

Allergen-induced impairment of bronchoprotective nitric oxide synthesis in asthma

Fabio L. M. Ricciardolo, MD, PhD,^{a,b} Mieke C. Timmers,^a Pierangelo Geppetti, MD,^c Annemarie van Schadewijk,^d Jozef J. Brahim, MD,^a Jacob K. Sont, PhD,^e Heidi W. F. M. de Gouw, PhD,^a Pieter S. Hiemstra, PhD,^a J. Han J. M. van Krieken, MD, PhD,^d and Peter J. Sterk, MD, PhD^a *Leiden and Nijmegen, The Netherlands, and Bergamo and Ferrara, Italy*

Background: Endogenous nitric oxide protects against airway hyperresponsiveness (AHR) to bradykinin in mild asthma, whereas AHR to bradykinin is enhanced by inhaled allergens. **Objective:** Hypothesizing that allergen exposure impairs bronchoprotective nitric oxide within the airways, we studied the effect of the inhaled nitric oxide synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA) on AHR to bradykinin before and after allergen challenge in 10 subjects with atopic asthma.

Methods: The study consisted of 3 periods (1 diluent and 2 allergen challenges). AHR to bradykinin (PD₂₀BK) was examined before and 48 hours after allergen challenge, both after double-blinded pretreatment with L-NMMA or placebo. The accompanying expression of the various NOS isoforms (ecNOS, nNOS, and iNOS) was examined by means of immunohistochemistry in bronchial biopsies obtained after diluent and allergen challenge.

Results: After placebo, AHR to BK worsened after allergen challenge in comparison with before allergen challenge (PD₂₀BK, 70.8 nmol [range, 6.3-331] and 257 nmol [35.5-2041], respectively; $P = .0004$). After L-NMMA, preallergen and postallergen PD₂₀BK values (50.1 nmol [1.8-200] vs 52.5 nmol [6.9-204]; $P = .88$) were similarly reduced ($P < .01$) and not different from the postplacebo/postallergen value ($P > .05$). After allergen challenge, the intensity of staining in bronchial epithelium decreased for ecNOS ($P = .03$) and increased for iNOS ($P = .009$). These changes in immunostaining were correlated with the accompanying worsening in AHR to BK ($R_s = -0.66$ and 0.71 ; $P < .04$).

Conclusions: These data indicate that allergen exposure in asthma induces increased airway hyperresponsiveness to

bradykinin through impaired release of bronchoprotective nitric oxide associated with downregulation of ecNOS. This suggests that new therapeutic strategies towards restoring the balance among the NOS isoforms during asthma exacerbations are warranted. (*J Allergy Clin Immunol* 2001;108:198-204.)

Key words: Asthma, airway hyperresponsiveness, allergen, ecNOS, nNOS, iNOS, nitric oxide, bradykinin

Airway hyperresponsiveness (AHR) to nonspecific bronchoconstrictor stimuli, a hallmark of asthma, is associated with the underlying inflammatory process of the disease.¹ Thus the understanding of the mechanism(s) of AHR might help to unravel the pathogenesis of asthma and contribute to the development of novel therapeutic strategies. Allergen exposure in individuals with atopic asthma causes bronchoconstriction, airway inflammation, and increased AHR to nonspecific stimuli,^{2,3} including bradykinin (BK).⁴ Allergen-induced bronchoconstriction is mostly characterized by biphasic responses, known as the early and the late asthmatic responses (EAR and LAR, respectively).² The LAR also shows airway inflammation because of an influx of eosinophils and lymphocytes,^{2,5} and it is accompanied by an increase in AHR.³ Eosinophil-derived proteins that can disrupt the epithelial barrier, hamper enzymatic degradation of mediators, or impair epithelium-derived bronchoprotective factors, including nitric oxide (NO), might contribute to allergen-induced increase in AHR.⁶⁻¹²

NO, a gaseous molecule, is endogenously generated during the conversion of the amino acid L-arginine to L-citrulline by a family of NO synthases (NOSs).¹³ Constitutive and inducible forms of NOS (cNOS and iNOS, respectively) have been described and are both present in the airways with divergent effects.¹³ iNOS expression is induced by proinflammatory cytokines and releases large quantities of proinflammatory NO in a sustained manner.¹⁴ However, Ca²⁺-dependent cNOS activity, represented by neuronal and endothelial NOS isoforms (nNOS and ecNOS, respectively), rapidly releases small amounts of bronchoprotective NO.¹⁵ BK is a mediator¹⁶ that in addition to activating neurally mediated bronchoconstriction stimulates the cNOS pathway.^{17,18} We have previously shown that pretreatment with a nonselective NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA),¹⁹ potentiates AHR to BK in mild,²⁰ but not in

From ^athe Department of Pulmonology, Leiden University Medical Center;

^bthe Department of Respiratory Disease, Bergamo General Hospital; ^cthe Department of Experimental and Clinical Medicine, University of Ferrara;

^dthe Department of Pathology, University Medical Center St Radboud, Nijmegen; and ^ethe Department of Medical Decision-making, Leiden University Medical Center.

Supported by a Research Fellowship of the European Respiratory Society and by the Netherlands Asthma Foundation (grant 96.10).

Received for publication December 26, 2000; revised April 17, 2001; accepted for publication April 17, 2001.

