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EXPRESSION OF INDUCIBLE NITRIC OXIDE IN HUMAN LUNG EPITHELIAL CELLS

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SUMMARY. Nitric oxide (NO) is increased in the exhaled air of subjects with several airway disorders. To determine if cytokines could stimulate epithelial cells accounting for the increased NO, the capacity of the proinflammatory cytokines (cytomix: tumor necrosis factor-α, interleukin-1ß, and interferon-γ) to increase inducible nitric oxide synthase (iNOS) was investigated in A549 and primary cultures of human bronchial epithelial cells. Cytomix induced a time-dependent increase in nitrite levels in culture supernatant fluids ($p<0.05$). Increased numbers of cells stained for iNOS and increased iNOS mRNA was detected in the cytokine-stimulated cells compared to control ($p<0.05$). Dexamethasone diminished the cytokine-induced increase in nitrite, iNOS by immunocytochemistry, and iNOS mRNA. These data demonstrate that cytokines, such as those released by mononuclear cells, can induce lung epithelial iNOS expression and NO release, and that this is attenuated by dexamethasone. © 1994 Academic Press, Inc.

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; TNF, Tumor necrosis factor-α; IL-1, interleukin-1ß; IFN, interferon-γ; DMEM, Dulbecco's modified Eagle's medium; dCTP, deoxycytosine triphospate; FCS, fetal calf serum; HBECs, human bronchial epithelial cells; HBSS, Hanks' balanced salt solution; L-NMMA, L-N^G-monomethyl-arginine; L-NAME, L-nitroarginine methyl ester; cNOS, endothelial constitutive nitric oxide synthase.

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Nitric oxide (NO) is formed when L-arginine is enzymatically converted to citrulline by nitric oxide synthase (NOS) (1). Several forms of NOS have been described, and an inducible NOS (iNOS) can be expressed by a variety of cells in response to proinflammatory cytokines, resulting in the formation of large amounts of NO (2). NO is a highly reactive gas that rapidly forms the more stable nitrite or nitrate in biological fluids (2).

NO has been detected in exhaled air and is increased in several inflammatory lung disorders including the airway diseases asthma and bronchiectasis (3,4). Although alveolar macrophages are a potential source of increased NO in exhaled air, another possible mechanism is the stimulation of airway epithelial cells to express iNOS and release NO by cytokines released from lung mononuclear cells (5).

To demonstrate the potential for airway epithelial cells to express iNOS and release NO, a human lung epithelial cell line, A549, and human bronchial epithelial cells were cultured in the presence of cytomix, a combination of tumor necrosis factor α (TNF), interleukin-1ß (IL-1), and interferon γ (IFN), which has been reported to markedly stimulate iNOS expression in other cells (5,6). Furthermore, we examined the effect of corticosteroids on the expression of iNOS in epithelial cells as dexamethasone attenuates iNOS expression in endothelial cells and murine epithelial cells (5,7).

Materials and Methods

Materials. Recombinant human TNF (50 U/ng) was purchased from British Bio-technology (Oxford, UK); Vitrogen 100 from Collagen Co; Ham's F-12 medium, Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, and L-glutamine from ICN Flow (High Wycombe, UK); fetal calf serum (FCS) from Sera Lab (Crawley Down, UK); 35 mm 6-well culture plates from Falcon (London, UK); [α-^{32}P]dCTP and Hybond-N filters from Amersham International (Amersham UK); agarose from Promega (Southampton, UK); water-saturated phenol from Rathburn Chemical Ltd (Walkerburn, UK); chloroform and isopropanol from BDH (Poole, UK); and the remaining reagents from Sigma (Poole, UK).

Culture of A549 cells. The human lung epithelial cell line, A549, was purchased from American Type Culture Collection

(Rockville, MD) (8) and grown on 35 mm 6-well culture plates in DMEM containing 10% FCS, L-glutamine (2 mM), and penicillin-streptomycin (100 U/ml-100 µg/ml). When confluent, the A549 cells were washed and incubated for 24 h in DMEM without FCS prior to cytokine stimulation.

