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Apocynin and 1400 W prevents airway hyperresponsiveness during allergic reactions in mice

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1 The contribution of reactive nitrogen species to the development of airway hyperresponsiveness in a mouse model of allergic inflammation was investigated by the use of selective inhibitors of nitric oxide and superoxide formation.

2 Sensitized mice, repeatedly challenged with ovalbumin showed a significant ($P<0.001$, $n=9$) increase in airway responsiveness measured using whole body plethysmography. This hyperresponsiveness was accompanied by an influx of eosinophils into the airway lumen and increased levels of ovalbumin-specific serum IgE.

3 Treatment of mice with the iNOS inhibitor 1400 W or the NADPH-oxidase inhibitor apocynin did not significantly alter cellular influx into the airway lumen nor serum ovalbumin specific IgE. In contrast, apocynin as well as 1400 W inhibited ovalbumin-induced airway hyperresponsiveness ($P<0.001$ and $P<0.05$ respectively, $n=9$). Furthermore, the airways of allergen challenged animals showed clear 3-nitrotyrosine staining, which was mainly located in eosinophils. Remarkably, treatment with apocynin or 1400 W did not alter 3-nitrotyrosine staining.

4 These data suggest that the development of airway hyperresponsiveness during the airway inflammation upon ovalbumin challenge is dependent on the release of both superoxide and nitric oxide and is therefore likely to be dependent on reactive nitrogen species. This mechanism, however, is not reflected by 3-nitrotyrosine formation in the airways.

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Abbreviations: AU, Arbitrary Units; FCS, Foetal Calf Serum; IFNγ, Interferon-γ; IL, interleukin; MNC, Mononuclear cells; n/I NOS, neuronal/inducible Nitric oxide synthase; NO, Nitric Oxide; Ova, Ovalbumin; PBS, Phosphate Buffered Saline; Penh, Enhanced Pause; $PGF_{2\alpha}$, Prostaglandin $F_{2\alpha}$; Sal, Saline; TBS, Tris Buffered Saline

Introduction

Asthma is a disease characterized by airway inflammation and hyperresponsiveness (Guidelines, 1991). The concentration of nitric oxide in exhaled air is increased in asthmatics and is thought to reflect the state of airway inflammation (Massaro *et al*., 1996; Dupont *et al*., 1998; ten Hacken *et al*., 1998). The role of nitric oxide in the airways, however, is ambiguous. Endogenously released nitric oxide is a bronchodilator (Lei *et al*., 1993; Nijkamp *et al*., 1993; Folkerts & Nijkamp, 1998). Nevertheless, high concentrations of nitric oxide putatively leading to reactive nitrogen species *via* interaction with radical oxygen species have previously been suggested to be important factors in the pathophysiology of allergic airway disease (Muijsers *et al*., 1997; Van der Vliet *et al*., 1999). This is supported by increased 3-nitrotyrosine staining (Saleh *et al*., 1998) and increased formation of superoxide upon allergen challenge (Calhoun *et al*., 1992) in human asthmatic airways.

In the present study, we investigated the contribution of the reactive nitrogen intermediate precursors nitric oxide and superoxide on the development of airway hyperresponsiveness and allergic inflammation of the airways was investigated in an established mouse model (de Bie *et al*., 1996; Hessel *et al*., 1997; Hofstra *et al*., 1998). The formation of nitric oxide and superoxide was prevented by the use of selective inhibitors of inducible nitric oxide synthase and NADPH-oxidase respectively (Muijsers *et al*., 2000). Furthermore, the airways of allergen challenged mice were immunohistochemically stained for 3-nitrotyrosine.

It is demonstrated that although airway eosinophilia and increased serum IgE is present, the development of airway hyperresponsiveness is reduced by inhibitors of either inducible NOS (1400 W) or NADPH-oxidase (apocynin).

Methods

Animals

Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Specific Pathogen-free male BALB/c mice (6 weeks) were

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obtained from the breeding colony of the Central Animal Laboratory (Utrecht, The Netherlands). The mice were housed in macrolon cages and were provided with food and water *ad libitum*.

Immunization protocol

Active sensitization was performed without adjuvant by seven i.p. injections of 10 μg ovalbumin (grade V) in 0.5 ml pyrogen-free saline on 7 alternate days. Three weeks after the last injection, the mice were exposed to an ovalbumin (2 mg ml^{-1}) or saline aerosol challenge for 5 min (once daily) on 8 consecutive days. The aerosols were performed in 5 l exposure chambers coupled to a jet nebulizer (particle size 2–3 μm) driven by compressed air at a flow rate of 6 l min^{-1}.

