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SPECIAL REPORT

Induction of calcium-independent nitric oxide synthase by allergen challenge in sensitized rat lung *in vivo*

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There is some evidence that nitric oxide synthase (NOS) is induced in the lungs of patients with allergic asthma, but the mechanism of this is not understood. The aim of the present study was to investigate whether the levels of NOS in rat lung could be altered by exposure of the animals to aerosols of allergen (ovalbumin). Brown-Norway rats were actively sensitized to ovalbumin, raising a mixed IgE/IgG antibody response. The levels of total and calcium-independent NOS in lung tissue homogenates were elevated at 6 h and 24 h after allergen exposure in sensitized rats but not in unsensitized rats. The induction was not due to contaminating lipopolysaccharide in the challenge solution. The allergen-induced increase in calcium-independent lung NOS was inhibited by pretreatment of the animals with the corticosteroid betamethasone (3 mg kg^{-1} i.p., 1 h prior to and 6 h after allergen). These results show that allergen challenge induces calcium-independent NOS in the lungs of sensitized rats, a process inhibited by an anti-inflammatory corticosteroid.

Keywords: Sensitization; allergic challenge; Brown-Norway rat; lung; nitric oxide synthase; induction; corticosteroid

Introduction The enzymes nitric oxide synthase (NOS) which produce NO from L-arginine exist in a number of isoforms (Knowles & Moncada, 1994) including a calcium-dependent, constitutively expressed form (cNOS) and a calcium-independent, inducible form (iNOS). The levels of iNOS can be increased in a wide variety of tissues, including lung, by exposure to certain inflammatory cytokines, immune complexes or lipopolysaccharide (LPS) (Salter *et al.*, 1991) and this induction can be blocked by corticosteroids (Rees *et al.*, 1990).

There is evidence that lung NOS levels are increased in patients with allergic asthma. The concentration of NO gas in exhaled air is greater in asthmatics and this is reduced by corticosteroid treatment (Kharitonov *et al.*, 1995). Furthermore, immunoreactive NOS is increased in lung tissue biopsies from asthmatics compared with controls (Hamid *et al.*, 1993).

In the studies described here, we have now investigated whether allergen exposure in previously sensitized rats could increase lung NOS levels, examined the calcium-dependency of lung NOS and tested the ability of a corticosteroid to inhibit these changes.

Methods Male, Brown-Norway rats (Harlan, U.K.; 200-300 g) were sensitized with ovalbumin (OVA, 100 μg) adsorbed onto aluminium hydroxide ($Al(OH_3)$, 1 mg) administered s.c. and i.p. in a divided dose (0.5 ml per site). Controls received only $Al(OH_3)$. In some animals, serum levels of antigen-specific IgE and IgG were measured 14-21 days later by using indirect ELISAs (Diaz-Sanchez & Kemeny, 1991). Other animals were placed in a chamber of 2 litre volume and exposed (6 min) to an aerosol of OVA generated from a solution (30 mg ml^{-1}) of OVA in saline, with a Pulmosonic nebuliser. The aerosol was drawn though the chamber at 20 ml s^{-1}. Unsensitized rats were also exposed to OVA as described. After 6 h and 24 h following OVA exposure, the animals were killed (pentobarbitone 120 mg kg^{-1} i.p.) and after perfusing the lungs free of blood and removing large vessels and airways, lung tissue samples were frozen at -20°C for later assay of NOS by using the conversion of [^{14}C]-arginine to citrulline (Rees *et al.*, 1995). Total NOS was defined as the difference in citrulline production in the presence and absence of the non-selective NOS inhibitor L-NMMA (300 μM). Ca^{2+}-independent NOS was defined as the difference between the total NOS and the activity persisting in the presence of EGTA (1 mM). The concentration of lipopolysaccharide (LPS) in solutions of OVA and of saline was determined by comparison with the Reference Standard Endotoxin, EC5, of the U.S. Pharmacopoiea. Sensitized rats were subsequently challenged with saline containing the concentration of LPS found in a 30 mg ml^{-1} solution of OVA. In separate experiments, betamethasone (3 mg kg^{-1} i.p.) or vehicle (PEG200) was given 1 h before and 6 h after OVA exposure. In some animals differential counting of leucocytes recovered from the lungs by bronchoalveolar lavage (BAL) was performed to characterize the inflammatory response. In a further group of animals, LPS (E. coli serotype 0111:B4) was administered (3 mg kg^{-1} i.v.) and lung samples were taken 5 h later. All data were calculated as the mean $\pm$ s.e.mean of 5 determinations unless otherwise stated, and comparisons were made by analysis of variance, where $P<0.05$ was taken as significant.

