Nitric oxide regulation of asthmatic airway inflammation with segmental allergen challenge

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Background: Despite evidence of increased nitric oxide (NO) in asthmatic compared with healthy individuals, the role of NO in airway inflammation is unclear.
Objective: The purpose of the study was to determine the in vivo effects of localized allergen challenge on airway NO levels and transcription factor activation.
Methods: In this study localized allergen challenge was used as a model of asthmatic exacerbation to determine the relationship of NO to airway inflammation.
Results: With allergen challenge, asthmatic patients had a rise in airway NO levels, whereas NO levels in healthy controls did not change. The increased NO in asthma with allergen challenge compared with healthy control subjects was associated with an increase in inflammatory cytokines (GM-CSF and macrophage inflammatory protein-1) in epithelial lining fluid and eosinophilic infiltrate in bronchoalveolar lavage fluid (BAL) and biopsy specimens. To investigate the mechanisms of cytokine gene expression, activation of the transcription factors activator protein-1 and nuclear factor-κB (NF-κB) in cells from BAL were evaluated. Activator protein-1 was not activated before or after local allergen challenge. In contrast, NF-κB activation was less in BAL cells from asthmatic patients with increased NO in comparison with controls.
Conclusion: Our studies are the first to suggest an inverse correlation between NF-κB and airway NO in a localized segmental allergen challenge model in allergic asthmatic patients. The current study demonstrates that activation of the inflammatory response (eg, cytokines, cellular infiltrate) in allergic asthmatic patients is temporally associated with increased airway NO. We propose that NO that is up-regulated by cytokines is part of an autoregulatory feedback loop (ie, allergen challenge stimulates inflammatory cytokine production, which in turn stimulates NO production, and NO down-regulates cytokine production). (J Allergy Clin Immunol 1999;104:1174-82.)

Key words: *Alveolar macrophage, asthma, nitric oxide, transcription factors, nuclear factor-κB, activator protein-1, segmental allergen challenge*

Abbreviations used

AP-1:	Activator protein-1
BAL:	Bronchoalveolar lavage
ELF:	Epithelial lining fluid
EMSA:	Electrophoretic mobility shift assay
MIP-1α:	Macrophage inflammatory protein-1α
NF-κB:	Nuclear factor-κB
NO:	Nitric oxide
PD_{20}:	Provocative dose causing a 20% reduction in FEV_1
PNU:	Protein nitrogen units
SAC:	Segmental allergen challenge
WCE:	Whole cell extract

Asthma is characterized by chronic airway inflammation, the pathogenesis of which is not well understood. One potential mediator in asthmatic inflammation is nitric oxide (NO), a freely diffusible molecule that is found in high levels in exhaled air of asthmatic individuals.[1] NO is important to the lung in smooth muscle relaxation, neurotransmission, and host defense.[1] However, when NO is produced in excess, NO or its reaction products may be cytotoxic.[2]

Despite clear evidence of increased NO in asthma, the function of NO as a toxic proinflammatory or protective anti-inflammatory agent is unresolved. Increased exhaled NO has been associated with active asthma under suboptimal control.[3,4] Therapies to reduce inflammation in asthma, such as glucocorticoids, also reduce airway NO levels.[5] Inhalation of NO has a small bronchodilating effect, and NO modulates bronchial tone in patients with asthma.[6] Also, inhalation of NO blunts methacholine-induced bronchospasm.[7]

Our previous in vitro studies have suggested that NO may function in an anti-inflammatory capacity through down-regulation of inflammatory cytokine secretion by human alveolar macrophages.[8] Cytokines down-regulated by NO include macrophage inflammatory protein-1α (MIP-1α) and GM-CSF.[8,9] Both these cytokines are among the chemoattractants for inflammatory cells such as eosinophils, basophils, and T lymphocytes implicated in asthmatic inflammation.[10,11] Furthermore, the transcription factor nuclear factor-κB (NF-κB), involved in inflammatory cytokine gene transcription, is decreased by NO in a dose-dependent manner in endotoxin-stimu-

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TABLE I. Characteristics of study population

Patient No.	Age (y)	Sex	Baseline FEV_1 (L)	Baseline FEV_1 (% predicted)[13]	A-PD_{20} by WLAC (PNU/mL)	Maximum Ag by WLAC (PNU/mL)	Fall in FEV_1 at maximum Ag (%)	Ag for SAC	SAC dose (PNU in 10 mL of NS)
Asthmatic									
1	29	F	2.89	88	1,000	1,000	–20	Grass	100
2	44	F	2.58	98	2,455	3,162	–21	Grass	300
3	40	F	3.05	100	232	316	–28	Grass	30
4	38	F	2.74	97	250	316	–25	Grass	100
5	31	F	3.37	96	1,441	3,162	–40	Grass	300
6	47	F	1.35	52	894	1,000	–24	Grass	100
7	37	M	3.68	86	9,464	10,000	–20	Ragweed	100
8	27	M	3.17	76	1,397	3,162	–35	Grass	300
Normal									
1	44	F	3.13	106	NA	10,000	4	Grass	100
2	26	F	2.83	84	NA	10,000	4	Grass	100
3	53	M	3.33	90	NA	10,000	2	Grass	100
4	41	F	2.85	104	NA	10,000	0	Grass	100
5	50	M	4.25	124	NA	10,000	–3	Grass	100
6	41	M	3.70	84	NA	10,000	–9	Ragweed	100

