

Short communication

Induction of nitric oxide synthase in a model of allergic occupational asthma

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We have studied the induction of nitric oxide during late allergic responses, using a guinea pig model of trimellitic anhydride (TMA)-induced airway allergy. TMA is a low molecular weight chemical which can cause occupational asthma. The activity of nitric oxide synthase (NOS) was investigated by the detection of ^3H -labeled citrulline formation from ^3H -labeled arginine. In sensitized animals, challenge with TMA conjugated to guinea pig albumin (TMA-GPSA) increased the activity of Ca^{2+} -independent NOS (inducible NOS; iNOS) in lung and bronchial tissues at 15–17 h after challenge compared to nonsensitized animals. The induction of iNOS activity was associated with an increased level of nitrite, an end metabolite of the L-arginine-NO pathway, in bronchoalveolar lavage fluid. In contrast to iNOS, the activity of Ca^{2+} -dependent NOS (constitutive NOS; cNOS) was not affected by the allergen challenge. These results demonstrate that iNOS in bronchial tissue is induced late after allergen challenge in sensitized guinea pig.

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Recent evidence indicates that the nitric oxide (NO) signaling pathway plays a direct role in the regulation of the contractile properties of airway smooth muscle *in vitro* and *in vivo* (13, 18). In addition to the modulatory function of NO under physiologic conditions, there is also accumulating evidence that NO may be involved in several pulmonary diseases. For example, a raised level of NO in exhaled breath has been found in asthmatics, and expression of inducible nitric oxide synthase (iNOS) was demonstrated in the epithelium of asthmatic patients, but not of healthy subjects (5). Furthermore, it was reported that the NO content in exhaled air from sensitized animals immediately increased following exposure to allergen challenge (15). This is probably due to the rapid, Ca^{2+} -dependent activation of the constitutive isoform of NOS (cNOS) (11). Moreover, the basal levels of NO in exhaled air are increased in untreated asthma (9, 16), probably due to induction of iNOS in the airways (5). Thus, NO is overproduced after allergen challenge and in chronic asthma, probably due to activation of both the cNOS and iNOS pathways. The mechanisms and kinetics of these responses are, however, poorly understood.

Trimellitic anhydride (TMA), a known cause of occupational asthma in man, has been successfully used to develop experimental animal models of allergic asthma (1, 7, 8). In the present experiments, we used guinea pigs sensitized to TMA. Three weeks after sensitization, the animals were exposed to TMA conjugated to guinea pig serum albumin (TMA-GPSA), which leads to an immediate airway response (1) and a later eosinophil influx and increase in bronchial hyperresponsiveness (14).

The aim of this study was to determine whether iNOS is present in the airway tissues late after TMA-exposure in sensitized guinea pigs. NOS activity in lung and bronchi was determined by formation of ^3H -labeled citrulline from ^3H -labeled arginine. Levels of nitrite, the end metabolite of NO, were also measured in bronchoalveolar lavage (BAL) in the same animals.

Material and methods

Sensitization

Thirty male Dunkin Hartley guinea pigs, 250–300 g body weight, were actively sensitized according to a

previously described method (1). Briefly, the animals were injected intradermally on two consecutive days with 0.1 ml of 0.3% (wt/vol) TMA suspended in corn oil. Unsensitized control animals were given two intradermal injections of 0.1 ml of corn oil alone. Three weeks later, the animals were placed in a chamber and challenged for 10 min with 0.1% of TMA-guinea pig serum albumin conjugate (TMA-GPSA) or saline aerosol generated by a nebulizer (Maxin[®], Lund, Sweden) driven by an airflow of 8 l/min. One nonsensitized group of animals ($n = 6$) was exposed to nebulized TMA-GPSA, a second group of sensitized animals was exposed to nebulized saline, and a third group of sensitized animals was exposed to nebulized TMA-GPSA. These three groups were killed 15–17 h after the exposure, and the lungs were removed for nitrite and NOS-activity measurements (see below). In three additional groups of sensitized animals, the lungs were removed 2, 8, and 24 h after exposure to TMA-GPSA.