Reprint requests: P. J. Sterk, MD, PhD, Department of Pulmonology, Lung Function Lab, C2-P, Leiden University Medical Center, Albinusdreef 2, PO Box 9600, NL-2300 RC Leiden, The Netherlands. (p.j.sterk@lumc.nl)

Copyright © 2001 by Mosby, Inc.

0091-6749/2001 \$35.00 + 0 1/81/116572

doi:10.1067/mai.2001.116572

Abbreviations used

NO:	Nitric oxide
AHR:	Airway hyperresponsiveness
BK:	Bradykinin
DD:	Doubling dilution
EAR:	Early asthmatic response
ecNOS:	Endothelial constitutive nitric oxide synthase
LAR:	Late asthmatic response
L-NMMA:	N ^G -monomethyl-L-arginine
PD ₂₀ BK:	Provocative dose producing a 20% fall in FEV ₁ to BK
nNOS:	Neuronal nitric oxide synthase
iNOS:	Inducible nitric oxide synthase

severe, asthma.²¹ These findings indicate that the airways are protected by their ability to release NO^{15,20} and that this protective effect is lost in severe asthma.²¹ It is unknown whether this loss can be driven by allergen. We postulated that the allergen-induced increase in AHR to BK⁴ is caused by impairment of cNOS-derived NO.

To test this hypothesis, we examined the effect of inhaled L-NMMA on AHR to BK before and after allergen challenge in patients with mild atopic asthma. As a secondary objective, we studied the effect of allergen on the expression of the 3 NOS isoforms (ecNOS, nNOS, and iNOS) in the epithelium of bronchial mucosal biopsies by means of immunohistochemistry.

METHODS

Subjects

Ten nonsmoking, house dust mite-atopic individuals with clinically stable asthma participated in the study (Table I). None of the participants were on regular medications, and all met the criteria of mild intermittent asthma¹ and were symptom-free at the time of the study. Their values of baseline FEV₁ had to be >70% of predicted,²² whereas the subjects had to be hyperresponsive to inhaled histamine (provocative concentration producing a 20% fall from baseline FEV₁ [PC₂₀] < 8.0 mg/mL).²³ None of the participants had a history of relevant allergen exposure or respiratory tract infection in the 6 weeks before and during the study. Inhaled short-acting β_2 -agonists and beverages containing xanthines were withheld for at least 12 hours before testing. The subjects were enrolled in the study provided that they had a documented EAR (fall in FEV₁ $\geq$ 20% from baseline 0 to 3 hours postallergen) and LAR (fall in FEV₁ $\geq$ 15% 3 to 7 hours) to inhaled house dust mite extract in the screening period.²³ The study was approved by the Medical Ethics Committee of the Leiden University Medical Center, and all patients gave written informed consent.

Study design

Each subject was seen within 1 week on 3 separate screening visits, during which the selection criteria were examined (Table II). A multidose skin prick test and a standardized allergen inhalation challenge with house dust mite extract were performed, as previously reported.^{23,24}

The study had a randomized, double-blinded, placebo-controlled, crossover design and consisted of 3 periods of 4 study days each, separated by wash-out intervals of 2 to 4 weeks. During each randomized study period, the participants underwent a BK

inhalation challenge before (day 3) and 48 hours after (day 10) a diluent (period 1) or allergen challenge (periods 2 and 3, day 8; Table III). Patients inhaled either placebo (periods 1 and 2) or L-NMMA (period 3) before each BK challenge. Tests were performed at the same time of day ($\pm$ 2 hours) in each subject.

Baseline FEV₁ had to be within 10% on each study day in all periods, and a control PC₂₀ histamine on day 1 of each study period had to remain within 1 doubling concentration. After baseline measurements of FEV₁, each participant inhaled an aerosol of either placebo or L-NMMA for 30 minutes. Then, 5 minutes after the end of the aerosol delivery, the participant started a BK challenge. In periods 1 and 2, bronchoscopy was performed in each patient 2 hours after the end of the second BK challenge (day 10), and 6 bronchial biopsies were taken.

Pretreatment

Freshly prepared L-NMMA (10 mg in 3 mL 0.9% NaCl) or placebo (3 mL 0.9% saline solution)^{20,21} were nebulized through use of a jet nebulizer (DeVilbiss 646, Somerset, Pa) operated by means of oxygen (output, 0.13 mL/min) and connected to the central chamber of an inspiratory-expiratory valve box with an expiratory aerosol filter (Ultipor BB50T; Pall, Portsmouth, United Kingdom). The aerosols were inhaled by tidal breathing through the mouth with the nose clipped for 30 minutes.^{20,21}

Bradykinin challenge

BK (0.0024–5.0 mg/mL; Clinalfa AG, L  ufelfingen, Switzerland) was freshly dissolved in 0.9% saline solution on each study day. Doubling doses of BK (from 0.25 nmol) were aerosolized at 5-minute intervals by a jet nebulizer connected to a dosimeter (Spirom, Helsinki, Finland) set to aerosolize for 2-second periods to deliver 16 μ L per breath.^{20,21} The patient, wearing a nose clip, slowly inhaled 7 breaths of each aerosolized solution from end-tidal volume to total lung capacity through a mouthpiece. FEV₁ was measured 1 and 3 minutes after each aerosolization until it dropped by $\geq$ 20% from baseline. The provocative dose of BK causing the 20% fall in FEV₁ was calculated by log-linear interpolation (PD₂₀BK).^{20,21}