A-PD_{20}, Allergen provocative dose to elicit 20% decrease in FEV_1, *WLAC,* whole lung allergen challenge; *PNU,* protein nitrogen units; *Ag,* allergen, *SAC,* segmental allergen challenge; *NS,* normal saline solution; *F,* female; *M,* male; *NA,* not available.

lated alveolar macrophages.[12] Taken together, these observations support the hypothesis that NO may have important anti-inflammatory effects in the airway, principally by functioning in an autoregulatory loop to down-regulate cytokine production. The purpose of the current study was to determine the in vivo effects of localized allergen challenge to confirm whether NO down-regulates inflammation.

METHODS

Subjects

Six healthy control subjects and 8 patients with mild asthma were recruited for the study at the Cleveland Clinic (Table I). Asthma was defined by the National Institutes of Health guidelines and included episodic respiratory symptoms, reversible airflow obstruction (documentation of variability of FEV_1 by 12% and 200 mL either spontaneously or after inhaled albuterol) or a positive methacholine challenge test.[14] All subjects underwent allergy prick skin testing with a standard panel of 10 aeroallergens (Bayer, Spokane, Wash), normal saline solution (negative control), and histamine (positive control). A positive reaction was defined as a wheal ≥3 mm in diameter. The prick needle was a 2-pronged lancet from Allergy Labs of Ohio. Skin tests were read after 15 minutes. Allergic asthmatic patients had 2 or more positive skin tests, one of which was ragweed or Timothy grass. Healthy controls were nonsmokers and skin test negative, and they had a negative methacholine provocation test (per the method of Chai et al[15], decrease in FEV_1 <20% with maximum concentration of methacholine of 25 mg/mL over 5 stages). All subjects had an FEV_1 ≥50% predicted, no asthma exacerbation, or respiratory tract infection within 1 month. Subjects were off inhaled anti-inflammatory agents for ≥6 weeks (except subject 5, who was off ≥2 weeks) and not taking oral steroids for ≥12 weeks. The study was approved by the Cleveland Clinic Institutional Review Board and patients gave written informed consent.

Study design

At an initial screening visit subjects were classified as allergic asthmatics or as nonallergic healthy controls. Each subject underwent a medical history, physical examination, and pulmonary function testing. Allergic asthmatic patients underwent whole-lung aerosol allergen challenge with ragweed or grass (grass was used in the ragweed season and ragweed was used in the grass season) to demonstrate antigen responsiveness and to determine the provocative dose that caused a 20% reduction in FEV_1 (antigen PD_{20}). A stock solution of allergen (Greer Laboratories, Lenoir, NC) (10,000 protein nitrogen units [PNU]/mL) was diluted with saline solution to produce 8 concentrations (1, 3.16, 31.6, 100, 1000, and 3162 PNU/mL). The allergen preparations at the concentrations used were all below the USP acceptable limit of endotoxin for injection (0.25 endotoxin units/mL) as determined by the limulus amebocyte lysate assay.[16] The patient took 5 breaths at each concentration per the method of Chai et al[15] through a Rosenthal-French dosimeter. The dose in PNU of allergen producing a 20% fall in FEV_1 (antigen PD_{20}) was determined. The dose used for segmental allergen challenge was 10% A-PD_{20} diluted into 10 mL of saline solution into each segment. The healthy nonallergic controls were challenged with 100 PNU allergen. At least 4 weeks after the aerosol challenge a bronchoscopy was carried out to obtain a baseline bronchoalveolar lavage (BAL). Three 50-mL aliquots of normal saline solution warmed to 37°C were instilled in the lingula and withdrawn by gentle hand aspiration. Processing of BAL was as described previously.[8] Allergen (in 10 mL of saline solution) was instilled into a segment of the right middle lobe. Subjects underwent a second bronchoscopy 48 hours later to obtain a BAL from the allergen-challenged segment. As an additional control, a sham challenge with saline solution in another segment was performed in 1 healthy control subject and in 2 asthmatic patients. Biopsy specimens were obtained from the allergen-challenged segment and in some subjects from another unchallenged segment at 48 hours.

Measurement of airway NO

NO was measured in lower airway gases sampled with use of a Teflon catheter inserted through the working channel of the bronchoscope, as previously described in detail.[17] Steady-state NO in the airway gases was quantified by chemiluminescence from the orifice of the lingula (control segment) at baseline and from right medial lobe (postallergen challenge) at 48 hours during a 10- to 15-second expiratory breathhold (Sievers NOA280, Boulder, Colo).