Isolation and culture of human bronchial epithelial cells. Trachea or main bronchi were obtained from heart or heart/lung transplantation donors and human bronchial epithelial cells (HBECs) prepared as described previously (9). Briefly, the cartilaginous portions were excised and incubated overnight at 4°C in 0.1% bacterial protease (*Streptomyces griseus* type XIV) in Ca^{++}/Mg^{++}-free Hanks' balanced salt solution (HBSS). Epithelial cells were isolated by repeatedly flushing the luminal surface with HBSS, and after washing, cultured on onto Vitrogen 100-coated 35 mm 6-well culture plates in serum-free, hormone-supplemented Ham's F12 medium containing penicillin-streptomycin, amphotericin B (2 µg/ml), L-glutamine, insulin (5 µg/ml), transferrin (5 µg/ml), epidermal growth factor (25 ng/ml), endothelial cell growth supplement (15 µg/ml), cholera toxin (10 ng/ml), and retinoic acid (0.1 µM). When the HBECs were nearly confluent, the cells were washed and incubated with Ham's F12 for an additional 24 h prior to cytokine stimulation.

The epithelial nature of the HBECs was confirmed using Immu-Mark Anti-cytokeratin Universal Kit (ICN Flow). More than 99% of the cells were stained with cytokeratin antibodies demonstrating the epithelial origin of the cells. Staining for vimentin and CD14 was also performed using a vimentin immunocytochemistry kit (Sigma) and a monoclonal human CD14 antibody (Sigma) to exclude the possibility of contamination by fibroblasts or monocytes. No significant staining was observed with either antibody.

Stimulation with cytokines. After incubating for 24 h in serum-free media, the A549 or HBECs were stimulated with cytomix. For most experiments, concentrations of 10 ng/ml of each cytokine were used. To investigate the effects of corticosteroids, dexamethasone was added to some cultures at the time of stimulation. Culture supernatant fluids were collected and either analyzed immediately or stored at -20°C for measurement of nitrite. Total cellular RNA was extracted from some layers of adherent cells.

Nitrite determinations. Nitrite concentrations were determined in the A549 cell culture supernatant fluids by the

Griess reaction (5). Nitrite concentrations in the HBEC culture supernatant fluids were below the level of detection of the Griess reaction, and for this reason, nitrite concentrations in HBEC culture supernatant fluids were determined by conversion of nitrite to NO, measuring NO using a chemiluminescence analyzer, and comparing the unknowns to the NO generated by known amounts of sodium nitrite (5,10).

Immunocytochemistry. A549 or HBECs were grown on tissue culture slides (NUNC, Labtek; ICN Biomedicals Ltd, UK). The cells were stimulated as above when subconfluent and fixed with freshly prepared 1% paraformaldehyde for 20 min, washed in buffered 15% sucrose solution, and permeabilized by 0.02% Triton X-100 solution. Inducible NOS was detected by staining with the avidin biotinylated-peroxidase complex (ABC; Vector Labs, Peterborough, UK) using two rabbit antisera, one to a peptide sequence of human iNOS that is known to react with human liver, and another to a peptide from the murine iNOS sequence (5,11).

Northern blot analysis. Total cellular RNA was extracted using a modification of the methods of Chomczynski and Sacchi (12). The RNA samples were applied to 1% denaturing agarose gels, electrophoresed, and transferred to Hybond-N filters. The filters were hybridized with a [^{32}P]-labelled human iNOS cDNA probe (1 x 10^6 cpm/ml) generated by random priming (Multiprime DNA labelling system, Amersham). The human iNOS cDNA probe used was the 0.9 kb cDNA fragment spanning the presumed coding region of hepatocyte iNOS cDNA clone (PvuII fragment) (13). The filters were washed at a final stringency of 0.2x SSC/0.1% SDS and 50°C and autoradiography performed for 3-7 days.

After autoradiography, the filters were stripped and re-hybridized with a [^{32}P]-labelled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (5). The GAPDH cDNA was the 1272-bp Pst I fragment from rat GAPDH cDNA. Autoradiographs were assessed by scanning laser densitometry. To control for any unequal loading of RNA, iNOS mRNA levels were expressed as the ratio of iNOS to GAPDH mRNA.