Allergen challenge

Allergen challenge was performed according to a standardized tidal breathing method.²³ Purified aqueous allergen extract (62.5–2000 BU/mL) of *Dermatophagoides pteronyssinus* (SQ 503; Vivo-diagnost, ALK, Benelux), with 0.5% phenol as a preservative, was prepared from the same production batch for both the multidose skin prick tests and the bronchoprovocation tests. Doubling concentrations of allergen were aerosolized by means of a DeVilbiss 646 nebulizer (output, 0.13 mL/min) and inhaled at 12-minute intervals by tidal breathing for 2 minutes with the nose clipped, until a fall in FEV₁ of $\geq$ 20% from baseline was reached. In periods 2 and 3, each subject inhaled the same 3 dilutions of house dust mite extract. The response to allergen was measured in duplicate 10 minutes after each inhalation of allergen and repeated at 20, 30, 40, 50, 60, 90, and 120 minutes and then hourly until 10 hours after the last inhalation.²³ The EAR and LAR were defined either as the maximum percentage fall from baseline FEV₁ or as the area under the time-response curve (AUC) from 0 to 3 hours (AUC₀₋₃) and from 3 to 10 hours (AUC₃₋₁₀) after allergen challenge, respectively.²³ The procedure for the diluent challenge was similar to that for the allergen challenge.

Bronchoscopy

Fiberoptic bronchoscopy (outer diameter, 6 mm; Pentax Optical Co, Tokyo, Japan) was carried out by experienced clinical investigators using a standardized protocol and safety procedures based on

TABLE I. Characteristics of participants

Patient no.	Sex	Age (y)	Atopic status*	FVC (% predicted)†	FEV ₁ (% predicted)†	PC ₂₀ FEV ₁ Histamine (mg/mL)‡
1	F	20	5	108	90	0.61
2	F	20	4	95	94	0.29
3	M	21	3	100	81	0.74
4	M	24	6	98	87	1.77
5	M	26	4	112	92	1.0
6	F	21	4	109	103	1.33
7	M	20	5	102	97	0.36
8	M	19	4	93	96	4.23
9	F	26	4	96	100	1.94
10	F	24	3	107	104	3.34

FVC, Forced vital capacity.

*As determined by the number of wheal responses to 10 common allergen extracts (Vivodiagnost, ALK, Benelux).

†Baselines in percentage of predicted values in the screening period.

‡Provocative concentrations of histamine causing a 20% fall in FEV₁ in the screening period.

TABLE II. Design schedule of measurements in the screening period

Screening item	Screening day		
	1	2	3
Questionnaire	×	—	—
Spirometry	×	—	—
Histamine challenge	×	—	—
Skin prick test	×	—	—
Diluent challenges	—	×	—
Multidose skin prick test	—	×	—
Allergen challenge	—	—	×

TABLE III. Design schedule of measurements in the study periods

Study period	Day 1	Day 3	Day 8	Day 10
1	Hist	Pla + BK	Diluent	Pla + BK*
2	Hist	Pla + BK	Allergen	Pla + BK*
3	Hist	L-NMMA + BK	Allergen	L-NMMA + BK

Hist, Histamine challenge; Pla, placebo pretreatment; BK, bradykinin challenge; L-NMMA, N^G-monomethyl-L-arginine pretreatment.

*Bronchoscopy: only in periods 1 and 2.

international guidelines, as have been used in previous studies in our department.⁷ Six bronchial biopsies were taken at the (sub)segmental level from either the right lung (right lower and middle lobe) or the left lung (lingula and left lower lobe) through use of a pair of cup forceps (Reda Ø 2.5 mm without spike; Reda, Tuttlingen, Germany). Alternate biopsy sites (right or left lung) were randomized over the 2 bronchoscopy visits.

Immunohistochemistry

Biopsy samples were immediately fixed in 4% formaldehyde/PBS (w/v) and embedded in paraffin. Four-micrometer-thick sections were made, and antigen retrieval was performed by means of microwave heating of the sections in 0.01-mol/L citrate/PBS (pH 6.0). The sections were incubated overnight with rabbit polyclonal antibodies for eNOS, nNOS, and iNOS (dilution, 1:80 in 1% BSA/PBS; Transduction Laboratories, Lexington, Ky). Next, the sections were incubated with biotinylated swine-anti-rabbit IgG (1:400 in 1% BSA/PBS; Dako A/S, Glostrup, Denmark) as a secondary anti-

body and with a tertiary complex of streptavidin-biotin conjugated to horseradish peroxidase (Dako), according to the manufacturer's instructions, in 1% BSA/PBS. After being rinsed in 0.1 mol/L sodium acetate (pH 5.0), the sections were stained for 7 minutes through use of 3-amino-9-ethyl-carbazole as chromogen. The sections were counterstained with Mayer's hematoxylin and mounted in Kaiser's glycerin. Incubation with PBS supplemented with 1% BSA instead of the primary antibody served as a negative control.

eNOS, nNOS and iNOS staining intensity in intact epithelium was assessed on coded material through use of light microscopy by semiquantitative analysis⁷ and expressed as follows: 0, absence of staining; 1, mild staining; 2, moderate staining; 3, intense staining. The scoring was done twice over a 2-day period by a single observer. Intraobserver repeatability was assessed by weighted κ (κ_w).