Treatment with apocynin and 1400 W

Mice were treated with either 1400 W (10 mg kg^{-1}) (Garvey *et al*., 1997) or apocynin (4 mg kg^{-1}) (Salmon *et al*., 1998) dissolved in 0.25 ml pyrogen-free saline by eight i.p. injections administered just prior to each ovalbumin challenge described in the previous section. Moreover, in a separate experiment mice were treated with apocynin dissolved in the drinking water (Hart 't *et al*., 1992) (0.16 or 26.6 μg ml^{-1}) during the challenge phase (9 days, starting 1 day before the first challenge). Mice received 4 mg kg^{-1} day^{-1} apocynin as calculated by measuring the amount of water consumed.

Airway responsiveness

Using barometric whole-body plethysmography (Buxco, Sharon, CT, U.S.A.), responses to inhaled methacholine, in conscious unrestrained mice were measured as described previously (Hamelmann *et al*., 1997; Hofstra *et al*., 1998). As an index of airway responsiveness, increases in enhanced pause (Penh) were measured. Penh data was statistically analysed using univariate repeated measures analysis of variance. Dose response curves to methacholine were analysed as repeated measures. Data were LOG transformed before analysis to equalize variances in all groups.

Serum levels of ovalbumin specific IgE

Directly after the dose response curve with methacholine the mice were injected with an overdose of pentobarbitone (0.5 g kg^{-1} body weight). Blood samples were obtained from the mice *via* a cardiac puncture, left at room temperature for 2 h and subsequently centrifuged for 10 min at 14,000 r.p.m. Serum was collected and samples were kept at -20°C until analysis. Ovalbumin specific IgE was measured using an ELISA method as described previously (Hessel *et al*., 1998). Levels were compared to a pooled standard serum and expressed as arbitrary units (au ml^{-1}).

Stimulation of thoracic lymph nodes and lung cells in vitro

Cytokine production by antigen-stimulated T cells derived from both thoracic lymph nodes and lung tissue was determined as described previously (Hofstra *et al*., 1998). Mice were injected with an overdose of pentobarbitone (0.5 g kg^{-1} bodyweight). The lungs were lavaged (as described below) and perfused *via* the right ventricle with 4 ml saline containing 100 u ml^{-1} heparin to remove any blood and intravascular leukocytes. The thoracic lymph nodes were isolated from the paratracheal and parabronchial regions. The lymph nodes were transferred to cold PBS and gently homogenized on a 70 μm cell strainer (Falcon, Lelystad, The Netherlands) in order to obtain a single cell suspension. The lymph node and lung cell suspensions were washed, resuspended in culture medium (RPMI 1640 containing 10% FCS, 1% glutamax I, 50 μg ml^{-1} gentamicin and 50 mM β-mercapto-ethanol) and total cell number counted. Cells (viability $>95\%$, 2×10^5 lymph node cells per well) were plated in round-bottom 96-well plates (Costar, Badhoevedorp, The Netherlands) in a volume of 200 μl. The cells were cultured for 5 days with ovalbumin (10 μg ml^{-1}) or medium only. The cells were cultured at 37°C with 5% CO_2 in humidified air. Each *in vitro* stimulation was performed in triplicate. Supernatants were harvested, pooled per stimulation and kept at -20°C until cytokine levels were determined by ELISA. The IFNγ, IL-4 and IL-5 ELISAs were performed according to the instructions of the manufacturer (Pharmingen, San Diego, CA, U.S.A.). The detection limits of the ELISAs were 156 pg ml^{-1} for IFNγ, 15.6 pg ml^{-1} for IL-4 and 31.3 pg ml^{-1} for IL-5.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed as described previously (Hessel *et al*., 1997). In short, the airways of the mice were cannulated and lavaged five times with 1 ml of pyrogen free saline (37°C). The total numbers of lavage cells were determined and cells in cytospin preparations were differentiated (Diff-Quick) into mononuclear cells, eosinophils and neutrophils by standard morphology. At least 200 cells were counted per cytospin preparation and the absolute number of each cell type was calculated.