Cytokine assays

Lavage fluid was concentrated by Ultrafree-15 centrifugal filter devices (Biomax-5, Millipore, Bedford, Mass) at least 20-fold. GM-CSF and MIP-1 were measured in the concentrated lavage fluid by ELISA (Endogen, Woburn, Mass, R&D Systems, Minneapolis, Minn). To correct for dilution of epithelial lining fluid (ELF) in BAL fluid, the concentration of urea was measured by blood urea nitrogen assay (Sigma Diagnostics, St Louis, MO) in the BAL fluid and was compared with the serum urea concentration.

Preparation of whole cell extracts

Extracts were prepared from freshly isolated BAL cells. For extraction, cells were resuspended in extraction buffer (20 mmol/L TRIS [pH 8.0], 150 mmol/L magnesium chloride, 1% Triton-X100) containing protease inhibitor cocktail and kept 20 minutes on ice. The samples were then centrifuged at 18,000*g* for 20 minutes at 4°C to clear debris, and supernatants representing WCE were collected. WCE were aliquoted in small volume to minimize repeated freeze thaw and kept at –80°C for further use. Protein content of WCE was determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill).

Electrophoretic mobility shift assay

For electrophoretic mobility shift assay (EMSA), 10 μg of the WCE was incubated in binding buffer (8 mmol/L HEPES, pH 7.0, 10% glycerol, 20 mmol/L potassium chloride, 4 mmol/L magnesium chloride, 1 mmol/L sodium pyrophosphate) containing 1.0 μg of poly-dI-dC and phosphorus 32–end labeled double-stranded oligonucleotide probe. The probes have sequences as follows:

NF-κB:
AACTCCGGGAATTTCCCTGGCCC
TTGAGGCCCTTAAAGGGACCGGG
Activator protein-1 (AP-1):
CGCTTGATGACTCAGCCGGAA
GCGAACTACTGAGTCGGCCTT

After incubation with the probe for 20 minutes, the reaction mixture was analyzed on a 4% nondenaturing acrylamide gel. The gels were then dried and exposed for autoradiography. When the activated transcription factors bind to the probe, the mobility is retarded (free probe runs faster). Retarded band(s) are detected by autoradiography. To demonstrate specificity of DNA-protein interaction, competition experiments with 1000-fold excess of unlabeled oligonucleotide or supershift of DNA-protein band by p65 or p50 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) were performed.

Immunofluorescence staining

The cell suspensions from washed lavage cells (100,000 cells/mL) were cytospun on slides and fixed in cold acetone for 2 minutes and stored at –20°C until stained. Before staining, slides were rinsed in PBS, pH 7.4, for 10 minutes. The slides were blocked with 2% goat serum for 15 minutes. Slides were briefly rinsed in PBS and stained with p65 antibody (Santa Cruz Biotechnology) for 60 minutes in a humid chamber. Slides were washed 3 times in PBS. Fluorescein goat antirabbit IgG in 2% goat serum was added, and the slides were incubated in a humid chamber for 45 minutes in the dark. Slides were washed 3 times in PBS and then coverslips were mounted on slides with Vectashield containing propidium iodide (Vector Laboratories, Burlingame, Calif) and sealed with clear lacquer. Simultaneous 2-color fluorescence images were collected on a Leica TCS-NT laser scanning confocal microscope. Nuclear p65 was quantified by the Bioquant True Color Windows 95, version 2.0 system (R & M Biometrics, Nashville, Tenn). This system provides advanced image analysis routines for morphometry. Two-color images from dual-labeled immunofluorescent staining (p65 antibody, fluorescein green; DNA, propidium iodide red) were used. The amount of red fluorescence in the cell was the area of the nucleus (denominator) and the amount of yellow (fluorescein green p65 antibody + propidium iodide red DNA) was the amount of antibody (p65) in the nucleus (numerator). Approximately 5 to 10 random fields containing 20 to 40 cells each were scanned for each sample. All samples from an individual control or asthmatic patient were stained and analyzed at the same time to avoid variations in staining intensity or laser intensity.

Statistical analysis

Healthy control subjects and asthmatic patients were compared with use of 1-sided *t* tests at baseline and at 48 hours with respect to the individual outcomes. The groups were compared with respect to the challenge effect (absolute difference) by either a *t* test for independent samples or by ANOVA, adjusting for baseline levels as a covariate if the baseline levels differed between the groups. We used paired *t* tests to study challenge effects within groups. Individual α levels of .05 were used to determine statistical significance, and 1-sided tests were considered appropriate for our directional hypothesis.

RESULTS

Inflammatory cells

At baseline the BALs from the healthy control subjects and the asthmatic patients were not different in total cell recovery, viability, or cellular differential (Table II). The allergen-challenged segment from the asthmatic group had a significant increase in the percent of eosinophils at 48 hours ($P = .02$, *t* test) and a corresponding decrease in the percent of macrophages. However, the absolute number of macrophages was slightly increased (Table II). Fig 1 shows the increase in eosinophils from baseline to 48 hours after allergen challenge for each asthmatic and healthy subject (5 of 6 healthy subjects showed no eosinophils at baseline or at 48 hours). The control group demonstrated no change in eosinophils from baseline. Biopsy specimen data confirmed the eosinophilic infiltrate (17.3 ± 7.3 cells/high power field asthmatic vs 2.3 ± 0.9 controls).

