\textsuperscript{26}. NO was detected with a chemiluminescence analyzer (Model 270B, Sievers, Boulder CO). Nitrite + nitrate was similarly measured except that nitrate was first converted to nitrite by incubating the sample with *E. coli* nitrate reductase(0.05 unit/ml, final concentration).

Nitrite was increased in the A549 culture supernatant compared to the AM culture supernatants. However, there was no increase compared to A549 cells cultured in media alone(Fig. 2). In contrast, nitrite + nitrate was markedly increased in the A549 supernatant fluids obtained from cells cultured with the AM supernatant compared to cells cultured in media alone.

LA-4 cells cultured for 24 hours had marked increases in nitrite and nitrite + nitrate compared to cells culture in media alone(Fig. 2).

Further experiments were done with the LA-4 cells to determine if macrophage derived cytokines such as TNF or IL-1 contributed to the AM-induced increases in nitrite. Antihuman TNFα and/or monoclonal mouse

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{Proposed role for NO in asthma. Alveolar macrophages are stimulated to release cytokines such as tumor necrosis factor α(TNF) and interleukin-1β(IL-1). These cytokines increase nitric oxide(NO) production by bronchial epithelial cells resulting in increased airway edema formation and increased NO detected in exhaled air.}
\end{figure}
antihuman IL-1β antibodies were preincubated with an AM supernatant fluid and the capacity of the fluid to stimulate NO production by the LA-4 cells evaluated by measuring nitrite concentrations in the supernatant fluids after 24 hours as above. In addition, the capacity of the competitive nitric oxide synthase inhibitor N6-monomethyl-L-arginine(L-NMMA) to inhibit the nitrite accumulation in the LA-4 culture
supernatant fluids was assessed as a negative control.

The antibody blocking experiments demonstrate that both the anti-TNF and/or anti-IL-1 antibodies reduced nitrite accumulation in the LA-4 culture supernatant fluids (Fig. 4). The reduction in nitrite seen with the antibody blocking experiments approximated the reduction seen with the NOS inhibitor, L-NMMA.

**CYTOKINE STIMULATION OF iNOS IN LUNG EPITHELIAL CELLS**

Based on the studies with the AM culture supernatant fluids, further studies were done with purified cytokines to determine if the increased accumulation of nitrite in the airway epithelial cell culture supernatant fluids was secondary to increased expression of iNOS\(^\text{260}\). LA-4 cells were stimulated with cytomix, a combination of IL-1\(\beta\), TNF\(\alpha\), and interferon-gamma, all at 10 ng/ml final concentration. Consistent with the experiments with the AM culture supernatant fluids, cytomix increased nitrite levels by 873% in the culture supernatant fluids. An increased number of cells were stained for iNOS on immunocytochemistry. An increase in iNOS mRNA was also observed (Fig. 5). Dexamethasone decreased the cytokine-induced increase in nitrite levels, NOS activity, iNOS immunoreactivity, and mRNA.

Similarly, cytomix increased inducible nitric oxide synthase(iNOS) expression in the human lung epithelial cell line, A549, and primary cultures of human bronchial epithelial cells\(^\text{27}\). Cytomix induced a time-dependent increase in nitrite levels in culture supernatant fluids\(^p < 0.05\). Increased iNOS mRNA level was detected in the cytokine-stimulated cells com-
pared to control (Fig. 6). Dexamethasone diminished
the cytokine-induced increase in nitrite, iNOS by
immunocytochemistry. and iNOS mRNA.

The above data demonstrate that cytokines which
may be released by cells within the lung can induce
lung epithelial iNOS expression and NO release
which is attenuated by dexamethasone.

EXHALED NITRIC OXIDE

The data above suggest that AMs releasing cyto-
kines such as TNF and IL-1 may induce lung ep-
ithelial cells to increase expression of iNOS resulting
in increased nitric oxide production. Asthma, but not
COPD, is a disorder which has been associated
with an increase in AM release of TNF and IL-1\textsuperscript{16,17}.
Therefore, we hypothesized that increased airway
epithelial cell NO production would be associated
with increased exhaled NO in asthmatics not recei-

![Graph](image1)

**Fig. 5.** Northern blot determination of iNOS mRNA levels from LA-4 cells cultured at 4, 8, or 24
hours in the presence of media, cytomicx, or cytomicx + dex (dexamethasone \(10^{-6}\) M) ex-
pressed as the ratio of optical density of scanning laser densitometry of iNOS mRNA
to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA.
\(n=3\) each data point. *p < 0.05 cytomicx compared to media and cytomicx compared to
cytomicx + dex. **p < 0.05 cytomicx compared to cytomicx + dex.

![Graph](image2)

**Fig. 6.** Northern blot determination of iNOS mRNA levels from A549
cells (Panel A) or primary cultures of human bronchial epithelial cells
(Panel B) cultured for 24 hours in the presence of media, cytomicx,
or cytomicx + dex (dexamethasone \(10^{-6}\) M). iNOS mRNA is
expressed as the ratio of optical density on scanning laser densi-
tometry of iNOS mRNA to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. \(n=3\) each data point.
*p < 0.05 cytomicx compared to media and ** cytomicx compared to
cytomicx + dex compared to cytomicx.
Fig. 7. Peak exhaled NO levels in normal, nonsmoking controls (normals, n=23), asthmatics not receiving corticosteroids (Asthma -steroids, n=7), asthmatics receiving corticosteroids (Asthma + steroids, n=11), and patients with chronic obstructive pulmonary disease (COPD, n=14). The asthmatics receiving corticosteroids had significantly greater exhaled NO levels than the other 3 groups (p<0.05). The asthmatics receiving corticosteroids and the COPD patients did not differ from the normal nonsmoking controls.

SUMMARY

Cytokine release from alveolar macrophages and subsequent interaction of these cytokines with the bronchial epithelium can induce epithelial cells to release inflammatory mediators. Nitric oxide (NO), a highly reactive gas formed from arginine by nitric oxide synthase (NOS), is known to be involved in inflammation and edema formation, and the inducible form of NOS (iNOS) can be increased by cytokines. In this context, we hypothesized that lung epithelial cells could be stimulated by cytokines released by alveolar macrophages to express iNOS. To test this hypothesis, the murine lung epithelial cell line, LA-4, or the human lung epithelial cell line, A549, were stimulated with culture supernatant fluids from alveolar macrophages. NO production was assessed by...
evaluating the culture supernatant fluids for nitrite and nitrate, the stable end products of NO. Both murine and human cell culture supernatant fluids demonstrated an increase in nitrite and nitrate which were time- and dose-dependent and attenuated by TNFα and IL-1β antibodies (p < 0.05, all comparisons). Consistent with these observations, cytotoxic a combination of TNFα, IL-1β, and γ-interferon, stimulated the lung epithelial cell lines as well as primary cultures of human bronchial epithelial cells to increase their NO production as evidenced by an increase in nitrite and nitrate in their culture supernatant fluids, an increase in the iNOS staining by immunocytochemistry, and an increase in iNOS mRNA by Northern blotting (p < 0.05, all comparisons). The cytokine effects on iNOS were all attenuated by dexamethasone. To determine if these in vitro observations are reflected in vivo, exhaled NO was measured and found to be increased in asthmatics not receiving corticosteroids. These data demonstrate that alveolar macrophage derived cytokines increase iNOS expression in lung epithelial cells and that these in vitro observations are mirrored by increased exhaled NO levels in asthmatics. Increased NO in the lung may contribute to edema formation and airway narrowing.

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