Immunohistochemistry

Lungs were perfused with PBS (37°C) *via* the right ventricle. Subsequently, the lungs were filled with a cryo-conservative (1 ml, Tissue-Tek) *via* the trachea, removed from the thorax, snap frozen and kept at -80°C until use. Transversal, 5 μm thick lung sections at the level of the tracheo-bronchial tree were prepared using a cryostate (Leica, Germany). Sections were fixated with acetone for 10 min, and washed with TBS for three times. A specific protein binding was blocked with 0.1% bovine serum albumin and 20% normal goat serum in TBS, washed and incubated overnight with a polyclonal rabbit antiserum against 3-nitrotyrosine (see Materials for details). Subsequently, the sections were washed and incubated for 1 h with a polyclonal goat alkaline phosphatase conjugated antiserum specifically directed against rabbit IgG, after which the sections were again washed and incubated for 20 min with a commercial fast red substrate chromogen solution containing levamisole to block endogenous phosphatase activity. The sections were counterstained with Mayer's Haematoxylin and evaluated. All incubations were done at room temperature. Seven saline and seven ovalbumin challenged mice from two independent experiments were stained.

Data analysis

Unless stated otherwise data are expressed as mean ± s.e.mean and evaluated using an analysis of variance followed by a *post-hoc* comparison between groups. A probability value $P<0.05$ was considered statistically significant.

Material

Anti-3-nitrotyrosine from Upstate Biotechnology Inc., Control serum: rabbit IgG (Vector, CA, U.S.A.). Chromogen (Fast Red) solution containing levamisole was obtained from DAKO (Denmark). Polyclonal goat alkaline phosphatase conjugated antiserum specifically directed against rabbit IgG was purchased at Pharmingen (U.S.A.). Chemicals and solutions: Ovalbumin (chicken egg albumin, crude grade V) from Sigma Chemical Company (St. Louis, MO, U.S.A.); PBS and FCS from Gibco Life Technologies (Merelbeke, Belgium); pentobarbitone (nembutal) from Sanofi Sante B.V. (Maassluis, The Netherlands); heparin from Leo Pharmaceuticals (Weesp, The Netherlands); saline from B. Braun Medical B.V. (Oss, The Netherlands) and methacholine (acetyl-ß-methylcholine) from Janssen Chimica (Beerse, Belgium). Diff-Quick from Merz & Dade A.G. (Düdingen, Switzerland).

Results

Airway responsiveness

Control mice challenged with ovalbumin showed a significantly increased responsiveness to methacholine (PENH) compared to saline challenged mice ($P<0.001$, $n=9$; Figure 1A,B). Treatment with either 1400 W ($P<0.05$, $n=9$; Figure 1A) or apocynin ($P<0.0001$, $n=9$, Figure 1B) inhibited ovalbumin-induced hyperresponsiveness. Moreover, oral administration of apocynin *via* the drinking water (p.o., 4 mg kg^{-1} day^{-1}) significantly inhibited ovalbumin-induced hyperresponsiveness ($P<0.05$, $n=6$, Figure 1C). Although less potent, a lower dose of apocynin (p.o., 0.02 mg kg^{-1} day^{-1}, $n=6$) still inhibited the bronchoconstriction by 38, 30 ($P<0.05$), and 18% in reponse to 12.5, 25 and 50 mg/ml methacholine respectively. Apocynin did not have an effect on the amount of water consumed (data not shown). Thus, ovalbumin-challenged mice show increased airway responsiveness to methacholine compared to saline-challenged mice, which can be inhibited by treatment with either 1400 W or apocynin.

3-Nitrotyrosine staining

Mice repeatedly challenged with ovalbumin showed a clear infiltration of inflammatory cells into the airways. The cells were mainly grouped in patchy cellular infiltrates, which are located around broncheoli. These cellular infiltrates were absent in saline challenged animals. 3-Nitrotyrosine staining was clearly present in the airways of all ovalbumin challenged animals (Figure 2B, $n=9$) and was absent in all saline challenged animals (Figure 2A, $n=9$). 3-Nitrotyrosine staining was mainly concentrated in eosinophils throughout the airways. Positive eosinophils were found around blood vessels and in bronchus-associated lymphoid tissue (Figure 2B). Treatment with either apocynin ($n=6$) or 1400 W ($n=4$) did not change eosinophil 3-nitrotyrosine staining in the airways (Figure 2C,D, respectively).