Increased NO in allergic asthmatic patients on allergen challenge

Airway NO levels were evaluated in asthmatic patients and in healthy controls before and after allergen challenge. NO was sampled at the time of the first bronchoscopy before the baseline BAL and at the second bronchscopy before the BAL in the allergen-challenged segment. Fig 2 shows the results from 7 asthmatic and 5 healthy subjects before and after allergen challenge. Asthmatic patients with low baseline airway NO levels demonstrated increased levels of NO with challenge. NO levels in those with high baseline levels showed minimal change. The asthmatic airway NO levels, adjusted for baseline NO levels, were significantly increased after allergen ($P = 0.05$, ANOVA).

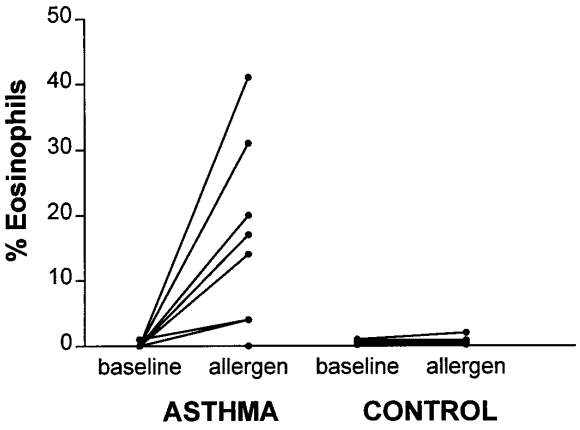

FIG 1. Increased eosinophils in BAL fluid with allergen challenge for asthmatic patients. Asthmatic patients demonstrate significant increase in eosinophils with allergen challenge (P = .02).

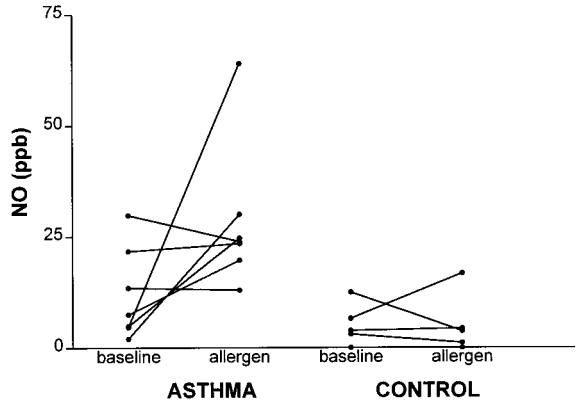

FIG 2. Increased airway NO levels after allergen challenge. NO levels of asthmatic patients are significantly increased with allergen challenge in comparison to healthy control subjects (P = .05). Asthmatic patients with low baseline airway NO levels demonstrated increased levels of NO with challenge. NO levels of asthmatic patients with high baseline levels showed minimal change.

Increased cytokine levels in BAL in asthmatic patients after allergen challenge

Concentrated BAL was analyzed for GM-CSF and MIP-1 levels. Fig 3 shows the GM-CSF and MIP-1 levels in the baseline ELF and the ELF from the allergen-challenged segment at 48 hours from both asthmatic and healthy control subjects. MIP-1 levels were significantly increased from baseline to 48 hours after allergen challenge in asthmatic patients (P = .01). Healthy control subjects did not demonstrate an increase in MIP-1 levels. Similarly, GM-CSF levels were increased from baseline to 48 hours after allergen in asthmatic but not healthy control subjects (P = .04).

Transcription factors

Because the transcription factors NF-κB and AP-1 are involved in inflammatory cytokine gene transcription,[18,19] we examined WCE from BAL cells for binding to consensus sequences of NF-κB and AP-1. Fig 4 shows results from extracts of baseline and 48-hour postallergen challenge lavages in 2 asthmatic and 2 healthy control subjects. Both asthmatic patients had a decrease in NFκB activation in BAL cells and an increase in airway NO levels. The 2 healthy control subjects demonstrated an increase in NF-κB binding between baseline and allergen challenge with one individual showing a slight increase in NO and the other individual showing no change in NO. In addition, the saline solution sham samples from the control and asthmatic subjects show an increase in NF-κB compared with baseline. From the healthy control subject (C5), the saline solution and allergen samples are not different, but from the asthmatic patient (A2) the saline solution sample shows an increase in NF-κB binding and the allergen sample shows a decrease (an additional asthmatic saline solution sample also demonstrated an increase over baseline binding [data not shown]). Supershift assays of both asthmatic and healthy control subject extracts confirmed the presence of both p50 and p65 in the complexes (Fig 5 shows the supershifts from an asthmatic patient). None of the subjects showed significant AP-1 activation in BAL cells and no change was demonstrated between baseline and 48 hours after challenge (data not shown).

