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Inducible nitric oxide synthase after sensitization and allergen challenge of Brown Norway rat lung

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1 We studied the effects of ovalbumin (OA) sensitization and challenge on inducible nitric oxide synthase (iNOS) gene and protein expression in Brown-Norway rats *in vivo*.

2 By use of Northern analysis, a 4.4-kb iNOS mRNA transcript was weakly observed in control rat lung but there was a 3 fold increase in lungs sensitized to OA alone ($P<0.05$). In sensitized rats, four hours after exposure to OA aerosol, there was a 6 fold increase in iNOS mRNA transcript ($P<0.05$), which returned to baseline at 24 h.

3 Immunostaining with an anti-mouse iNOS antibody revealed some patchy staining of airway epithelium in naïve rats. There were no changes in sensitized rats exposed to saline, but sensitized and OA-exposed rats showed increased expression in iNOS staining in macrophages.

4 Electrophoretic mobility shift assays of lung nuclear extracts showed a marked increase in nuclear factor-κB (NF-κB)-binding activity at 2 h after allergen exposure with return to baseline at 6, 12 and 24 h.

5 We concluded that there is increased iNOS gene and protein expression associated with increased NF-κB DNA-binding in lungs of sensitized and challenged rats. The increase in iNOS expression may underlie the increase in exhaled NO found after allergen challenge and may contribute to the development of allergen-induced airway hyperresponsiveness.

Keywords: Nitric oxide; ovalbumin; inducible nitric oxide synthase; nuclear factor-κB; bronchial hyperresponsiveness

Introduction

Nitric oxide synthase (NOS) catalyses the conversion of L-arginine to citrulline and the highly reactive free radical species, nitric oxide (NO) (Moncada *et al*., 1991). Two major isoforms of the enzyme have been identified, namely the constitutively-expressed form (cNOS) and a calcium-independent cytokine-inducible isoform, iNOS (Forstermann *et al*., 1994). iNOS is the most likely to be involved in pathophysiological conditions (Nathan, 1992; Forstermann *et al*., 1994) and it leads to the production of high levels of NO which can be cytotoxic to cells. Excess production of NO has been implicated in the pathogenesis of several inflammatory diseases including asthma (Barnes & Belvisi, 1993). Increased concentrations of NO have been shown in exhaled air of patients with asthma (Kharitonov *et al*., 1994) and immunoreactive iNOS has been localized to the airway epithelium of such patients (Hamid *et al*., 1993), indicating that airway epithelial cells may be the source of excess NO in asthma. In more recent studies, allergen challenge of allergic asthmatic patients led to a delayed rise in NO levels in exhaled air, indicating that iNOS may have been induced (Kharitonov *et al*., 1995).

In order to obtain direct evidence that an increase in iNOS activity is the mechanism by which excessive NO is produced in the airways during antigen-induced immunological responses, we undertook studies in the IgE-ovalbumin sensitized Brown-Norway rat model (Elwood *et al*., 1991; Haczku *et al*., 1995). In this model, sensitization followed by allergen challenge induces bronchial hyperresponsiveness associated with airway inflammation characterized by eosinophil and T-cell influx (Haczku *et al*., 1995). We examined the effects of sensitization and ovalbumin challenge on iNOS mRNA expression in the lungs of these rats; in addition, we performed immunohistochemical localization for iNOS. Because the transcriptional activity of the iNOS gene has been shown to be dependent on the transcriptional regulating factor, nuclear factor-κB (NF-κB) in its promoter region (Lowenstein *et al*., 1993; Adcock *et al*., 1994), we also determined NF-κB DNA-binding activity using electrophoretic mobility shift assay on nuclear lung extracts.

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Methods

Ovalbumin challenge

We studied 5 groups of inbred male Brown-Norway rats weighing 250 to 300 g. Three groups of rats were actively sensitized by i.p. injection of 1 ml suspension of 1 mg ovalbumin and 100 mg of aluminium hydroxide [$AL(OH)_3$] in saline for 3 consecutive days. Control animals were injected with saline alone. Twenty-one days after the initial ovalbumin injection, animals were divided into 3 groups. Animals in sensitized groups were either not exposed or exposed to an aerosol of saline or 1% ovalbumin for 15 min. At 4 or 24 h after exposure, the animals were killed and lungs were removed for RNA extraction and immunohistochemistry. The last group included non-sensitized animals challenged with ovalbumin.