Bronchoalveolar lavage

The number of cells in the bronchoalveolar lavage fluid was used as a measure for the infiltration of cells into the airways. Ovalbumin challenge resulted in an influx of eosinophils into the airway lumen (Table 1). No eosinophils could be detected in saline challenged mice. Furthermore, the number of neutrophils and mononuclear cells were significantly greater in ovalbumin challenged animals than in saline challenged animals. Treatment with 1400 W and apocynin did not affect the cellular influx induced by ovalbumin (Table 1). Moreover,

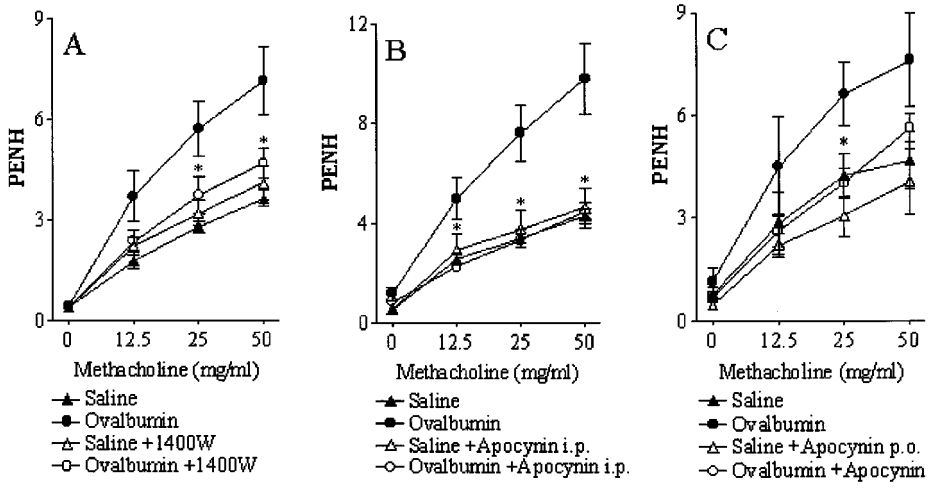

Figure 1 Apocynin and 1400 W inhibit airway hyperresponsiveness. Effect of i.p. 1400 W (A), i.p. apocynin (B), and p.o. apocynin (C) treatment on ovalbumin induced hyperresponsiveness to methacholine in sensitized mice (▲ = saline, ● = ovalbumin, = saline + inhibitor, ○ = ovalbumin + inhibitor). Data represent mean ± s.e.mean. *$P<0.05$ (1400 W i.p., $n=9$), $P<0.0001$ (Apocynin i.p., $n=9$) and $P<0.05$ (Apocynin p.o., $n=6$) compared to ovalbumin challenge.