Results Rats immunised with OVA/$Al(OH_3)$ developed a mixed OVA-specific serum antibody response, concentrations being 1100 ± 520 μg ml^{-1} and 0.94 ± 0.13 μg ml^{-1} for IgG and IgE, respectively. There were no detectable OVA-specific antibodies in animals given $Al(OH_3)$ alone. Before challenge, 99% of leucocytes in BAL were monocytes, and this was not changed 2 h after challenge with OVA in unsensitized animals or with saline in sensitized animals. In sensitized rats, however, the numbers of both eosinophils and neutrophils in BAL were increased at 6 h (0.12 ± 0.03 and $0.40\pm0.09\times10^6$ per rat, respectively) and 24 h (1.0 ± 0.33 and $2.4\pm0.36\times10^6$ per rat, respectively) after OVA. In animals pretreated with betamethasone then challenged with OVA, the numbers of eosinophils and neutrophils in BAL 24 h later were reduced compared with those in vehicle-treated animals (95%: $P<0.001$ and 93%: $P<0.01$, respectively).

In unsensitized, unchallenged rats there was detectable calcium-dependent NOS activity but no significant Ca^{2+}-independent NOS activity and this was unaltered at 24 h after allergen challenge (Figure 1). NOS activity in lungs was minimal in sensitized, unchallenged rats, being not significantly different from that in unsensitized rats, but was significantly increased both at 6 h and at 24 h after a single OVA exposure (Figure 1). The increase in total NOS at 24 h was pre-

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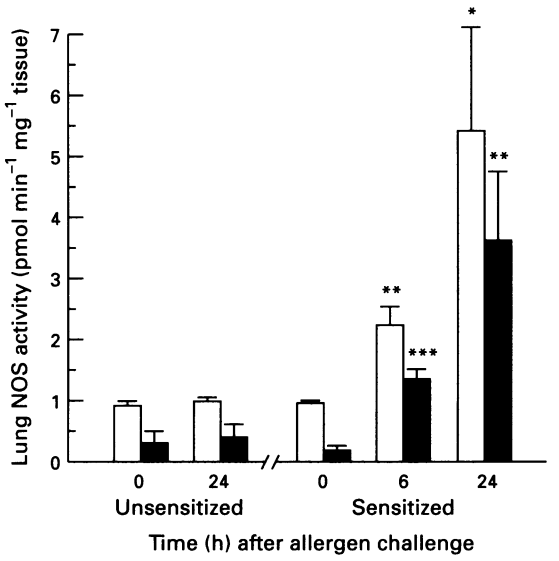

Figure 1 Effect of inhalation challenge with ovalbumin in unsensitized and sensitized Brown-Norway rats on lung nitric oxide synthase (NOS) activity. Lung tissue was taken at the times indicated and total (open columns) and Ca^{2+}-independent (solid columns) NOS activity determined by monitoring the conversion of [^{14}C]-L-arginine to citrulline. Increased total NOS activity in the lungs of sensitized rats was largely due to changes in the levels of Ca^{2+}-independent activity. Columns show the mean ± s.e.mean NOS activity, $n=5$ (*$P<0.05$, **$P<0.02$, ***$P<0.001$ greater than control values; analysis of variance).

dominantly (77%) accounted for by an increase in Ca^{2+}-independent NOS. Both total and Ca^{2+}-independent NOS were increased following near-maximal challenge with LPS (to 13.8 ± 1.24 and 9.1 ± 0.62 pmol min^{-1} mg^{-1} tissue, respectively, $n=3$).

In sensitized animals pretreated with betamethasone, the increase in Ca^{2+}-independent NOS in lung at 24 h after OVA was inhibited by 77% (from 1.67 ± 0.24 to 0.74 ± 0.14 pmol min^{-1} mg^{-1} tissue; $P<0.01$).

The concentration of LPS was determined to be 3.6 ng ml^{-1} in the OVA solution and 0.05 ng ml^{-1} in the saline vehicle. iNOS activity was not significantly altered in the lungs of sensitized rats at 24 h after challenge with saline containing 3.6 ng ml^{-1} LPS compared with the activity in unchallenged animals (0.97 ± 0.17 and 0.65 ± 0.10 pmol min^{-1} mg^{-1} tissue, respectively, $P>0.05$).