Preparation of cDNA probes

The iNOS and glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probes were amplified by polymerase chain reaction (PCR) as previously described (Liu *et al*., 1993). Two micrograms of total RNA from lungs of control (for GAPDH) or endotoxin-treated (for iNOS) Wistar rats were reverse-transcribed (RT) to cDNA by use of a reverse transcription kit (Promega, Southampton, U.K.). A 741 base pair (bp) iNOS cDNA and a 309-bp GAPDH cDNA fragment were amplified from RT-generated cDNA by designed primers corresponding to the published murine iNOS (Xie *et al*., 1992) and rat

GAPDH (Fort *et al*., 1985) cDNA sequences. The sense and antisense primers for iNOS and GAPDH respectively were: CACAAGGCCACATCGGATTTC, TGCATACCACTTCAACCCGAG, and TCCCTCAAGATTGTCAGCAA, AGATCCACAACGGATACATT. The PCR-amplified cDNA fragments were purified by gel electrophoresis and eluted from the gels by use of a Geneclean II kit (Stratech Scientific, Luton, U.K.). Authenticities of the PCR products were confirmed by dideoxy chain termination sequencing (Sambrook *et al*., 1989). The cDNA probes were labelled with [α^{32} P]-dCTP by use of a random labelling kit (Amersham U.K.).

Northern blot analysis

Lungs were ground and homogenized. Total RNA was extracted following the method described by Chomczynski and Sacchi (1987) (Haddad *et al*., 1995). Poly-$(A)^+$mRNA was isolated by use of a Poly (A) mRNA isolation kit (Promega, Southampton, U.K.). Approximately 3 μg mRNA from each sample was separated in a 1.2% denaturing agarose gel and transferred onto Hybond-N nylon filter (Amersham, U.K.). The filter was incubated at 42°C for at least 4 h in a prehybridization buffer which consisted of: 50% formamide, 5X Denhardt's solution, 5X standard saline citrate (SSC), 5 mM sodium phosphate, 200 μg ml^{-1} sonicated denatured salmon sperm DNA, and 0.1% sodium dodecyl sulphate (SDS). The filter was then hybridized with ^{32}P-labelled probes at the concentration of 0.5 to 1.0×10^6 c.p.m. ml^{-1} at 42°C for 14–16 h. The blots were washed twice in $2\times$SSC/0.1% SDS at 42°C for 20 min, once in $0.5\times$SSC/0.1% SDS at 50°C for 20 min, in $0.2\times$SSC/0.1% SDS at 55°C for 20 min and then in $0.1\times$SSC/0.1% SDS for 20 min. Blots were exposed to Kodax X-OMAT S film in the presence of an intensifying screen for 2–3 days. Each filter was first hybridized to the iNOS probe. After film exposure, the filters were washed at 70°C in 50% formamide and 10 mM Na_2HPO_4 buffer to remove the iNOS probes, and were subsequently rehybridized to GAPDH probes. The iNOS bands were quantified by laser densitometry (Howtek, Hudson, NH, U.S.A.) linked to a computer analysis system (PDI, Huntington Station, NY, U.S.A.). The relative RNA levels of iNOS were expressed as percentages of their corresponding GAPDH bands (iNOS/GAPDH ratio).