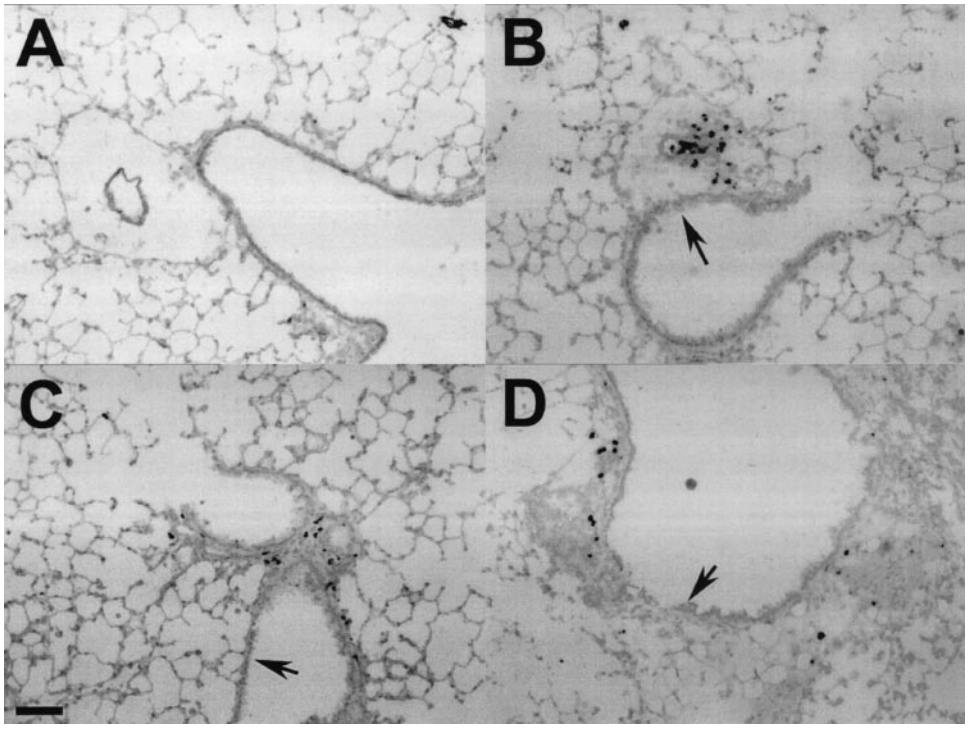

Figure 2 Immunohistochemical demonstration of 3-nitrotyrsosine residues (black spots) in the airways of ovalbumin sensitized mice challenged with either ovalbumin or saline. Arrowheads indicate airway epithelium. (A) Saline challenged animal. (B) Ovalbumin challenged animal. (C) Ovalbumin challenged animal treated with apocynin, (D) Ovalbumin challenged animal treated with 1400 W. Calibration bar in C represents 100 μm.

Table 1 Effect of i.p. apocynin and 1400 W treatment on ovalbumin challenge induced cell influx into the airway lumen and serum ovalbumin specific IgE

Treatment	*IgE* (AU ml^{-1})	*Total cells* ($\times 10^3$)	*Eosinophils* ($\times 10^3$)	*Neutrophils* ($\times 10^3$)	*MNC* ($\times 10^3$)	n
A						
Sal	79±14	248±24	0.0±0.0	6.9±3.8	241±22	9
Sal+Apocynin	84±6	222±20	3.0±1.6	13±5.7	222±20	9
Ova	770±86*	882±83*	466±58*	66±13*	350±18*	9
Ova+Apocynin	851±113*	840±126*	418±86*	63±17*	359±43*	9
B						
Sal	54±8	185±26	0.0±0.0	1.7±0.5	183±26	9
Sal+1400 W	68±6	168±16	0.0±0.0	1.4±0.5	166±16	9
Ova	387±60*	498±80*	213±52*	26±8.7*	257±22*	9
Ova+1400 W	531±97*	436±99*	243±84*	29±12*	263±48*	9

*$P<0.05$ compared to saline challenge, AU: arbitrary units of ovalbumin specific IgE compared to a standard reference serum set at 1000 AU ml^{-1}. Number of cells in bronchoalveolar lavage fluid per mouse. (MNC: Mononuclear cells).

administration of the inhibitors to saline challenged animals did not significantly change absolute BAL-cell numbers or the differential counts.

Th_2 cytokine release by thoracic lymph node cells

Unstimulated lymph node cells produced undetectable levels of IL-4 and IL-5 and very low levels of IFNγ. *In vivo* challenge with ovalbumin followed by *in vitro* stimulation by ovalbumin significantly and greatly enhanced production of IL-4 and IL-5 compared to unstimulated cells and cells from saline challenged animals (Table 2). Treatment of both saline- and ovalbumin-challenged animals with either 1400 W or apocynin did not significantly affect the cytokine levels produced by thoracic lymph node cells *in vitro* (Table 2). IFNγ production was significantly greater by cells from ovalbumin challenged animals than from saline challenged animals. *In vitro* ovalbumin stimulation did not significantly change IFNγ production. Treatment with 1400 W did not alter IFNγ release. Furthermore, apocynin treatment slightly inhibited IFNγ production by cells from ovalbumin challenged animals that were not stimulated with ovalbumin *in vitro*, although this difference was not statistically significant.

Immunoglobulin E

IgE levels were determined in serum samples taken 24 h after the last challenge. Repeated challenge with ovalbumin

Table 2 Effect of i.p. apocynin and 1400 W treatment *in vivo* on IL-4 and IL-5 production by thoracic lymph node cells stimulated *in vitro* with ovalbumin obtained from either saline or ovalbumin challenged animals

Treatment	*Challenge*	In vitro *stimulus*	*IL-4* (pg ml^{-1})	*IL5* $\times 10^2$ (pg ml^{-1})	*IFN*γ (pg ml^{-1})	n
Control	Saline	Medium	0.0±0.0	0.0±0.0	4.0±4.0	6
	Saline	Ovalbumin	71±49	68±34	108±54	6
	Ovalbumin	Medium	0.0±0.0	1.0±0.7	595±327*	4
	Ovalbumin	Ovalbumin	668±12*	334±22*	643±95*	4
Apocynin	Saline	Medium	0.0±0.0	0.0±0.0	18±18	5
	Saline	Ovalbumin	0.0±0.0	31±25	52±23	5
	Ovalbumin	Medium	0.0±0.0	0.4±0.3	186±84*	5
	Ovalbumin	Ovalbumin	611±57*	344±68*	570±72*	5
1400 W	Saline	Medium	0.0±0.0	0.0±0.0	2.0±2.0	5
	Saline	Ovalbumin	20±20	21±21	86±73	5
	Ovalbumin	Medium	0.0±0.0	0.0±0.0	648±279*	5
	Ovalbumin	Ovalbumin	479±80*	273±73*	658±91*	5