Statistics. All results are reported as mean ± SEM. Statistical analysis was made using the two-tailed Student's t test with significance defined as $p<0.05$.

Results

Nitrite determinations. A549 and primary cultures of HBECs contained increased amounts of nitrite in their culture

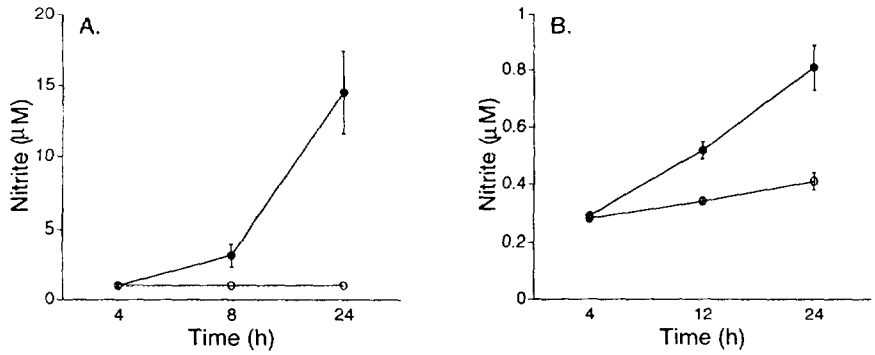

Figure 1. Nitrite levels in A549 (Panel A) or primary human bronchial epithelial cell (Panel B) culture supernatant fluids. In each panel, nitrite concentrations are on the vertical axis and the time after addition of either fresh serum-free media with or without a cytokine mixture (cytomix, TNF, IL-1, and INF at 10 ng/ml) is on the horizontal axis (n=3, each time point). Nitrite levels were below the lower limit of detection by the Griess reaction in supernatant fluids obtained A549 cells cultured in media alone but was detectable after 8 and 24 hours in the A549 supernatant fluids obtained from the cells cultured with cytokines. Nitrites were significantly increased after 12 and 24 hours in the primary human bronchial epithelial cell supernatant fluids obtained from cells cultured with cytomix compared to those cultured in media alone ($p<0.05$, both comparisons).

supernatant fluids compared to controls cultured in media alone (Figure 1). Dexamethasone at a concentration of 10^{-6}M significantly decreased the nitrite concentration in both the A549 and HBEC culture supernatant fluids (Figure 2, $p<0.05$ both comparisons). L-N^G-monomethyl-arginine (L-NMMA, 10^{-4}M) and L-nitroarginine methyl ester (L-NAME, 10^{-4}M) significantly decreased the cytokine-induced nitrite concentrations in the A549 and HBEC

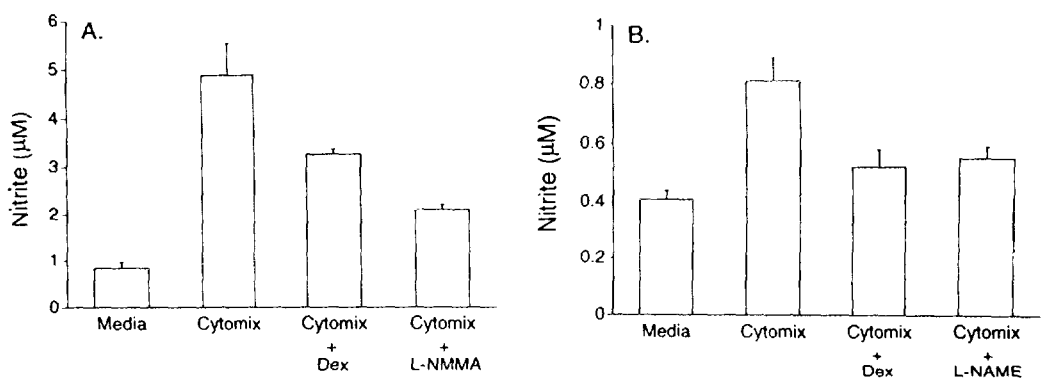

Figure 2. Nitrite levels in A549 (Panel A) or primary human bronchial epithelial cell (Panel B) culture supernatant fluids after cytokine stimulation (TNF, IL-1, and INF at 10 ng/ml). Dexamethasone (Dex, 10^{-6}M), L-NMMA 10^{-4}M, or L-NAME 10^{-4}M added at the time of cytokine stimulation significantly decreased the nitrite levels (n=3, $p<0.05$ all comparisons).