Electrophoretic mobility shift assays

Lung tissue was finely chopped in Buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.1% Nonidet P40). After centrifugation the nuclear pellet was lysed with Buffer B (20 mM HEPES, 1.5 mM $MgCl_2$, 0.42 mM NaCl, 0.5 mM DTT, 25% glycerol, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 0.2 mM EDTA). The subsequent soluble fraction was mixed with buffer C (20 mM HEPES, 50 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA) as previously described (Adcock *et al*., 1995). Double-stranded oligonucleotides encoding the consensus target sequence of NFκB (5′-AGTTGAGGGGACTTTCCCAGG-) were end-labelled with [α-^{32}P]-ATP and T4 polynucleotide kinase. Binding for the non-inducible transcription factor Oct-1 by use of the consensus sequence 5′-TGTCGAATGCAAATCACTAGAA-3′ was used as an internal standard. Ten micrograms of nuclear protein from each sample was incubated with 50,000 c.p.m. of labelled oligonucleotide in 25 μl incubation buffer (4% glycerol, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM Tris-HCl, pH 7.5, 0.8 mg ml^{-1} sonicated salmon sperm DNA) for 20 min at 25°C. Protein-DNA complexes were separated on a 6% polyacrylamide gel with $0.25\times$ Tris-Borate-EDTA running buffer. The retarded band was detected by autoradiography and quantified by laser densitometry. The results were expressed as a ratio of NF-κB to Oct-1 retarded band. Specificity was determined by addition of excess unlabelled double-stranded oligonucleotides.

Immunohistochemistry

We examined the distribution of iNOS by immunohistochemistry and compared the staining obtained in allergen-challenged rats 24 h post-challenge ($n=6$) to that observed in naïve and sensitized and saline challenged rats or rats that were treated with lipopolysaccharide (15 mg kg^{-1}, i.p.) and killed at 6 h. Other experimental groups were: control saline-injected ($n=3$), non-sensitized ovalbumin-challenged ($n=3$) and sensitized saline-challenged rats ($n=4$). Frozen sections (6 μm) of lung tissues were cut on a cryostat and placed on poly-l-lysine-coated microscope slides. Sections were fixed in cold acetone for 10 min and excess acetone was allowed to evaporate. Endogenous peroxidase was blocked by incubating slides in 1% hydrogen peroxide and 0.02% sodium azide in tris-buffered saline (TBS) for one hour followed by washing twice in TBS. Permeabilization of the sections was carried out by immersion for one hour in PBS containing 0.4% Triton X100, followed by washing in TBS. The rabbit anti-mouse iNOS polyclonal antibody (TCS Biologicals, Lake Placid, New York), which cross-reacts with rat iNOS, was applied at a dilution of 1:200 in antibody diluent (Dako Ltd, High Wycombe, Bucks) for one hour at room temperature, followed by washing in TBS. Following the primary antibody, biotynilated swine anti-rabbit immunoglobulin (Dako Ltd) at 1:200 dilution was applied for 45 min, followed by further washing in TBS. Sections were then incubated with horse-radish peroxidase-avidin conjugate (Dako Ltd) at a dilution of 1:500 for 45 min. After being washed with TBS, the primary antibody-biotin-avidin complex was visualized with chromogen-fast diaminobenzidine for 5 min and the slides were counterstained with haematoxylin and mounted on mounting medium (DPX, Raymond Lamb, London).

For the quantification of changes in immunmohistochemical localization of iNOS protein, two sections of lung tissue were prepared immunohistochemically from each animal of the experimental groups. Manual eye-counts were performed on a light microscope at a final magnification of $\times400$. A total of twenty consecutive fields were examined and the number of positively-stained macrophages were recorded by an observer unaware of the treatment received by the rats.

Data analysis

Data are presented as mean $\pm$ s.e.mean. Statistical analysis of results was performed by Kruskal-Wallis rank test followed by Mann-Whitney U-test for stepward comparison.

Results

iNOS gene expression

We detected a single band of approximately 4.4-kb iNOS message in RNA from all lungs studied by the murine iNOS cDNA probe (Figures 1 and 2). The iNOS band was weak in RNAs of control lungs, but increased by approximately 3-fold in lungs of animals sensitized with ovalbumin and challenged with saline (Figure 2, Sens). The iNOS signal was not significantly different in sensitized and saline-challenged animals at 4 and 24 h post-challenge (data not shown). Challenge of the sensitized animals with aerosol ovalbumin caused a further significant increase in the iNOS mRNA transcript ($P<0.05$). Four hours after aerosol ovalbumin challenge (Figure 2, Ov-4), the relative iNOS mRNA level increased significantly to approximate 6 fold above that of control rats ($P<0.05$). The iNOS mRNA level in lungs 24 h after aerosol ovalbumin challenge was higher than in control, but lower than that in sensitized rats (Figures 1 and 2). The iNOS mRNA expression in non-sensitized and ovalbumin-exposed animals was similar to that seen in control saline-injected and saline-challenged animals (data not shown).