Statistical analysis

All data are reported as means and SEs unless otherwise noted. The value of PD₂₀BK was log-transformed for statistical analysis, and the corresponding geometric means were calculated. A repeated measures analysis of variance (MANOVA) was applied to test whether there were any differences in baseline FEV₁ and logPD₂₀BK between the study days. Two-tailed paired Student *t* tests were applied to explore the differences. In addition, the difference in logPD₂₀BK, expressed in terms of doubling dilutions (DDs), was calculated in each individual by means of the formula previously described.²⁰ Reproducibility of the allergen and BK challenges was examined by means of intraclass correlation coefficients (*R*_i). Nonparametric statistical analysis (Wilcoxon rank test) was applied to examine the effect of allergen on NOS immunostaining. Correlation analyses were made by means of Spearman rank correlation testing (*R*_s). Statistical significance was accepted for *P* values less than .05.

RESULTS

The stability of asthma between the challenge days

Baseline FEV₁ was not different between the diluent/allergen days (mean $\pm$ SEM): 3.83 $\pm$ 0.17 L (period 1), 3.78 $\pm$ 0.15 L (period 2), 3.83 $\pm$ 0.18 L (period 3), respectively (MANOVA, *P* = .42). Baseline FEV₁ did not change significantly between the BK challenge days and was not affected by placebo or L-NMMA pretreatment (Table IV).

TABLE IV. FEV₁ values (L) at baseline and after placebo/L-NMMA pretreatment on all bradykinin days of each study period

	Period 1		Period 2		Period 3	
	Day 3	Day 10	Day 3	Day 10	Day 3	Day 10
Baseline	3.86 ± 0.21*	3.84 ± 0.22*	3.87 ± 0.17*	3.77 ± 0.20*	3.83 ± 0.20*	3.74 ± 0.19*
Placebo/L-NMMA	3.84 ± 0.21	3.82 ± 0.21	3.84 ± 0.16	3.78 ± 0.21	3.82 ± 0.20	3.72 ± 0.20
P value	.17	.19	.22	.53	.59	.23

*MANOVA, $P = .13$.

The stability of the response to allergen

The response to allergen was well reproducible between the 2 challenges. The R_i values for AUC₀₋₃, AUC₃₋₁₀, and total AUC were 0.82, 0.92 and 0.9, respectively. The maximum percentage fall in FEV₁ from baseline during the EAR (mean ± SEM) was 43.7% ± 3.6% in period 2 and 41.4% ± 3.8% in period 3 ($P = .44$), whereas AUC₀₋₃ of the 2 allergen responses was 51.5% ± 6.4% fall · h in period 2 and 48.7% ± 8.3% fall · h in period 3 ($P = .56$). All subjects had an LAR (mean ± SEM) with 33.2% ± 4.5% fall in period 2 and 32.1% ± 5.4% fall in FEV₁ in period 3 ($P = .69$), and the AUC₃₋₁₀ was 175.2% ± 29.9% fall · h (period 2) and 176.9% ± 31.0% fall · h (period 3; $P = .89$). In period 1, diluent did not affect FEV₁ at each time point (data not shown).

Bradykinin hyperresponsiveness

BK inhalation caused a dose-related bronchoconstriction on all study days, and a measurable PD₂₀BK was obtained in every case. In period 1, PD₂₀BK values before (day 3: 182 nmol [range, 46.8-676.1 nmol]) and after diluent (day 10: 195 nmol [range, 45.7-832 nmol]) were not different ($P = .13$) and were highly reproducible, as expressed by an R_i of 0.98. Those values of PD₂₀BK were also not significantly different from the postplacebo/preallergen value in period 2 (MANOVA, $P = .56$). As expected, PD₂₀BK after allergen challenge (70.8 nmol [range, 6.3-331.1 nmol]) was significantly lower than the postplacebo/preallergen value in period 2 (257 nmol [range, 35.5-2042 nmol]; $P = .0004$; Fig 1), the mean difference being 1.8 ± 0.38 DDs. However, when L-NMMA pretreatment was used in period 3, PD₂₀BK did not differ between before allergen challenge (50.1 nmol [range, 1.8-200 nmol]) and after allergen challenge (52.5 nmol [range, 6.9-204 nmol]; $P = .88$; Fig 1). In fact, both PD₂₀BK values after L-NMMA pretreatment in period 3 were significantly reduced in comparison with the postplacebo/preallergen value in period 2 ($P < .01$; DDs, 2.36 ± 0.69 and 2.29 ± 0.33, respectively; Fig 1), whereas they did not differ from the postplacebo/postallergen value in period 2 ($P > .05$).

NOS immunostaining

Positive immunoreactivity for all NOS isoforms was observed in both columnar and basal cells of the airway epithelium and was mainly evident on the apical surface of columnar cells (Fig 2). The intraobserver repeatability,

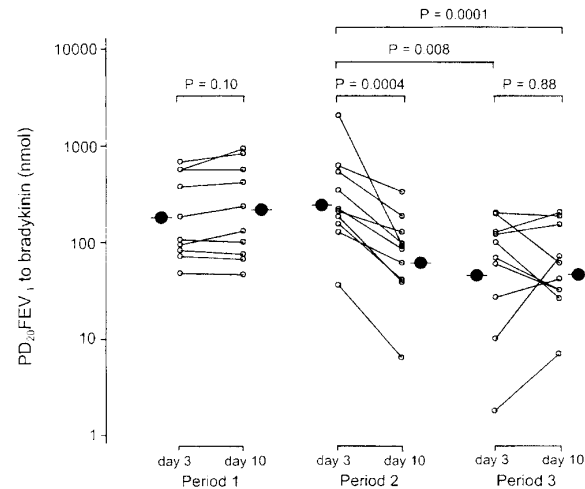


FIG 1. Individual changes in PD₂₀FEV₁ to inhaled BK within the 3 periods of the study in 10 patients with atopic asthma. Period 1: BK challenge with placebo pretreatment, before (day 3) and 48 hours after (day 10) diluent challenge. Period 2: BK challenge with placebo pretreatment, before and after allergen challenge. Period 3: BK challenge with L-NMMA pretreatment, before and after allergen challenge. Each bar indicates the geometric mean of PD₂₀FEV₁ to BK (nmol) on the respective day, and P values have been added.