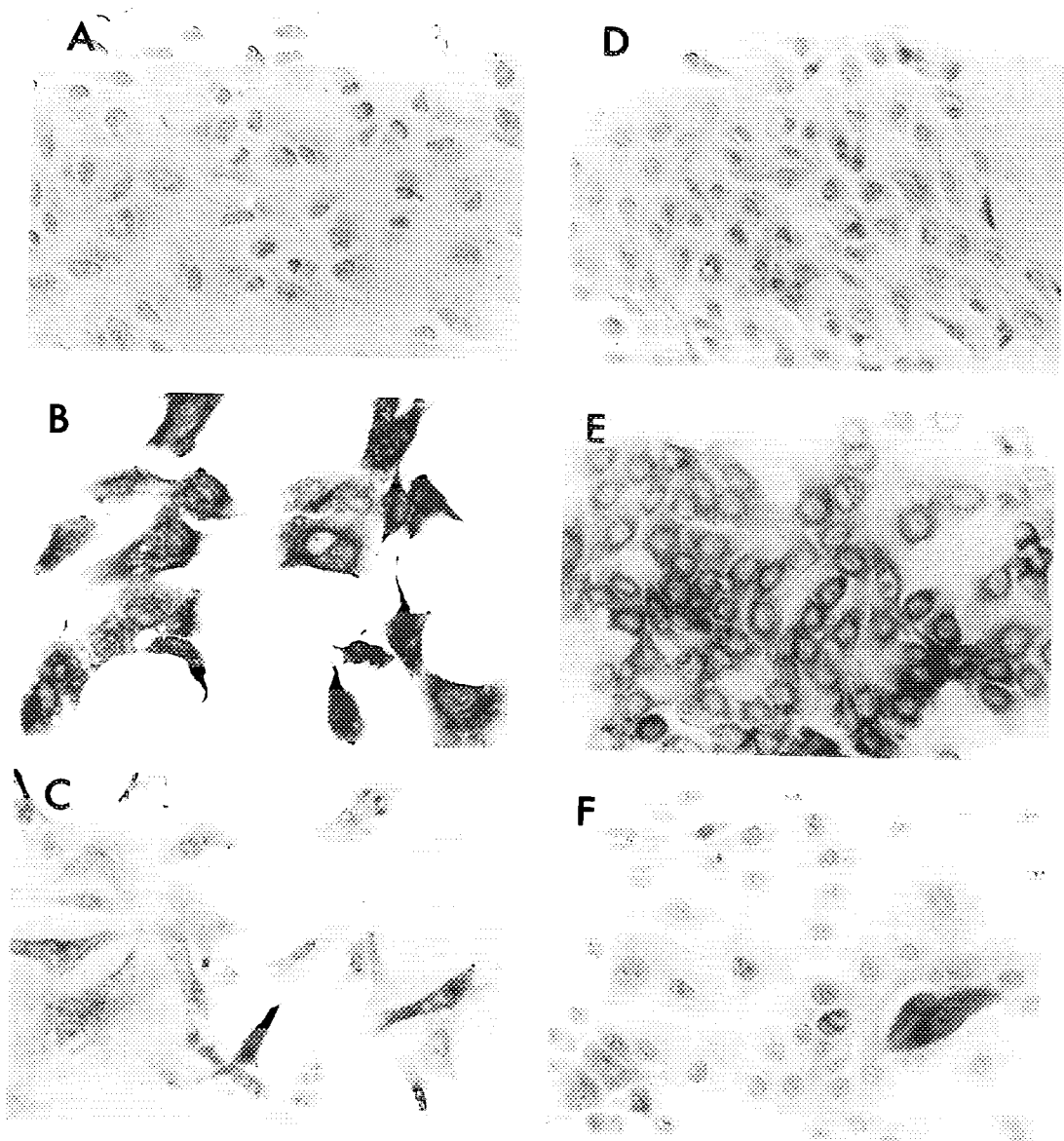

Figure 3. Immunocytochemistry for iNOS using the murine iNOS antisera. Panels A, B, and C represent A549 cells and D, E, and F HBECs cultured for 24 h in the presence of media, cytomix, or cytomix + dexamethasone 10^{-6} M, respectively. Identical results were obtained using the human iNOS antisera.