Immunohistochemistry

Immunohistochemistry with the anti-iNOS antibody showed positive staining of macrophages within alveolar spaces with some lesser staining in vascular endothelial cells in lung parenchyma of sensitized and allergen-exposed rats killed at 24 h post-challenge (Figure 3), while control or sensitized saline-exposed rats or non-sensitized ovalbumin-exposed rats showed no immunostaining in the lungs or airways. There was also no immunostaining observed in lungs or airways of rats treated with aluminium hydroxide i.p. alone ($n=2$) or in those treated with aluminium hydroxide i.p. followed by ovalbumin exposure 21 days later ($n=2$). In all groups of rats, there was occasional staining in airway epithelium with no differences between the groups. In the sensitized ovalbumin-challenged rats, there were 40.8 ± 9.4 positively-staining macrophages per 20 high-power fields, while there were no positively-staining

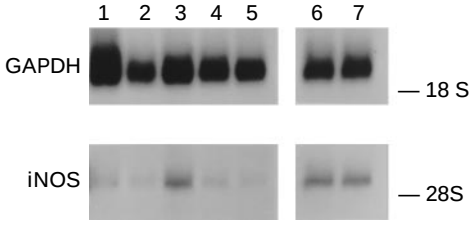

Figure 1 Northern blot autoradiogram showing the effects of sensitization with ovalbumin and aerosol ovalbumin challenge after sensitization on the expression of inducible nitric oxide (iNOS) mRNA in the lungs. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA served as an internal control. Sensitization was achieved by intraperitoneal injection of 1 mg ovalbumin and 100 mg aluminium hydroxide [$AL(OH)_3$] in 1 ml of saline for 3 consecutive days. Some of the sensitized animals were challenged with aerosol ovalbumin (1%) 4 and 24 h (Ov-4 and Ov-24 groups) before the animals were killed and lungs were removed. Lanes 1 and 2, control; lane 3, sensitized only; lanes 4 and 5, 24 h after aerosol challenge in sensitized animals; and lanes 6 and 7, 4 h after aerosol challenge in sensitized animals. The ribosomal RNA 18S and 28S are also shown.

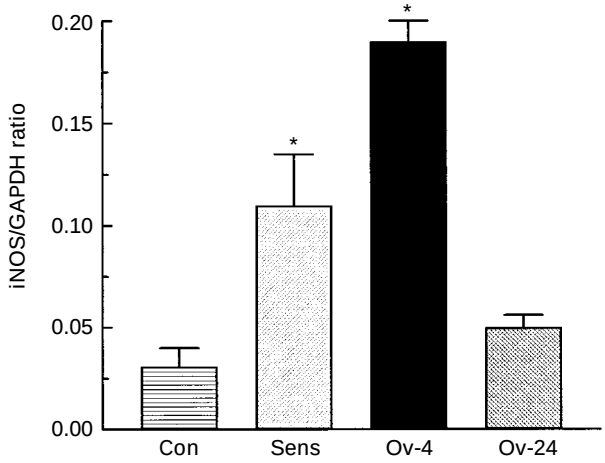

Figure 2 The relative iNOS mRNA level in the lungs from animals that were saline-treated (Con), sensitized only (Sens), and 4 (Ov-4) or 24 h (Ov-24) after aerosol ovalbumin challenge following ovalbumin sensitization. The relative iNOS mRNA levels were quantified by densitometry and expressed as iNOS/GAPDH optical density ratio. $*P<0.05$, compared with control; $\#P<0.05$, compared with Ov-4. Data presented as mean $\pm$ s.e.mean of 4–6 animals.