*$P<0.05$ compared to saline/medium.

resulted in increased levels of ovalbumin-specific IgE compared to saline challenge ($P<0.0001$) (Table 1). Treatment with 1400 W or apocynin did not influence the allergen-induced increase in serum-IgE, although 1400 W treatment slightly enhanced IgE concentrations in ovalbumin-challenged mice (Table 1).

Discussion

Increasing evidence exists for the involvement of reactive nitrogen species in the pathobiology of inflammatory airway disease (Van der Vliet *et al*., 1999). Although the formation of reactive nitrogen species during allergic asthma is well-established (Saleh *et al*., 1998), little is known about whether and how reactive nitrogen species contribute to the characteristics of asthma, i.e. airway hyperresponsiveness, airway eosinophilia and specific IgE production. The present study demonstrates that inhibition of inducible nitric oxide synthase or inhibition of NADPH oxidase prevents allergen-induced hyperresponsiveness but not 3-nitrotyrosine formation, eosinophil influx or IgE production. These data suggest that nitric oxide, superoxide and possibly reactive nitrogen species released in the airways are involved in the development of airway hyperresponsiveness by an as yet unresolved mechanism. 3-Nitrotyrosine formation by eosinophils, however, does not reflect this mechanism.

Interestingly, the putative peroxynitrite footprint 3-nitrotyrosine is readily formed by an nitric oxide independent process mediated by myeloperoxidase, with hydrogen peroxide and the nitric oxide metabolite nitrite as substrates (Kettle *et al*., 1997; Eiserich *et al*., 1998). Since only 10–15% of the BAL cells consisted of neutrophils only a small part of the 3-nitrotyrosine staining can be attributed to this cell type. Eosinophil peroxidase is an even stronger promoter of 3-nitrotyrosine formation (Wu *et al*., 1999). The fact that 3-nitrotyrosine was mainly present in eosinophils and could not be inhibited by the NOS inhibitor 1400 W strongly suggest that allergen induced 3-nitrotyrosine formation in eosinophils is completely mediated by a NOS independent process. Moreover, eosinophil peroxidase deficient mice, unlike wild type mice, do not show 3-nitrotyrosine staining in the airways upon allergen challenge, despite the influx of eosinophils (Duguet *et al*., 2000). It remains unclear why the NADPH oxidase inhibitor apocynin also failed to inhibit 3-nitrotyrosine formation. This is not likely to be due to insufficient dosages, since substantially lower doses than those used in the present study were able to prevent ulcerative skin lesions in rats (Hart 't *et al*., 1992). The relevance of eosinophil peroxidase mediated 3-nitrotyrosine formation in the development of airway hyperreactivity is highly disputable, since 1400 W and apocynin inhibited airway hyperresponsiveness but did not affect 3-nitrotyrosine formation. However, reactive nitrogen species may not lead to 3-nitrotyrosine formation in all circumstances. Further, 3-nitrotyrosine residues may be denitrated by enzymatic activity (Kuo *et al*., 1999), leaving them undetected or are simply formed in amounts to low to be detected by immunohistochemistry. Moreover, reactive nitrogen species may release other mediators, such as F2-isoprostanes, that are formed during oxidative stress *via* a cyclo-oxygenase independent pathway (Roberts & Morrow, 2000). Interestingly, it was recently demonstrated that a strong hyperresponsiveness could be induced by very low amount of 8-epi-$PGF_{2\alpha}$ administered to the airways of mice *in vivo* or *in vitro* (Held *et al*., 1999; Held & Uhlig, 2000). Although apocynin and 1400 W did not alter the number of eosinophils, it cannot be excluded that these inhibitors effected the activity and mediator release of the inflammatory cells resulting in less oxidative stress. Apocynin irreversibly inhibits the assembly of the NADPH-oxidase complex on the granulocyte membrane (Simons *et al*., 1990; Stolk *et al*., 1994), thereby preventing the formation of superoxide. Moreover, apocynin is a potent inhibitor of not only superoxide but also peroxynitrite formation by murine macrophages *in vitro* (Muijsers *et al*., 2000). Apocynin, injected intraperitoneally or dissolved in the drinking water, inhibits allergen-induced hyperresponsiveness without affecting the number of eosinophils. Recently, comparable results were obtained by Larsen *et al*. (2000) in mice that overexpress Cu/Zn superoxide dismutase. These animals were protected against allergen-induced tracheal hyperresponsiveness although an increase in the number of eosinophils in the lungs was still observed. Apocynin could have a therapeutic