culture supernatant fluids (Figure 2, $p<0.05$ both comparisons). Nitrite concentrations in culture supernatant fluids of A549 cells cultured with the with TNF, IL-1, and INF at a concentration of 1 ng/ml was less than with the cytokines added at 10 ng/ml (1.9 $\pm$ 0.2 vs. 2.9 $\pm$ 0.2 μM, n=3). A549 cells cultured for 24 h with the cytokines added at 0.1 ng/ml or with each cytokine added alone

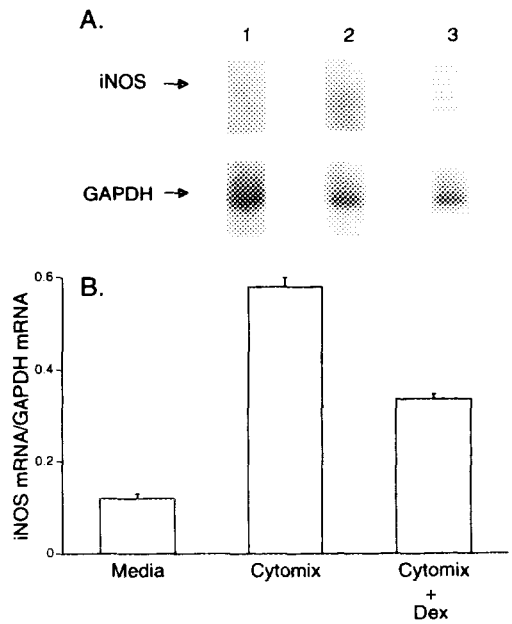

Figure 4. Northern blot analysis for iNOS mRNA in A549 cells. A representative Northern blot analysis is Panel A showing iNOS mRNA levels in A549 cells cultured for 24 hours in media alone (Lane 1), cytomix (Lane 2; TNF, IL-1, and IFN at 10 ng/ml), or cytomix with dexamethasone (Lane 3; 10^{-6}M). Below is the GAPDH mRNA of the same lanes. In Panel B is the summary data (n=3) with iNOS mRNA on the vertical axis expressed as ratio to GAPDH mRNA. The cytokines stimulated iNOS which was significantly reduced by dexamethasone ($p<0.05$, both comparisons).

were below the lower limit of detection by the Griess reaction (≈0.5-1.0 μM).

Immunocytochemistry. Strong immunoreactivity for iNOS was seen in most A549 and HBEC cells cultured for 24 h with cytomix (each cytokine 10 ng/ml, Figure 3, panels A,B,D,E). Stimulation with the combination of cytokines at concentrations of 1 ng/ml resulted in less pronounced staining for iNOS and cells cultured with each cytokine individually resulted in only weak staining seen in a small proportion of cells (data not shown). Dexamethasone 10^{-6}M attenuated the cytokine-induced increase in iNOS (Figure 3, panels C,F). Identical results were obtained with the two antisera used.

Northern blot analysis. iNOS mRNA was increased by stimulation with the combination of cytokines for 24 h in A549 (Figure 4) and HBECs (Figure 5). Dexamethasone 10^{-6}M decreased the cytokine-induced increase in iNOS mRNA from both cell types (Figures 5 and 6, p,0.05 both comparisons). Dexamethasone dose-responsively inhibited the cytokine-induced increase in iNOS mRNA in A549 cells (Figure 6).