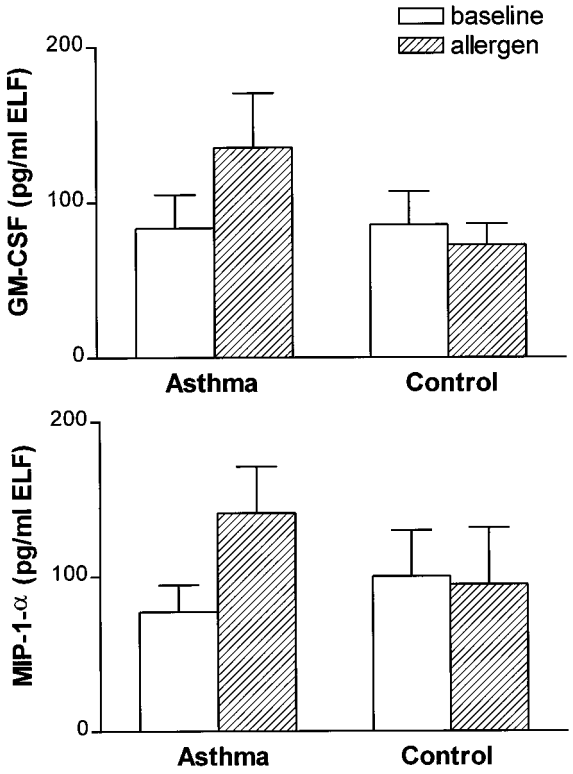

FIG 3. Increased GM-CSF and MIP-1 in BAL after allergen challenge. GM-CSF levels are significantly increased after allergen challenge (P = .04). Similarly, MIP-1 levels in BAL from asthmatic subjects are increased after allergen challenge (P = .01).

TABLE II. Leukocytes in BAL fluids after segmental allergen challenge

	Asthmatics		Controls	
	Baseline control	48-h Allergen challenge	Baseline control	48-h Allergen challenge
Total cell No. ($\times 10^6$)	6.5 ± 0.8	9.5 ± 2.5	7.2 ± 1.6	7.0 ± 1.6
Viability (%)	92 ± 2	92 ± 2	93 ± 2	93 ± 1
Alveolar macrophage (%)	96.1 ± 1.0	74.5 ± 5.0*	96.0 ± 0.8	89.0 ± 3.8
Lymphocytes (%)	3.1 ± 0.7	4.1 ± 0.8	3.0 ± 1.0	4.8 ± 1.7
Polymorphonuclear leukocytes (%)	0.6 ± 0.4	5.0 ± 1.8	0.8 ± 0.6	5.8 ± 3.5
Eosinophils†	0.1 ± 0.1	16.4 ± 5.0*	0.2 ± 0.2	0.3 ± 0.3

*Significantly changed from baseline (P <.05).
†Change from baseline differed significantly between asthmatics and controls (P < .05).

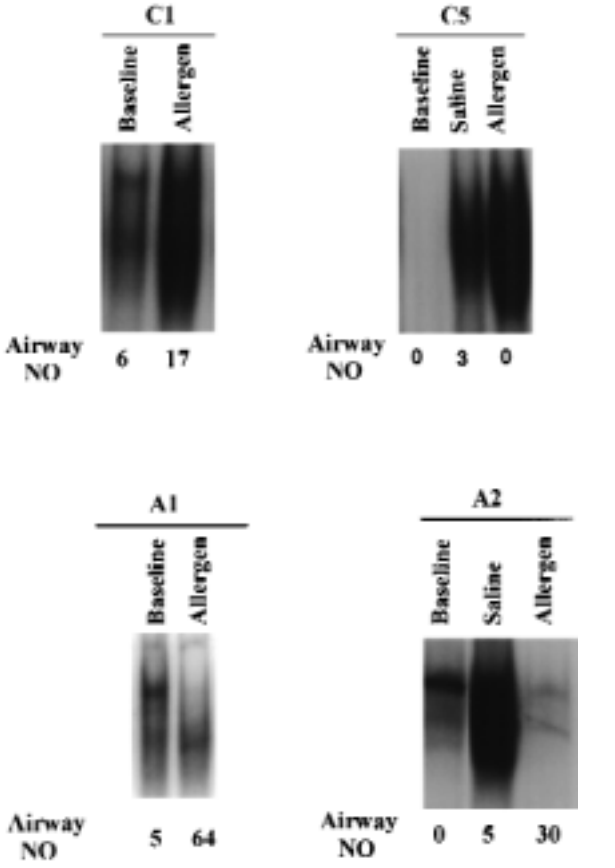

FIG 4. NF-κB activation in WCE from BAL cells and airway NO levels of 2 healthy control and 2 asthmatic subjects at baseline and 48 hours after allergen challenge. Both asthmatic patients demonstrate decrease in NF-κB activation in BAL cells and increase in airway NO levels. The 2 healthy control subjects demonstrated increase in NF-κB activation between baseline and allergen challenge, with one individual showing slight increase in NO and other individual showing decrease in NO. Sham saline solution samples demonstrated increased NF-κB activation in comparison to baseline for both healthy control (C5) and asthmatic (A2) subjects.