value by limiting the formation of not only reactive oxygen species, but also reactive nitrogen species.

A number of studies report a putative role for nitric oxide as a mediator of T-cell response (Liew, 1995). Nitric oxide exposure skews the Th_1/Th_2 balance into the direction of a Th_2-response (Chang *et al*., 1997). Interestingly, iNOS knockout mice show decreased allergic inflammation compared to their wild-type littermates (Xiong *et al*., 1999), which is suggested to be due to an enhanced production of the Th_1 cytokine IFNγ. Nonetheless, the *in vivo* treatment with 1400 W did not enhance IFNγ production by thoracic lymph node cells *in vitro* and 1400 W did not significantly alter serum IgE or IL-5 production by thoracic lymph node cells. Treatment with 1400 W did slightly, but not significantly, inhibit IL-4 production by thoracic lymph node cells but the physiological relevance is questionable since an IL-4 neutralizing antibody did not affect the asthmatic phenotype in mice (Hessel *et al*., 1997).

Controversy exists as to which isoform of nitric oxide synthase is the main source of nitric oxide during allergic airway inflammation. Although NOS2 (iNOS) is upregulated in asthmatic airways (Saleh *et al*., 1998), the role for this enzyme in animal models of allergic airway inflammation remains unclear. Feder *et al*. (1997) found no increase in NOS2 protein nor mRNA upon allergic challenge in mice. Xiong *et al*. (1999) reported inhibition of allergic airway inflammation in NOS2-knockouts, but unaltered development of airway hyperresponsiveness, whereas de Sanctis *et al*. (1999), showed that knocking out the NOS2 gene neither affects allergic inflammation nor airway hyperresponsiveness. Interestingly, allergen induced airway hyperresponsiveness is completely abolished in NOS1 (nNOS) knockouts (de Sanctis *et al*., 1999). Based on the fact that 1400 W is at least a 200 fold more potent inhibitor of iNOS compared to nNOS (Garvey *et al*., 1997), iNOS is put forward as the most likely candidate in our model.

The cellular and molecular mechanisms leading to increased nitric oxide synthesis and reactive nitrogen species formation upon allergen challenge are not exactly known. In asthmatic subjects, iNOS protein is upregulated in the airway epithelium due to transcriptional regulation (Guo *et al*., 1997; 2000). IFNγ is likely to play an important role in the induction of iNOS during asthma, since the cytokine is essential for iNOS expression in human airway epithelial cells *in vitro* (Punjabi *et al*., 1994; Robbins *et al*., 1994). Furthermore, the concentration of IFNγ is increased in the epithelial lining fluid of asthmatics (Guo *et al*., 2000). Interestingly, the development of allergen induced hyperresponsiveness in the murine model described in this study is dependent on IFNγ (Hessel *et al*., 1997). Therefore, IFNγ could also be the cytokine responsible for iNOS induction during allergic airway inflammation. A large population of the T-lymphocytes present in the airways after allergen challenge is non-antigen specific and release IFNγ (Ying *et al*., 1995). Indeed, the present data show that thoracic lymph node cells from ovalbumin challenged animals release increased amounts of IFNγ independently of *in vitro* ovalbumin stimulation.

Conclusion

Inhibitors of either iNOS (1400 W) or NADPH oxidase (apocynin) reduce the development of airway hyperresponsiveness without an effect on airway inflammation and serum IgE levels. Furthermore, the formation of 3-nitrotyrosine in eosinophils appears to be mediated by eosinophil peroxidase rather than by nitric oxide synthase related processes and does not seem to reflect a pathophysiological mechanism leading to airway hyperresponsiveness. Both nitric oxide synthase and NADPH oxidase inhibition prove to be promising therapeutic targets to prevent the development of allergen induced airway hyperresponsiveness.

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