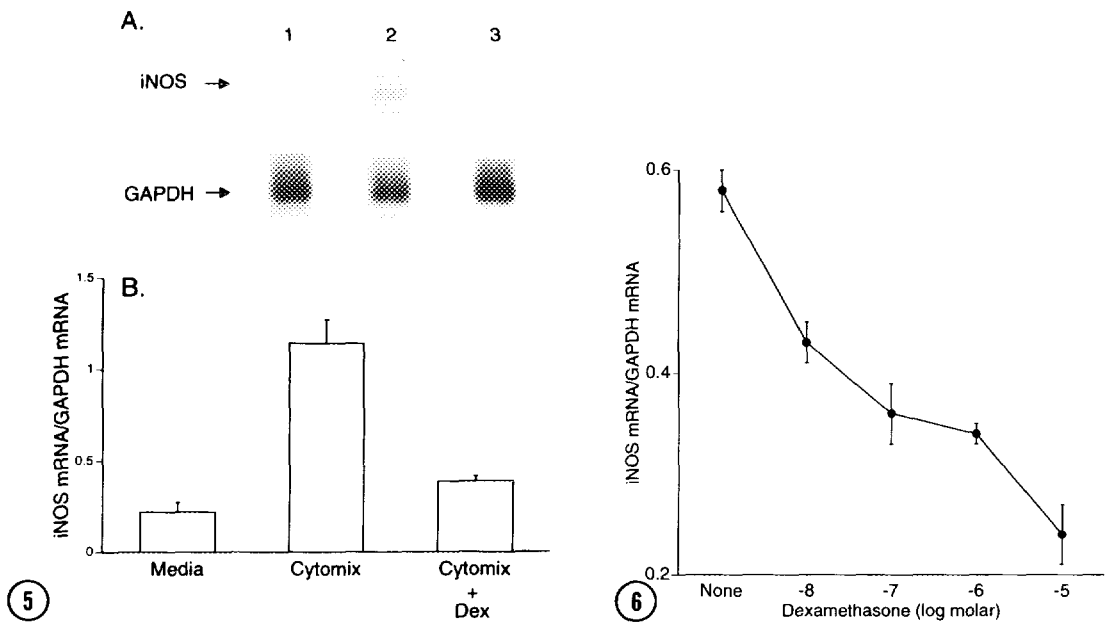

Figure 5. Similar to Figure 4 except human bronchial epithelial cells cultured 24 h were studied (n=3, each data point). Again, the cytokines stimulated iNOS which was significantly reduced by dexamethasone ($p<0.05$, both comparisons).

Figure 6. Inhibition of iNOS mRNA by dexamethasone in A549 cells. Cells were stimulated with cytomix (TNF, IL-1, and IFN 10 ng/ml) for 24 h and iNOS mRNA was quantified by Northern blot analysis and expressed as a ratio to GAPDH mRNA. Dexamethasone at 10^{-5}, 10^{-6}, and 10^{-7} significantly reduced the cytokine-induced increase in iNOS ($p<0.05$).

Discussion

These studies demonstrate that human lung epithelial cells possess the capacity to release NO with cytokine stimulation. This was determined by detecting increased levels of the end-product of NO production, nitrite, in culture supernatant fluids of A549 and HBECs after cytokine stimulation. The increase in nitrite was consistent with the observed increase in iNOS protein and mRNA demonstrated by immunocytochemistry and Northern blot analysis. Furthermore, corticosteroids which are known to attenuate iNOS induction, but not the constitutive endothelial NOS (cNOS), decreased nitrite concentrations in the supernatant fluids and iNOS protein and mRNA in the lung epithelial cells.

NO can be detected in the exhaled air of animals and humans and is increased in several respiratory disorders including asthma and bronchiectasis (3,4). Although the cellular source of the increase in exhaled NO is unknown in these disorders, several

lines of evidence suggest that increased airway epithelial cell expression of iNOS might contribute to the increase in exhaled NO. The high levels of NO in the exhaled air and the high reactivity of NO, suggest that the cellular source likely resides within the airways. Immunohistochemistry has demonstrated increased iNOS in bronchial epithelial cells of subjects with asthma, one disorder associated with high exhaled NO levels (3,14). Studies in a murine lung epithelial cell line, LA-4, demonstrate similar results to the human epithelial cells (5). Corticosteroids, which attenuate iNOS, but not cNOS, decrease exhaled NO levels in asthmatic subjects but not in normal, non-asthmatic controls (3,7,15).