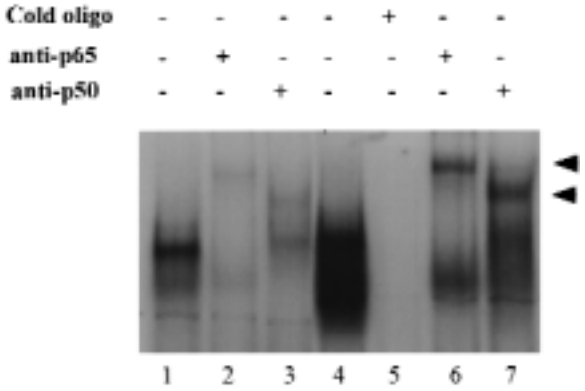

FIG 5. NF-κB complex contains both p50 and p65. EMSA of WCE from positive control subject *(lanes 1-3)* and asthmatic patient *(lanes 4-7)* demonstrated that both p50 and p65 are contained in the NF-κB complex.

To confirm the translocation of p65 to the nucleus and to quantify the change in NF-κB activation, immunofluoresence and Bioquant image analysis were carried out on cytospin preparations from both healthy control and asthmatic subjects (Fig 6, *A* to *D*). The healthy control subject demonstrated an increase in nuclear p65 with allergen challenge compared with baseline, whereas the asthmatic patient shows a decrease.

The EMSA and confocal results as well as mean airway NO levels are summarized in Fig 7. All 5 control subjects demonstrated an increase in NF-κB activation by EMSA or immunofluorescence and no change in mean airway NO levels. An increase in eosinophils in BAL is a marker of a delayed allergic response after allergen challenge. None of the 5 controls demonstrated BAL eosinophilia. The 3 asthmatic patients with an eosinophil response demonstrated decreased NF-κB activity after allergen challenge. The 2 asthmatic patients without the eosinophil response demonstrated an increase in NF-κB from baseline to allergen challenge, similar to the controls with minimal change in mean NO levels.

DISCUSSION

The relationship between NO and asthma has not been conclusively defined. Airway NO levels in asthmatic patients are heterogeneous, with some patients in the same range as healthy control subjects. Whether this variation in airway NO levels corresponds to disease activity is not known. All the patients in the current study had mild asthma. Allergen-induced late asthmatic reactions have been associated with increased exhaled NO.[20]