expressed as κ_w of immunohistochemical analysis, was good for all of the NOS isoforms (ecNOS, 0.83; nNOS, 0.72; iNOS, 0.71). The intensity of the staining for iNOS in the epithelium of the bronchial biopsies was significantly higher 48 hours after allergen inhalation (period 2; score [mean ± SEM], 2.1 ± 0.18) in comparison with biopsies taken 48 hours after diluent exposure (period 1; 1.3 ± 0.15; $P = .009$). The intensity of the staining for ecNOS was, however, lower after allergen (1.3 ± 0.3) than after diluent exposure (2.1 ± 0.18; $P = .03$). In contrast, staining for nNOS in the epithelium was not different between postallergen exposure (1.8 ± 0.2) and postdiluent exposure (2.2 ± 0.13; $P = .084$).

Relationship between BK hyperresponsiveness and NOS immunostaining

The difference between postdiluent and postallergen PD₂₀BK (periods 1 and 2), expressed in DDs (1.5 ± 0.04; $P = .007$), was inversely correlated with the accompanying decrease in ecNOS immunostaining ($R_s = -0.66$; $P = .039$; Fig 3) and positively correlated with the increase in iNOS immunoreactivity ($R_s = 0.71$; $P = .024$).

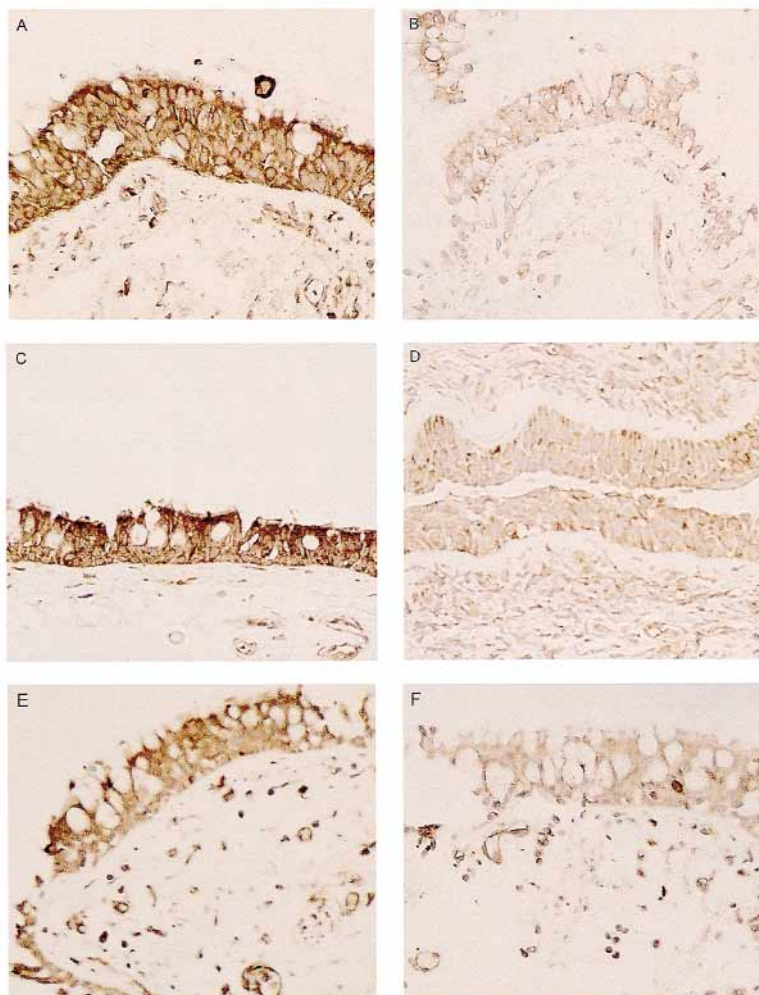


FIG 2. Immunohistochemical staining for eNOS (A and B), nNOS (C and D), and iNOS (E and F) at the highest (A, C, and E) and lowest (B, D, and F) intensity scores in the epithelium of 4- μ m sections of formalin-fixed, paraffin-embedded bronchial biopsies of patients with mild atopic asthma taken 48 hours after diluent (A, C, and F) and allergen (B, D, and E) challenges. Original magnification: 200 $\times$.