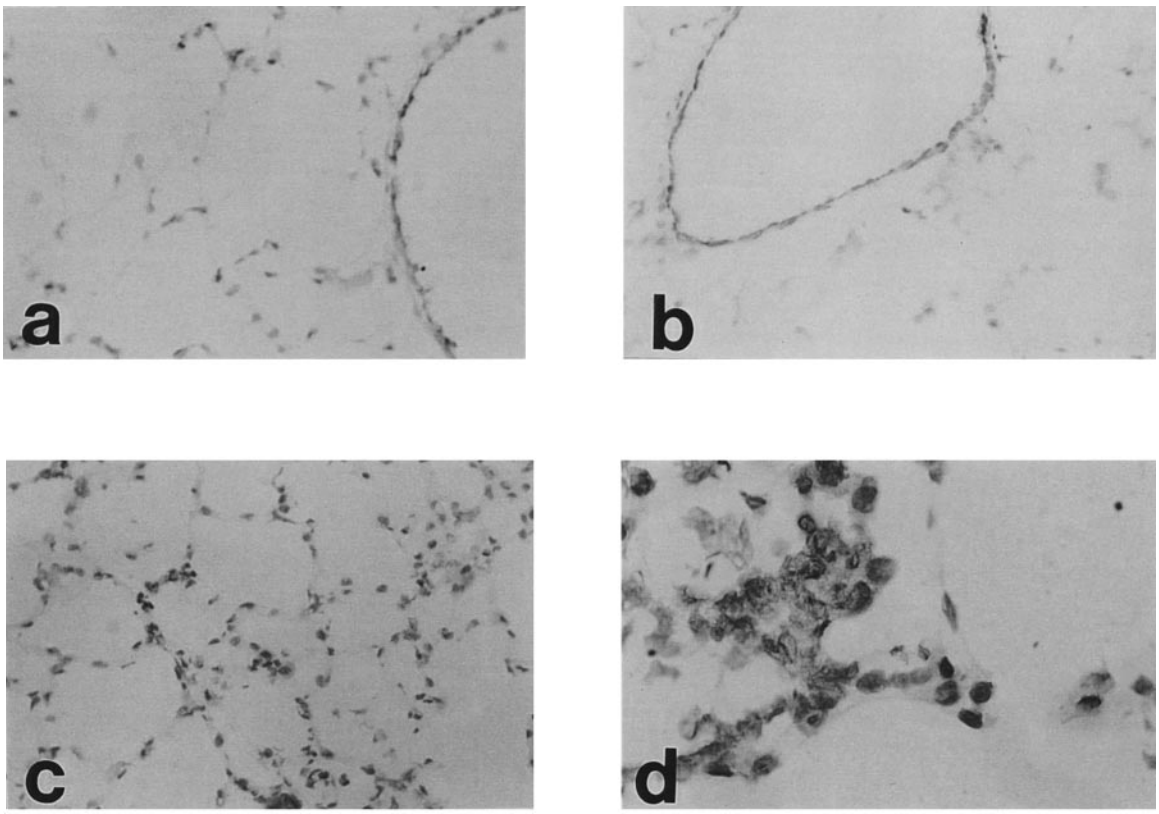

Figure 3 Immunohistochemical staining for iNOS with a rabbit anti-mouse iNOS antibody in rat lung. (a) Section through the lung of a naïve rat with no detectable staining (magnification: $\times 400$). (b) Section through the lung of a sensitized rat exposed to saline showing no staining in lung parenchyma and some patchy staining in airway epithelium. ($\times 400$). (c) Section through the lung of a sensitized rat exposed to ovalbumin and killed 24 h post-challenge showing staining of alveolar macrophages and of vascular endothelial cells ($\times 400$). (d) High power photomicrograph of a cluster of positively-staining macrophages in lung parenchyma, with lighter staining of vascular endothelial cells ($\times 1000$).

macrophages observed in the sections from the other 3 groups of rats. By contrast, the endotoxin-treated rats showed staining in both alveolar macrophages and infiltrating neutrophils and vascular endothelium.

NF-κB-binding activity

Electrophoretic mobility shift assays on nuclear extracts showed a marked increase in NF-κB DNA-binding activity expressed as a ratio of Oct-1 binding following allergen challenge of sensitized rats at 2 h (Figure 4). This activity declined towards baseline values at 6, 12 and 24 h. Saline challenge of sensitized rats did not result in NF-κB-binding activity.