The present study supports the concept that alveolar macrophages are important in regulating NO production within the airways. However, this study does not exclude production of NO by activated human macrophages within the lung, but rather suggests, that activated mononuclear cells may also play a role by releasing cytokines which stimulate NO release by lung epithelial cells. Consistent with this concept, alveolar macrophages are known to release increased amounts of TNF and IL-1 in asthma (16,17).

Increased NO production may have functional significance in airway disorders, such as asthma. NO is a potent bronchial vasodilator in animal airways (18). It is feasible that NO-mediated dilatation of bronchial blood vessels could account, at least in part, for the edema seen in narrowing of the airway in conditions such as asthma. In support of this concept, NO inhibitors reduce neurogenic plasma exudation in guinea pig airways (19). Inhaled corticosteroids which are highly effective in controlling airway inflammation may work, at least in part, by inhibiting the production of NO by cytokine-activated airway epithelial cells.

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References

1. Palmer, R. M. J., Ashton, D. S., and Moncada, S. (1988) Nature 333, 664-666.
2. Moncada, S., and Higgs, E. A. (1993) N. Engl. J. Med. 329,2002-2012.

3. Kharitonov, S. A., Yates, D., Robbins, R. A., Logan-Sinclair, R., Shinebourne, E. A., Barnes, P. J.(1994) Lancet 343, 133-135.
4. Kharitonov, S. A., Wells, A. U., O'Connor, B. J., Cole, P. J., and Barnes, P. J. (1994) Am. J. Respir. Crit. Care Med. 149, A198.
5. Robbins, R. A., Springall, D. R., Warren, J. B., Kwon, O. J., Buttery, L. D. K., Wilson, A. J., Adcock, I. M., Riveros-Moreno, V., Moncada, S., Polak, J., and Barnes, P. J. (1994) Biochem. Biophys. Res. Commun. 198, 835-843.
6. Nakayama, D. R., Geller, D. A., Lowenstein, C. J., Davies, P., Pitt, B. R., Simmons, R.L., and Billiar, T. R. (1992) Am. J. Respir. Cell Mol. Biol. 7, 471-476.
7. Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 10043-10049.
8. Lieber, M., Smith, G. P., Szakal, A., Nelson-Rees, W., and Todaro, G. (1976) Int. J. Cancer 17, 62-70.
9. Kwon, O. J., Au, B. T., Collins, P. D., Mak, J. C., Robbins, R. A., Chung, K. F., and Barnes, P. J. Am. J. Physiol. (in press).
10. Archer, S. A. (1993) FASEB J. 7, 349-360.
11. Evans, T. J., Buttery, L. D. K., Uwechue, N., Springall, D. R., Cohen, J., Polak, J. M. (1993) Endothelium 1(suppl), s27.
12. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
13. Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., DiSilvio, M., Wang, S. C., Nakyama, D., et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 3491-3495.
14. Hamid, Q., Springall, D. R., Riveros-Moreno, V., Chanez, P., Howarth, P., Redington, A., Bousquet, J., Godard, P., Holgate, S., and Polak, M. (1993) Lancet 342, 1510-1513.
15. Kharitonov, S. A., Yates, D. H., Robbins, R. A., Thomas, P. S., and Barnes, P. J. (1994) Am. J. Respir. Crit. Care Med. 149, A201.
16. Gosset, P., Tsicopoulos, A., Wallaert, B., Vannimenus, C., Joseph, M., Tonnel, A. B., and Capron, A. (1991) J. Allergy Clin. Immunol. 88, 561-571.
17. Borish, L., Mascali, J. J., Dishuck, J., Beam, W. R., Martin R. J., and Rosenwasser, L. J. (1992) J. Immunol. 149, 3078-3082.
18. Alving, K., Fornhem, C., Wietzberg, E., and Lundberg, J. M. (1992) Acta Physiol. Scand. 146, 407-408.
19. Kuo H.-P., Liu, S., Barnes, P.J. (1992) Eur J Pharmacol 221, 177-182.