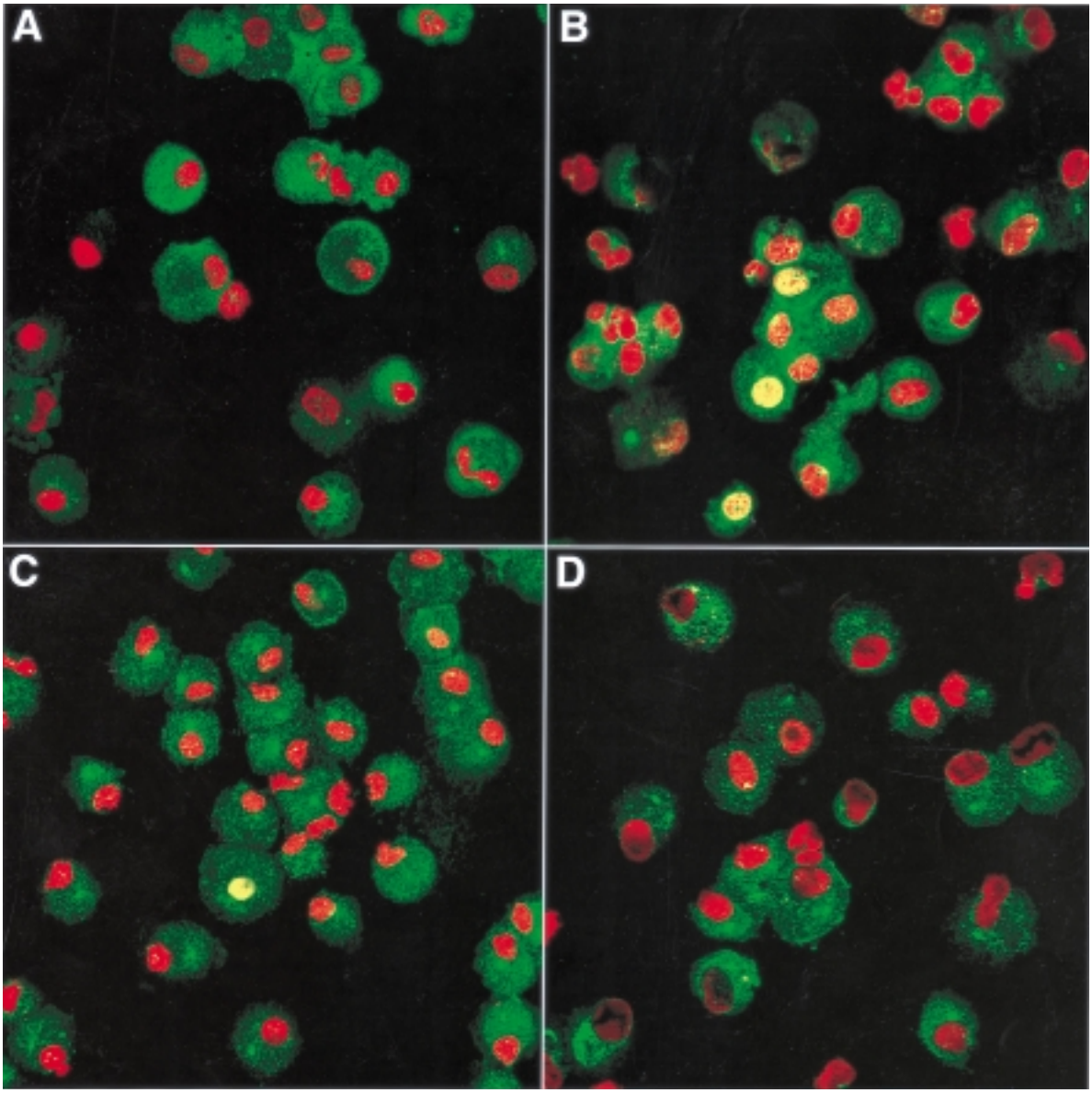

FIG 6. Intracellular localization of p65. BAL cytospin preparations were stained with p65 antibody. Coverslips were mounted on cytospin preparations with mounting media containing propidium iodide, which stains the nucleus red. If NF-κB is not activated, p65 staining is localized to cytoplasm. When activated, NF-κB migrates to nucleus and nuclei appear yellow. Healthy control subject demonstrated increase in nuclear staining with allergen challenge **(B)** compared with baseline **(A)** (6-fold increase as determined by Bioquant). Asthmatic patient demonstrated decrease in nuclear staining with allergen challenge **(D)** compared with baseline **(C)** (2-fold decrease as determined by Bioquant).

Glucocorticoids decrease exhaled NO.[5] Beneficial effects of exogenous NO (bronchodilation) and detrimental effects with NO inhibitors (bronchoconstriction) have been reported.[7,21] In contrast, a recent study reported no effect on early and late asthmatic responses of L-N^G-nitroarginine methyl ester.[22] In vitro studies have shown inhibition of inflammatory cytokine production by NO in human alveolar macrophages and a decrease in NF-κB activation.[8,12]

Segmental allergen challenge (SAC) coupled with bronchoscopy provides a valuable model to investigate inflammatory mechanisms in allergic asthma.[16,23] The main advantage of SAC over simple observational studies of bronchoscopy in chronic stable asthma is the ability to determine the temporal relationship between the exacerbating factor and the onset of the inflammation in a controlled setting. Numerous post-SAC studies to evaluate the late asthmatic response have been performed at various time points including 19, 24, 48, and 72 to 96 hours.[16,23] Studies have repeatedly shown maximal BAL

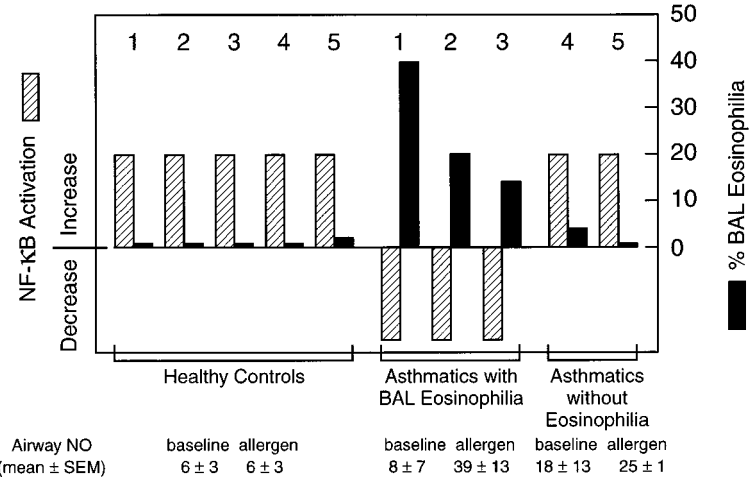

FIG 7. Summary of NF-κB activation as determined by EMSA or immunofluorescence in healthy control and asthmatic BAL samples baseline and after allergen challenge. NF-κB activation is shown as an increase or decrease from baseline to allergen challenge. Of 10 samples evaluated for NF-κB activation, 4 control and 2 asthmatics samples were evaluated by both EMSA and immunofluorescence. All samples demonstrating increase or decrease (2- to 16-fold change) by quantitative method of immunofluorescence coupled with Bioquant analysis also demonstrated increase or decrease by qualitative EMSA method (Table III). All 5 controls demonstrated increase in NF-κB activation by EMSA or immunofluorescence. Increase in eosinophils in BAL is marker of delayed allergic response after allergen challenge. None of 5 controls demonstrated BAL eosinophilia. Three asthmatic patients with eosinophil response demonstrated decreased NF-κB activity after allergen challenge, with increased airway NO levels, and 2 asthmatic patients without eosinophil response and minimal change in NO levels demonstrated increase in NF-κB from baseline to allergen challenge, similar to that of control subjects.