Discussion

We have shown that there is enhanced iNOS gene expression following ovalbumin sensitization alone, followed by a further increase in gene expression at 4 h, with return towards baseline values by 24 h after exposure to ovalbumin aerosol. Immunohistochemical examination of the lungs revealed that the expression was predominantly in macrophages but not in airway epithelium. In addition, the increase in iNOS mRNA expression was preceded by an increase in NF-κB DNA-binding in the lung. Our data concerning the expression of iNOS following allergen challenge are complementary to those of Yeadon and Price (1995), who demonstrated that allergen challenge in the same rats induced increased levels of calcium-independent NOS activity in lung tissue at 6 and 24 h after allergen exposure. Therefore, enhanced production of NO following allergen challenge is likely to be the result of an increase in iNOS mRNA and protein expression, together with increased NOS activity, particularly in macrophages. This increase in iNOS mRNA expression may be dependent on increased NF-κB binding.

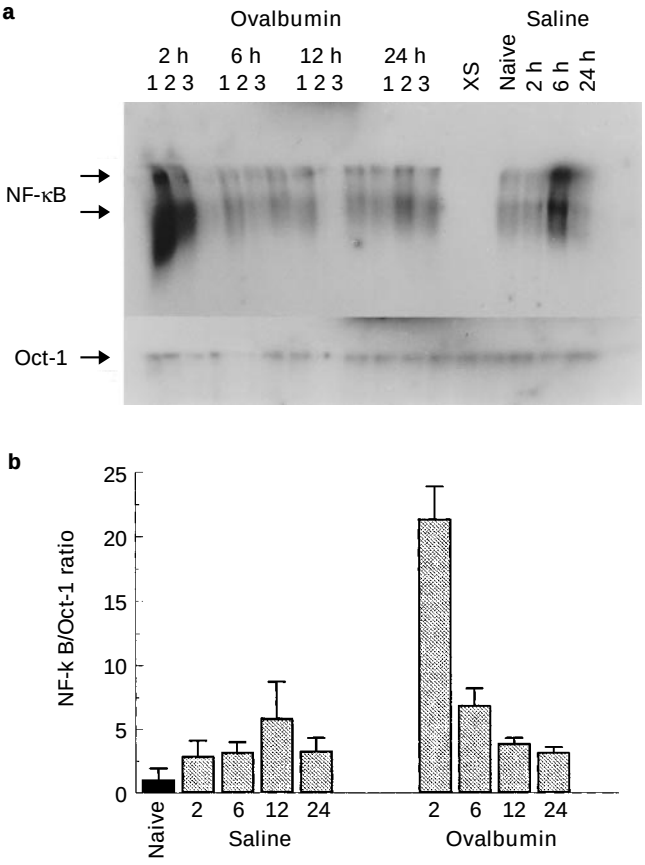

Figure 4 Effect of exposure to ovalbumin or saline of sensitized rats on NF-κB-binding activity. (a) An electrophoretic gel mobility shift assay showing NF-κB-binding activity in rat lung cell nuclear proteins from individual sensitized rats at various time-points after exposure to ovalbumin. Three rats were examined at each of the time-points. Results of single sensitized rats at 2, 6 and 24 h after exposure to saline are shown, in addition to that of one non-sensitized non-exposed (naïve) rat. The specificity of the binding was confirmed by adding 100 fold excess unlabelled NF-κB oligonucleotide (XS). The corresponding gel shift assays with Oct-1 oligonucleotide is also shown. (b) The time-course of densitometric scanning of the band shift data expressed as a ratio of NF-κB to Oct-1-binding activity ($n=3$ for each group). The numbers are hours after either saline or ovalbumin challenge. There was an increase in NF-κB-binding at 2 h after allergen exposure with return towards baseline values at 12 h. There was no effect of saline exposure. Naïve rats were non-sensitized and non-exposed. Data shown as mean ± s.e.mean.