Discussion The results of the present study demonstrate that allergen challenge increases the levels of NOS in the lungs of sensitized Brown-Norway rats. That the majority of the activity was calcium-independent and that its appearance was inhibited by a glucocorticosteroid strongly suggests that this is the inducible isoform, iNOS. The mechanism of induction is unlikely to reflect LPS contaminations of the ovalbumin, since lung NOS activity was unchanged in similarly exposed unsensitized rats. Furthermore, the sustained induction of iNOS by allergen contrasts with the transient induction obtained with LPS (Salter *et al.*, 1991). In addition, challenge of sensitized rats with a solution containing LPS at the concentration found in the OVA solution failed to increase NOS activity in the lung.

It has been shown that exposure of sensitized Brown-Norway rats to aerosol allergen upregulates the low affinity IgE receptor, FcεRII (CD23) on alveolar macrophages (Mencia-Huerta *et al.*, 1991). This observation suggests a possible mechanism for the results presented here, since in human peripheral blood monocytes, CD23 upregulation by IL-4 followed by exposure to IgE/anti-IgE complexes induces nitrite production which is blocked by the NOS inhibitor, L-NMMA (Paul-Eugene *et al.*, 1995).

NO gas is detectable in the exhaled air of laboratory animals and also in man. In healthy animals, the majority of the NO production in lung is calcium-dependent (Persson *et al.*, 1994) and thus is likely to be derived from constitutively expressed NOS. In contrast, the levels of NO in exhaled air in allergic asthmatics is greater than in healthy individuals and is reduced by corticosteroid treatment (Kharitonov *et al.*, 1995), suggesting the presence of an inducible NOS in asthmatic airways.

In conclusion, we have shown that inhalation challenge with allergen in sensitized rats induces a Ca^{2+}-independent isoform of NOS in the lung. As found in the present study where betamethasone inhibited the induction of NOS following inhalation challenge in rats, it is possible that inhibition by corticosteroids of iNOS expression in the lung may contribute to their therapeutic activity in asthma. If this were to be the case, then selective inhibitors of iNOS may also be of clinical value in the treatment of asthma.

References

DIAZ-SANCHEZ, D. & KEMENY, D.M. (1991). Generation of a long-lived IgE response in high and low responder strains of rat by coadministration of ricin and antigen. *Immunology*, **72**, 297–303.

HAMID, Q., SPRINGALL, D.R., RIVEROS-MORENO, V., CHANEZ, P., HOWARTH, P., REDINGTON, A., BOUSQUET, J., GODARD, P., HOLGATE, S. & POLAK, J.M. (1993). Induction of nitric oxide synthase in asthma. *Lancet*, **342**, 1510–1513.

KHARITONOV, S.A., YATES, D.H. & BARNES, P.J. (1995). Regular inhaled budesonide decreases nitric oxide concentration in the exhaled air of asthmatic patients. *Am. J. Resp. Crit. Care Med.*, **151**, A387.

KNOWLES, R.G. & MONCADA, S. (1994). Nitric oxide synthases in mammals. *Biochem. J.*, **298**, 249–258.

MENCIA-HUERTA, J.M., DUGAS, B., BOICHOT, E., PETIT-FRERE, C., PAUL-EUGENE, N., LAGENTE, V., CAPRON, M., LIU, F.T. & BRAQUET, P. (1991). Pharmacological modulation of the antigen-induced expression of the low-affinity IgE receptor (FcεRII/CD23) on rat alveolar macrophages. *Int. Arch. Allergy Appl. Immunol.*, **94**, 295–298.

PAUL-EUGENE, N., KOLB, J.P., SARFATI, M., AROCK, M., QUAAZ, F., DEBRE, P., MOSSALAYI, D.M. & DUGAS, B. (1995). Ligation of CD23 activates soluble guanylate cyclase in human monocytes via an L-arginine-dependent mechanism. *J. Leukoc. Biol.*, **57**, 160–167.

PERSSON, M.G., MIDTVEDT, T., LEONE, A.M. & GUSTAFSSON, L.E. (1994). Ca^{2+}-dependent and Ca^{2+}-independent exhaled nitric oxide, presence in germ-free animals inhibition by arginine analogues. *Eur. J. Pharmacol.*, **264**, 13–20.

REES, D.D., CELLEK, S., PALMER, R.M.J. & MONCADA, S. (1990). Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxin shock. *Biochem. Biophys. Res. Commun.*, **173**, 541–547.

REES, D.D., CUNHA, F.Q., ASSREUY, J., HERMAN, A.G. & MONCADA, S. (1995). Sequential induction of nitric oxide synthase by *Corynebacterium parvum* in different organs of the mouse. *Br. J. Pharmacol.*, **114**, 689–693.

SALTER, M., KNOWLES, R.G. & MONCADA, S. (1991). Widespread tissue distribution, species distribution and changes in activity of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthases. *FEBS Lett.*, **291**, 145–149.

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