The animals were killed by an overdose of barbiturate anesthetic at the designated time after the saline or TMA-GPSA exposure. BAL was performed by twice gently flushing with 5 ml of prewarmed saline (37°C). The recovery volume was 6.4–7.2 ml. The BAL fluid was centrifuged at 500 g for 10 min at 4°C, and the supernatant was then removed and stored at –70°C until assayed. Organs were removed and stored at –70°C until NOS activity was measured. The total number of inflammatory cells in the lavage fluid was counted with a Bürker chamber.

The bronchial tissue was separated from lung parenchymal tissue by fixing the trachea and gently scraping off the lung tissue, leaving central and segmental bronchi connected to the trachea (10). The segmental bronchi seem to be maintained with the tracheobronchial preparation at the level of at least two bifurcation, or more. The lung tissue was stored at –70°C. The trachea was separated from the bronchial tissue, which was stored at –70°C.

NOS assay

Enzyme activity was determined by formation of ³H-labeled citrulline from ³H-labeled arginine as described (17). In brief, tissues were homogenized in 5 volumes (w/v) of ice-cold buffer containing 50 mM Tris/HCl, 320 mM sucrose, 0.1 mM EDTA, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM DL-dithiothreitol, 10 µg/ml soybean trypsin inhibitor, and 2 µg/ml aprotinin (pH 7.4 at 4°C). Homogenates were centrifuged at 100 000 g for 30 min at 4°C, and the obtained supernatants were used in the enzyme assays.

NOS activity was measured by the ability of homogenates to convert ³H-labeled L-arginine to ³H-labeled citrulline, the coproduct of nitric oxide

(NO) formation. Standard reaction mixtures contained 40 µl of the supernatant, 10 µM L-arginine/³H-labeled L-arginine (100 000 cpm/tube), 2 mM CaCl₂, 1 mM NADPH, 60 mM L-valine, and 1 µM tetrahydrobiopterin in a final volume of 100 µl obtained by the addition of 10 mM HEPES buffer (pH 7.2). After a 20-min incubation at 30°C, the assays were terminated with 1 ml of 20 mM HEPES (pH 5.5), and ³H-labeled citrulline was quantified after separation from ³H-labeled arginine by a Dowex 50 W rinse (200–400, 8% cross-linked Na⁺ form). The activity of Ca²⁺-independent NOS was the difference between the samples containing 1 mM EGTA and samples containing 1 mM EGTA and 2 mM L-NAME. Protein was determined by the Bio-Rad method.

Nitrite assay

Nitrite in the supernatants of BAL was determined by a spectrophotometric method based on the Griess reaction. Briefly, 1 ml of BAL sample was lyophilized, and then mixed with 200 µl of Griess' reagent (1% sulfanilamide, 0.1% naphthylethylenediamide-dihydrochloride, and 2.5% H₃PO₄) and incubated for 10 min at room temperature. Nitrite concentrations in samples were determined using different concentrations of sodium nitrite as a standard curve. The detection limit of the assay is about 1 µM.

L-³H-labeled arginine and L-³H-labeled citrulline were purchased from Amersham International (Amersham, Buckinghamshire, UK). Other reagents were obtained from Sigma Chemical Co (St Louis, MO, USA).

Statistical analysis

Values are presented as mean $\pm$ SEM. Comparisons among groups were performed by the Kruskal-Wallis test, and specific significance tests between groups were determined using the Mann-Whitney U-test.