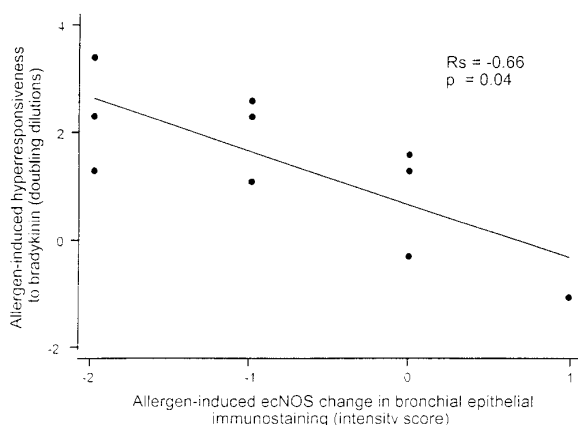


FIG 3. Correlation of the change in AHR to inhaled BK as induced by allergen challenge (change in PD₂₀FEV₁ between day 10 of periods 1 and 2), expressed in DD, with the accompanying allergen-induced fall in the expression of bronchial epithelial eNOS, expressed as intensity score. The regression line has been drawn. R_s , Spearman rank correlation coefficient with corresponding P value.

DISCUSSION

The present study shows that increased AHR to BK, induced by allergen exposure in asthma, is due to impaired production of bronchoprotective NO, a phenomenon that is associated with downregulation of eNOS and upregulation of iNOS within the airway epithelium. These findings underscore the relevance of bronchoprotection by endogenous NO to limit AHR in asthma and warrant the development of treatment strategies to restore eNOS activity during asthma exacerbations.

This message is derived from the following observations. First, we confirmed that allergen exposure leads to increased AHR to BK,⁴ suggesting that allergen might either increase bronchoconstrictor or reduce bronchoprotective mechanisms. Second, L-NMMA potentiated AHR to BK in subjects with mild asthma unexposed to allergen, as previously observed,²⁰ suggesting that in mild asthma BK not only induces bronchoconstriction²⁵ but also mobilizes bronchoprotective NO.²⁰ Third, there are the novel observations that the magnitudes of the

potentiation of BK-induced bronchoconstriction caused by L-NMMA and by allergen challenge were not different and, more important, that the potentiation of BK-induced bronchoconstriction caused by allergen was not further increased by L-NMMA. The latter suggests that allergen and L-NMMA share a final common pathway to exaggerate AHR to BK, consisting of the ability to reduce the release of bronchoprotective NO.

Utmost care was taken to ensure optimization of the method used in this study. We selected a homogenous group of nonsmoking subjects with mild atopic asthma who were not on glucocorticoids, which are known to be iNOS inhibitors,²⁶ before or during the experiments. The results in the present study were obtained by applying a double-controlled design (diluent/allergen and placebo/L-NMMA) and carefully validated methods for inhalation challenge,²³ bronchoscopy,⁷ immunostaining of biopsies,²⁷ and intensity scoring.⁷

NO, derived from neural nNOS, is the mediator of the nonadrenergic, noncholinergic neural relaxation in the airways of several species of mammal, including human beings.²⁸ Recently, it has been proposed that NO released from the epithelium limits bronchoconstriction to spasmogens.¹⁵ Neurally mediated bronchoconstriction by BK^{16,25} is limited by several epithelium-derived factors, including peptidases,²⁹ prostaglandin E₂,^{29,30} and, more importantly, NO.^{8,18,20} Thus, blockade of NOS pathways in guinea pigs potentiated BK-induced bronchoconstriction¹⁷ and changed the BK-induced and epithelium-dependent relaxation of an isolated trachea into a contraction.⁸ In addition, BK caused a Ca²⁺-dependent rapid (cNOS-derived) release of NO from guinea pig airway segments.¹⁸ Finally, allergen (or LPS) increased AHR in guinea pigs during the LAR by cNOS impairment,^{9-11,31,32} whereas after the LAR the partial reversal of AHR is iNOS-dependent.^{31,32} Our study suggests that iNOS-derived bronchoprotective NO in humans is uncertain.

The capability of BK to induce bronchoconstriction in individuals with asthma but not in normal individuals¹⁶ might in part be explained by the shedding of airway epithelium present in those with asthma. For instance, eosinophil-derived epithelial damage might result in reduced peptidase activities and subsequent uncontrolled kinin levels³³ with altered AHR³⁴ or in exposure of sensory nerve endings to noxious agents, including kinins, that might more easily activate bronchomotor neural reflexes.^{16,35} In addition to these mechanisms, we have here provided evidence for an additional epithelial-dependent pathway, which, via an altered expression of NOS isoforms in airway epithelium, results in increased AHR to BK in asthma.

Eosinophil-derived toxic proteins, proinflammatory cytokines, and other mediators formed during allergic inflammation contribute to a change in airway epithelium toward a proinflammatory phenotype,³⁶ which is characterized by downregulation of protective (cNOS)^{9,12} and upregulation of detrimental NOS pathways (iNOS),^{36,37} among other features. Increased expression of iNOS is a relatively long-term process that leads to the release of

large quantities of Ca²⁺-independent proinflammatory NO.¹⁴ Findings obtained after allergen exposure showing downregulation and upregulation of epithelial ecNOS and iNOS, respectively, point out that changed expression of ec/iNOS isoforms is the underlying cause of allergen-induced increase in AHR to BK. This is further supported by the correlations between physiologic and immunohistochemical findings. However, despite the fact that nNOS expression in airway epithelium was not affected by allergen exposure, we cannot rule out a deficiency of nNOS activity in airways with allergic inflammation.³⁸