Lung macrophages appear to be an important source of iNOS following allergen challenge and are known to express iNOS and release NO following stimulation with endotoxin or various cytokines including interferon γ (IFNγ), interleukin-iβ (IL-1β) and tumour necrosis factor-α (TNF-α) (Stuehr & Marletta, 1987; Nathan & Xie, 1994; Modolell *et al*., 1995). However, there is no direct evidence that macrophages release NO on direct activation with allergen. Exposure of sensitized Brown-Norway rats to inhaled allergen increases the number of low affinity IgE receptor FCεRII (CD23) on alveolar macrophages (Mencia Huerta *et al*., 1991), and effect probably mediated by the release of IL-4 (Paul-Eugene *et al*., 1993). Exposure of macrophages with unregulated CD23 expression on exposure to IgE/anti-IgE complexes induces nitrite production (Paul-Eugene *et al*., 1994; Becherel *et al*., 1994), supporting a direct effect of allergen in inducing iNOS expression in these cells. Although airway epithelial cells can be induced to express iNOS mRNA and to release nitrite on exposure to cytokines (Robbins *et al*., 1994), we found no increase in expression of iNOS following either allergen or endotoxin exposure in these cells. It is of interest that the pattern of expression of immunoreactive iNOS in the rats after endotoxin exposure is different from that of allergen exposure.

The upstream portion of the murine iNOS gene contains sequences that bind to the transcription factor NF-κB, and this has been shown to be essential for the transcription of murine iNOS gene (Lowenstein *et al*., 1993; Xie *et al*., 1994). Our demonstration that NF-κB binding was markedly increased at 2 h after allergen exposure, preceeding the increase in iNOS mRNA expression, supports the involvement of NF-κB in this process. Transcription of iNOS mRNA in an epithelial cell line was dependent on NF-κB activation that was induced by oxidative mechanisms (Adcock *et al*., 1994). However, the mechanisms by which NF-κB activation occurs after allergen challenge may involve the release of early cytokines such as IL-1β or TNF-α, but further work is needed.

The role of iNOS expression leading to the subsequent generation of NO during allergic lung responses is unclear. Recent studies point to an involvement of iNOS in modulating interactions between Th-1 and Th-2 cells. First, NO may exert a self-regulatory effect on Th-1 cells that are implicated in immunopathological situations. Thus, high concentrations of NO can inhibit the secretion of IFNγ and IL-2 by Th-1 T-cells, while having no effect on IL-4 production by Th-2 cells (Taylor-Robinson *et al*., 1994). This reduction in IL-2 and IFNγ production would result in increased antigen-driven proliferation of Th-2 cells as IFN-γ from Th-1 T-cells can inhibit Th-2 T-cells (Gajewski & Fitch, 1988). On the other hand, Th-2 cytokines such as IL-4 and IL-10 can inhibit the induction of iNOS and in this way may allow some activity of Th-1 cells (Liew *et al*., 1991). We have previously found that the sensitized and challenged Brown-Norway rat lung expresses the Th-2 cytokines, IL-4 and IL-5 with a reduction in the expression of the Th-1 cytokine, IFNγ (Haczku *et al*., 1996). Thus, it is conceivable that the inhibition of IFN-γ expression in sensitized and exposed rats may be due to enhanced iNOS activity, in addition to the enhanced IL-4 expression in rat lung

(Haczku *et al*., 1996). Thus, iNOS may be involved in the complex balance between Th-1 and Th-2 cells in immune and inflammatory states, which ultimately favours a Th-2 cell outcome.

The pathophysiological consequences of enhanced iNOS expression remain unclear. NO has been shown to be a bronchodilator (Dupuy *et al*., 1992) and inhibitors of nitric oxide synthesis increase airway responsiveness to exogenous bronchoconstrictors (Nijkamp *et al*., 1993). In addition, these inhibitors markedly increase antigen-induced bronchoconstriction in the guinea-pig (Persson *et al*., 1993). Thus, endogenous excessive NO production may protect the airway from excessive bronchoconstriction. The modulatory effects of endogenous NO on the recruitment of inflammatory cells such as eosinophils to the airways following an allergic response are not known.

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