Table 1. Determination of iNOS and cNOS in lung and bronchi in sensitized guinea pigs exposed to TMA-GPSA at different times after exposure

Time	<i>n</i>	Lung		Bronchi	
		cNOS	iNOS (nmol/min per mg protein)	cNOS	iNOS
2 h	4	2.96 $\pm$ 0.86	0.13 $\pm$ 0.08	1.02 $\pm$ 0.32	0.11 $\pm$ 0.04
8 h	5	1.97 $\pm$ 0.32	0.09 $\pm$ 0.02	1.54 $\pm$ 0.29	0.11 $\pm$ 0.02
16 h	5	2.04 $\pm$ 0.29	0.17 $\pm$ 0.02	0.86 $\pm$ 0.19	0.43 $\pm$ 0.13
24 h	5	2.97 $\pm$ 1.63	0.19 $\pm$ 0.07	0.95 $\pm$ 0.32	0.23 $\pm$ 0.08

iNOS: inducible nitric oxide synthase, measured in absence of Ca²⁺; cNOS: constitutive nitric oxide synthase, measured in presence of Ca²⁺.

Data are shown as mean $\pm$ SEM.

Statistical significance was established at $P < 0.05$. Evaluation of the time-course of NOS activity is presented using descriptive statistics.

Results

In bronchial tissue from TMA-sensitized animals exposed to TMA-GPSA, the Ca^{2+} -dependent NOS activity (cNOS) was unaffected 15–17 h after exposure, whereas the Ca^{2+} -independent NOS activity (iNOS) was substantially increased (Kruskal-Wallis test: $P < 0.05$) (Fig. 1a and c). In lung tissue from allergen-exposed sensitized animals exposed to TMA-GPSA, cNOS was unaffected 15–17 h after exposure, whereas iNOS was slightly increased (Kruskal-Wallis test: $P < 0.05$) (Fig. 1b and d).

The concentration of nitrite in BAL 17 h after challenge of sensitized and TMA-GPSA-challenged animals was significantly increased compared to the two control groups (Kruskal-Wallis test: $P < 0.05$)

(Fig. 2a). However, the concentration of nitrite was even further increased 24 h after allergen challenge (Fig. 2b). The maximal levels of iNOS activity in the bronchial tissue were found at 15–17 h (Fig. 2c). cNOS did not seem to be affected to any great degree by allergen challenge in either bronchial or lung tissue at any time after allergen exposure (Table 1).

The total number of inflammatory cells in the BAL 17 h after challenge was increased in the TMA-GPSA-exposed sensitized animals ($2.6 \times 10^6 \pm 0.4 \times 10^6$), compared to the TMA-GPSA-exposed non-sensitized ($1.40 \times 10^6 \pm 0.17 \times 10^6$) and the saline-exposed sensitized animals ($1.41 \times 10^6 \pm 0.16 \times 10^6$).

Discussion

In the present study, we have found that allergen exposure in sensitized guinea pigs results in a clear increase in iNOS-activity in bronchial tissue at approximately 16 h after challenge. This increase of