What are the clinical implications of these findings? Increased AHR to BK in individuals with severe asthma is likely due to the failure of BK to release bronchoprotective NO.²¹ The present study shows that such failure to activate NO-dependent bronchoprotection can be induced by allergens. Allergen exposure is thought to be a major factor in the development,³⁹ maintenance,⁴⁰ and exacerbation⁴¹ of asthma. Current therapy is almost exclusively directed at opposing bronchoconstrictor or proinflammatory stimuli.¹ It can be questioned whether this is the most sensible approach and whether restoring impaired endogenous protection should not have at least a similar priority. Our results demonstrate the functional significance of endogenous bronchoprotective NO in conjunction with ecNOS expression within the airways. This warrants the development of treatment strategies, such as the use of calciumlike peptides⁴² and ecNOS gene transfer,⁴³ aimed at the restoration of downregulated ecNOS in asthma. Such therapeutic targeting might not only be beneficial for patients with severe asthma²¹ but also afford protection during exacerbations of the disease.^{44,45}

REFERENCES

1. National Heart, Lung and Blood Institute. Expert panel report 2. Guidelines for the diagnosis and management of asthma. Bethesda (MD): National Institutes of Health; 1997. NIH publication no. 97-4051.
2. O'Byrne PM, Dolovich J, Hargreave FE. Late asthmatic responses. *Am Rev Respir Dis* 1987;136:740-51.
3. Cockcroft DW, Ruffin RE, Dolovich S, Hargreave FE. Allergen-induced increase in non-allergic bronchial reactivity. *Clin Allergy* 1977;7:503-13.
4. Berman AR, Togias AG, Skloot G, Proud D. Allergen-induced hyperresponsiveness to bradykinin is more pronounced than that to methacholine. *J Appl Physiol* 1995;78:1844-52.
5. Bentley AM, Kay AB, Durham SR. Human late asthmatic reactions. *Clin Exp Allergy* 1997;27(Suppl 1):71-86.
6. Gleich GJ, Flavahan NA, Fujisawa T, Vanhoutte PM. The eosinophil as a mediator of damage to respiratory epithelium: a model for bronchial hyperreactivity. *J Allergy Clin Immunol* 1988;81:776-81.
7. Sont JK, van Krieken JH, van Klink HCJ, Roldaan AC, Apap CR, Willems LNA, et al. Enhanced expression of neutral endopeptidase (NEP) in airway epithelium in biopsies from steroid- versus non-steroid treated patients with atopic asthma. *Am J Respir Cell Mol Biol* 1997;16:549-56.
8. Figini M, Ricciardolo FLM, Javdan P, Nijkamp FP, Emanuelli C, Pradelles P, et al. Evidence that epithelium-derived relaxing factor released by bradykinin in the guinea-pig trachea is nitric oxide. *Am J Respir Crit Care Med* 1996;153:918-23.
9. De Boer J, Meurs H, Coers W, Koopal M, Bottone AE, Visser AC, et al. Deficiency of nitric oxide in allergen-induced airway hyperreactivity to contractile agonists after the early asthmatic reaction: an ex vivo study. *Br J Pharmacol* 1996;119:1109-16.
10. Mehta S, Drazen JM, Lilly CM. Endogenous nitric oxide and allergic bronchial hyperresponsiveness in guinea pigs. *Am J Physiol* 1997;273:L656-L662.