TABLE III. Comparison of NF-κB activation determined by EMSA and Bioquant

	EMSA*	Bioquant (fold change)†
Healthy controls		
1	+	ND
2	+	6
3	+	6
4	+	3
5	+	3
Asthmatics		
1	–	–2
2	–	ND
3	–	ND
4	+	16
5	ND	2

ND, Not determined.
*EMSA results are reported as increase (+) from baseline to allergen challenge or a decrease (–).
†Bioquant results are reported as fold change in amount of nuclear yellow from baseline to allergen challenge (see Methods for details).

eosinophilia, cyokine release, and cell activation both at 24 and 48 hours with greater tissue eosinophilia at 48 hours.[16,23] We chose 48 hours to avoid the early neutrophilia frequently noted at 24 hours and to maximize tissue inflammation.

In the current study results from 8 asthmatic and 6 healthy control subjects undergoing localized endobronchial allergen challenge demonstrate a rise in airway NO levels, particularly in patients with low baseline NO levels. This increase in NO is associated with an increase in inflammatory cytokines in ELF as well as inflammatory cellular infiltrate in both BAL and biopsy specimens. Increased MIP-1 levels were also reported by Cruikshank et al[24] at 6 hours after challenge. GM-CSF levels increased in asthmatic patients from baseline to 48 hours after allergen challenge. Significant increases in GM-CSF have been reported in other studies.[25,26] Thus increased NO levels after allergen challenge are associated with increases in inflammatory markers (eg, cytokines, cellular infiltrate).

Our previous in vitro studies in alveolar macrophages demonstrated a decrease in NF-κB activation with increasing NO levels.[12] Preliminary data from the WCEs of BAL cells from the 3 asthmatic patients with increased airway NO and BAL eosinophilia with allergen challenge show decreased NF-κB activation, whereas the 2 patients with no eosinophilia did not demonstrate a decrease in NF-κB. In contrast, the samples from the 5 healthy control subjects showed increased NF-κB with challenge, although their airway NO levels did not increase. Despite the increase in NF-κB in healthy control subjects, cytokine levels in ELF were unchanged with allergen challenge. This observation is consistent with the BAL itself inducing macrophage priming. This effect was also shown with the saline solution sham controls where 2 asthmatic and 1 healthy control subject

demonstrated an increase of NF-κB over baseline (Fig 5). An example of priming is demonstrated by the in vitro studies of Haskill et al[27] with monocytes. Adherence was shown to activate NF-κB and cytokine messenger RNA but cytokine product was not made. A second signal is necessary for messenger RNA translation. The small number of subjects prohibits definitive conclusions regarding the in vivo relationship of NO and cytokine gene transcription. However, these data are consistent with the hypothesis that NO may have important anti-inflammatory effects in the airway principally by functioning in an autoregulatory loop to down-regulate cytokine production. In previous studies we demonstrated high levels of NFκB activation in freshly obtained BAL cells from asthmatic patients with low airway NO levels in comparison to healthy control subjects and asthmatic patients with high airway NO levels.[12] If NO regulates NF-κB in vivo in asthma, the cytokine levels should decrease in patients with increasing NO and decreasing NF-κB levels. We propose that NO, which is up-regulated by cytokines, is part of an autoregulatory feedback loop (ie, allergen challenge stimulates inflammatory cytokine production, which in turn stimulates NO production, and NO down-regulates cytokine production). Future studies should evaluate post-SAC BAL at several other time points to further define the kinetics of NF-κB activation and cytokine production. Studies evaluating the effect of pretreatment with exogenous NO on these parameters are also needed.

Our studies are the first to demonstrate the inverse correlation between NF-κB and airway NO in a localized SAC model in allergic asthmatic patients. Previous studies dealt with whole lung allergen challenge and measures of NO at the mouth without BAL. Kharitonov et al[20] demonstrated increased NO in dual responders between 10 and 21 hours after challenge. Taylor et al[22] reported that exhaled NO in dual responders increased at 21 hours after challenge and that treatment with the NO inhibitor L-N^G-nitroarginine methyl ester prevented the increase in NO. Hart et al[28] reported NF-κB in lung cells from pooled samples from multiple asthmatic patients without allergen challenge and NO was not measured. Furthermore, our work would suggest that with the heterogeneity among patients pooling of samples would not provide valid indicators of activation.

In summary, with this model we have shown that an increase in airway NO levels occurs with localized allergen challenge, particularly in asthmatic patients with low baseline airway NO levels. Inflammatory parameters as measured by eosinophilic infiltrate and cytokine levels in BAL fluid also increased with challenge. This preliminary study shows an association between airway NO and established parameters of inflammation. Our studies are the first to suggest an inverse correlation between NF-κB and airway NO in a localized segmental allergen challenge model in allergic asthmatic patients. Additional studies manipulating NO levels by exogenous NO or NO blockers with the SAC model and samplings at multiple time points are necessary to establish whether NO is anti-inflammatory or proinflammatory in allergic asthma.

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