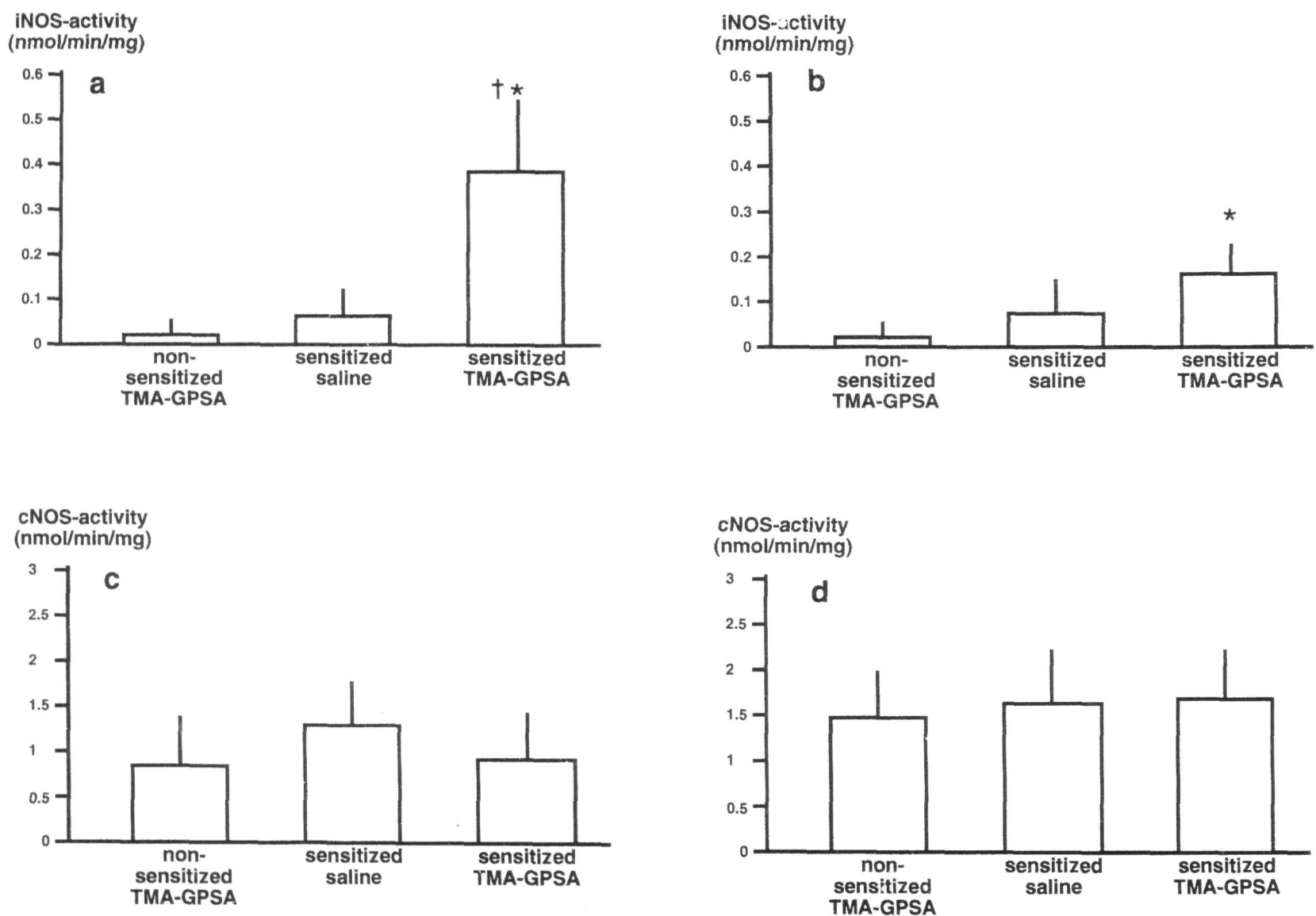


Fig. 1. Levels of iNOS activity in bronchial preparations (a) and lung preparations (b) 15–17 h after TMA-GPSA exposure or saline exposure in unsensitized animals or animals previously sensitized to free TMA (0.3% on two consecutive days 3 weeks previously). Levels of cNOS activity in bronchial preparations (c) and lung preparations (d) 15–17 h after TMA-GPSA exposure or saline exposure in unsensitized animals or sensitized animals. TMA-GPSA exposure increased level of iNOS activity in both bronchial and lung preparations, whereas cNOS was unaffected. Data are shown as means $\pm$ SEM. * $P < 0.01$ between TMA-GPSA-exposed sensitized and nonsensitized animals. [†] $P < 0.01$ between sensitized animals exposed to either saline or TMA-GPSA.