11. Schuilting M, Zuidhof, Bonouvrie MAA, Venema N, Zaagsma J, Meurs H. Role of nitric oxide in the development and partial reversal of allergen-induced airway hyperreactivity in conscious, unrestrained guinea-pigs. *Br J Pharmacol* 1998;123:1450-6.
12. Meurs H, Schuurman FE, Duyvendak M, Zaagsma J. Deficiency of nitric oxide in polycation-induced airway hyperreactivity. *Br J Pharmacol* 1999;126:559-62.
13. Zapol WM, Bloch KD. Nitric oxide and the lung. New York: Marcel Dekker; 1997.
14. Morris SM, Billiar TR. New insights into the regulation of inducible nitric oxide synthesis. *Am J Physiol* 1994;266:E829-E839.
15. Folkerts G, Nijkamp FP. Airway epithelium: more than just a barrier! *Trends Pharmacol Sci* 1998;19:334-41.
16. Barnes PJ. Bradykinin. In: Barnes PJ, Grunstein MM, Leff AR, Woolcock AJ, editors. *Asthma*. New York: Lippincott-Raven; 1997. p. 577-84.
17. Ricciardolo FLM, Nadel JA, Yoshihara S, Geppetti P. Evidence for reduction of bradykinin-induced bronchoconstriction in guinea-pigs by release of nitric oxide. *Br J Pharmacol* 1994;113:1147-52.
18. Ricciardolo FLM, Vergnani L, Wiegand S, Ricci F, Manzoli N, Fischer A, et al. Detection of nitric oxide release induced by bradykinin in guinea-pig trachea and main bronchi using a porphyrinic microsensor. *Am J Respir Cell Mol Biol* 2000;22:97-104.
19. Reif DW, McCreedy SA. N-nitro-L-arginine and N-monomethyl-L-arginine exhibit a different pattern of inactivation toward the three nitric oxide synthases. *Arch Biochem Biophys* 1995;320:170-6.
20. Ricciardolo FLM, Geppetti P, Mistretta A, Nadel JA, Sapienza MA, Bellofiore S, et al. Randomised double-blind placebo-controlled study of the effect of inhibition of nitric oxide synthase in bradykinin-induced asthma. *Lancet* 1996;348:374-7.
21. Ricciardolo FLM, Di Maria GU, Mistretta A, Sapienza MA, Geppetti P. Impairment of bronchoprotection by nitric oxide in severe asthma. *Lancet* 1997;350:1297-8.
22. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault J-C. Lung volumes and forced ventilatory flows. *Eur Respir J* 1993;6(Suppl 16):5-40.
23. Sterk PJ, Fabbri LM, Quanjer PH, Cockcroft DW, O'Byrne PM, Anderson S, et al. Airway responsiveness: standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. *Eur Respir J* 1993;6(Suppl 16):53-83.
24. Cockcroft DW, Murdock KY, Kirby J, Hargreave F. Prediction of airway responsiveness to allergen from skin sensitivity to allergen and airway responsiveness to histamine. *Am Rev Respir Dis* 1987;135:264-7.
25. Ichinose M, Nakajima N, Takehashi T, Yamauchi H, Inoue H, Takishima T. Protection against bradykinin-induced bronchoconstriction in asthmatic patients by neurokinin receptor antagonist. *Lancet* 1992;340:1248-51.
26. Di Rosa M, Radomski M, Camuccio R, Moncada S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive nitric oxide synthase in vascular endothelial cells. *Proc Natl Acad Sci U S A* 1990;87:10,043-9.
27. Coers W, Timens W, Kempinga C, Klok PA, Moshage H. Specificity of antibodies to nitric oxide synthase isoforms in human, guinea pig, rat, and mouse tissues. *J Histochem Cytochem* 1998;46:1385-92.
28. Belvisi MG, Stretton CD, Yacoub M, Barnes PJ. Nitric oxide is the endogenous neurotransmitter of bronchodilator nerves in humans. *Eur J Pharmacol* 1992;210:221-2.
29. Frossard N, Stretton CD, Barnes PJ. Modulation of bradykinin responses in airway smooth muscle by epithelial enzymes. *Agents Actions* 1990;31:204-9.
30. Leikauf GD, Ueki IF, Nadel JA, Widdicombe JH. Bradykinin stimulates Cl secretion and prostaglandin E2 release by canine tracheal epithelium. *Am J Physiol* 1985;248:F48-F55.
31. Schuilting M, Meurs H, Zuidhof AB, Venema N, Zaagsma J. Dual action of iNOS-derived nitric oxide in allergen-induced airway hyperreactivity in conscious, unrestrained guinea pigs. *Am J Respir Crit Care Med* 1998;158:1442-9.
32. Toward TJ, Bradley KJ. Airway reactivity, inflammatory cell influx and nitric oxide in guinea-pig airways after lipopolysaccharide inhalation. *Br J Pharmacol* 2000;131:271-81.
33. Christiansen SC, Proud D, Sarnoff RB, Jurgens U, Cochrane CG, Zuraw BL. Elevation of tissue kallikrein and kinin in the airways of asthmatic subjects after endobronchial allergen challenge. *Am Rev Respir Dis* 1992;145:900-5.
34. Roisman GL, Lacroque JG, Desmazes-Dufeu N, Carre C, Le Cae A, Dusser DJ. Airway responsiveness to bradykinin is related to eosinophilic inflammation in asthma. *Am J Respir Crit Care Med* 1996;153:381-90.
35. Barnes PJ. Asthma as an axon reflex. *Lancet* 1986;i:242-5.
36. Holgate ST. The cellular and mediator basis of asthma in relation to natural history. *Lancet* 1997;350(Suppl 2):5-9.
37. Xiong Y, Karupiah G, Hogan SP, Foster PS, Ramsay AJ. Inhibition of allergic airway inflammation in mice lacking nitric oxide synthase 2. *J Immunol* 1999;162:445-52.
38. De Sanctis GT, MacLean JA, Hamada K, Mehta S, Scott JA, Jiao A, et al. Contribution of nitric oxide synthases 1, 2, and 3 to airway hyperresponsiveness and inflammation in a murine model of asthma. *J Exp Med* 1999;189:1621-30.
39. Sporik R, Holgate ST, Platts-Mills TAE, Cogswell JJ. Exposure to house-dust mite allergen (Der p 1) and the development of asthma in childhood—a prospective study. *N Engl J Med* 1990;323:502-7.
40. Platts-Mills TAE, Carter MC. Asthma and indoor exposure to allergens. *N Engl J Med* 1997;336:1382-4.
41. Djukanovic R, Feather I, Gratzou C, Walls A, Peroni D, Bradding P, et al. Effect of natural allergen exposure during the grass pollen season on airways inflammatory cells and asthma symptoms. *Thorax* 1996;51:575-81.
42. Villain M, Jackson PL, Manion MK, Dong WJ, Su Z, Fassina G, et al. De novo design of peptides targeted to the EF hands of calmodulin. *J Biol Chem* 2000;275:2676-85.
43. Lund DD, Faraci FM, Miller FJ, Heistad DD. Gene transfer of endothelial nitric oxide synthase improves relaxation of carotid arteries from diabetic rabbits. *Circulation* 2000;101:1027-33.
44. Gaston B, Sears S, Woods J, Hunt J, Ponaman M, McMahon T, et al. Bronchodilator S-nitrosothiol deficiency in asthmatic respiratory failure. *Lancet* 1998;351:1317-9.
45. Folkerts G, van der Linde HJ, Nijkamp FP. Virus-induced airway hyperresponsiveness in guinea pigs is related to a deficiency in nitric oxide. *J Clin Invest* 1995;95:26-30.