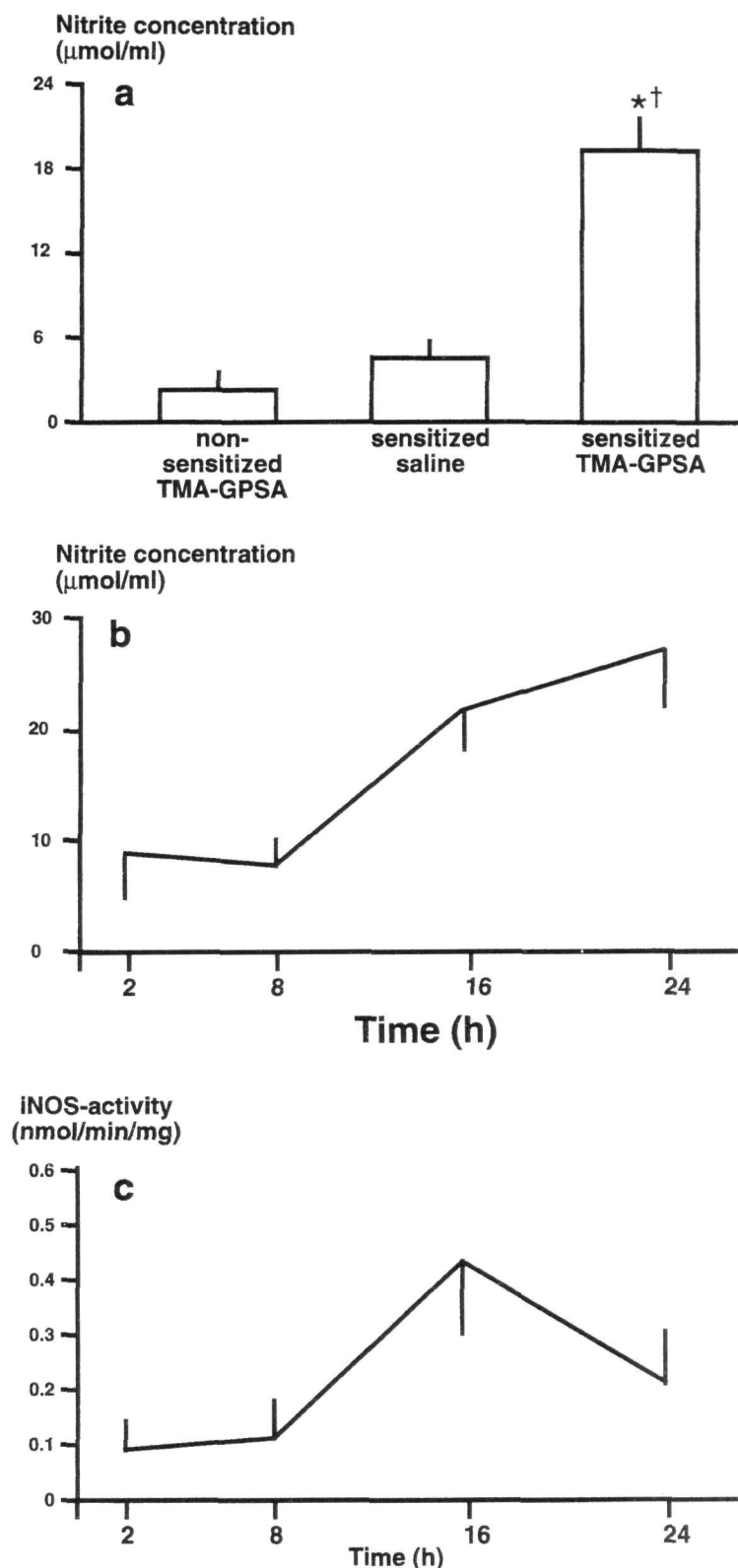


Fig. 2. a) Nitrite concentrations in BAL fluid 15–17 h after TMA-GPSA exposure or saline exposure in unsensitized animals or animals previously sensitized to free TMA (0.3% on two consecutive days 3 weeks previously). Nitrite levels were increased in sensitized and allergen-exposed animals. Data are shown as means $\pm$ SEM. Time-course of iNOS activity in bronchial preparation after TMA-GPSA exposure in animals sensitized to free TMA: maximal level of iNOS activity was found 15–17 h after allergen exposure, and levels were maintained slightly increased also at 24 h. Data are shown as means $\pm$ SEM. * $P < 0.01$ between TMA-GPSA-exposed sensitized and nonsensitized animals. † $P < 0.01$ between sensitized animals exposed to either saline or TMA-GPSA. b) Time-course of nitrite concentrations in BAL fluid after TMA-GPSA exposure in animals sensitized to free TMA. Nitrite was increased 15–17 h after allergen exposure and further increased also at 24 h. Data are shown as means $\pm$ SEM. c) Time-course of iNOS activity in bronchial preparation after TMA-GPSA exposure in animals sensitized to free TMA. Maximal level of iNOS activity was found 15–17 h after allergen exposure, and levels were maintained slightly increased also at 24 h. Data are shown as means $\pm$ SEM.

iNOS activity was associated with an increased level of an end metabolite of NO, nitrite in BAL fluid, further indicating that the expressed enzyme was active *in vivo*.

Three NOS are now distinguished by cloning of their complementary and genomic DNA in several species (12, 13). Two constitutive isoforms, which were first described in endothelial cells and neurons, respectively, are both Ca^{2+} and calmodulin dependent (cNOS). The third isoform (iNOS) is independent of the intracellular Ca^{2+} level. According to these concepts, we detected NOS activity in the presence and absence of Ca^{2+} to estimate the activity of cNOS and iNOS, respectively. In the present study, we detected an increase in activity of Ca^{2+} -independent NOS in lung and bronchi of sensitized and allergen-exposed animals, suggesting that iNOS was induced. Furthermore, the change in activity of Ca^{2+} -independent NOS was associated with a long-lasting and clear increase in nitrite concentration in BAL from sensitized animals, indicating an increased output of NO in the airways from these animals. Since cNOS activation by acetylcholine, bradykinin, and other substances leads only to short-lived production of NO in picomolar amounts (11), it is unlikely that cNOS could contribute to this observed increase in nitrite levels. iNOS, on the other hand, is able to generate NO in the nanomolar range for prolonged periods of time (11). Therefore, it is likely that the large increase in nitrite concentrations was due to iNOS induction after allergen exposure.

iNOS reached a peak at 17 h after allergen challenge, and the activity decreased after that time. At similar time points, a late reaction of allergic asthma has been found in guinea pigs sensitized to TMA, a response which is associated with nonspecific bronchial hyperresponsiveness and eosinophil infiltration (14). In this study, we observed an overproduction of an end metabolite of NO (nitrite) 17–24 h following the allergen challenge, implying that iNOS may play a role in modulating the late response. Although NO has been demonstrated to play a role in the host defense, excessive production of NO may result in deleterious consequences by promoting the inflammatory process (3).

The cellular source of iNOS in the allergen-exposed bronchi is not clear from the present experiments. However, in the airways, at least two classes of cells may be sources of iNOS in response to inflammatory or immunologic stimuli. The first class includes many structural cells, such as epithelial cells, smooth-muscle cells, and endothelial cells (4, 6, 11). The alternative sources are macrophages, mast cells, neutrophils and other infiltrating inflammatory cells (11). Regarding the mechanism underlying iNOS induction in these experiments, we suggest that allergen exposure causes the release of

proinflammatory cytokines locally, and that the subsequent infiltration of inflammatory cells into the bronchial wall triggers the induction of iNOS (12). In fact, we found an increased total amount of inflammatory cells in the BAL fluid. The iNOS may be present in these inflammatory cells, but may also be expressed in parenchymal cells, such as those of the airway epithelium, secondary to cytokine release from the inflammatory cells. Major asthma-associated cytokines, such as tumor necrosis factor- α , interleukin-1, and interferon- γ , have the ability to induce iNOS expression in many cells (2, 3). However, in a clinical bronchoscopy study in asthmatic patients, iNOS was found in the epithelial cells, which may be the most important source (2, 5).

We found a small increase in iNOS activity in the lung tissue specimens. However, the increase of iNOS at this airway level may still be due to iNOS activity in bronchial and bronchiolar tissues, an activity remaining in the lung tissue preparation. To find the exact cellular source of iNOS in this tissue preparation, studies using immunohistochemical techniques or *in situ* hybridization are required.

In summary, our data demonstrate that iNOS in bronchi is induced in response to allergen challenge in sensitized animals. Induction of iNOS is associated with a high production of NO in lung. Further studies are required to find the cellular source and to determine the exact role of NO in the late allergic response.

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