Inhibition of nitric oxide synthesis attenuates thermally induced asthma

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Kotaru, C., M. Skowronski, A. Coreno, and E. R. McFadden, Jr. Inhibition of nitric oxide synthesis attenuates thermally induced asthma. *J Appl Physiol* 91: 703–708, 2001.—To determine whether the inhibition of nitric oxide (NO) synthesis attenuates thermally induced obstruction, we had 10 asthmatic volunteers perform isocapnic hyperventilation with frigid air after inhaling 1 mg of N^G-monomethyl-L-arginine (L-NMMA) or isotonic saline in a blinded fashion. The challenges were identical in all respects, and there were no differences in baseline lung function [1-s forced expiratory volume (FEV_1); saline 2.8 ± 0.3 liters; L-NMMA 2.9 ± 0.3 liters; $P = 0.41$] or prechallenge fractional concentration of nitric oxide in the exhaled air (F_{ENO}) [saline 23 ± 6 parts/billion (ppb), L-NMMA 18 ± 4 ppb; $P = 0.51$]. Neither treatment had any impact on the FEV_1, pulse, or blood pressure. After L-NMMA, F_{ENO} fell significantly ($P < 0.0001$), the stimulus-response curves shifted to the right, and the minute ventilation required to reduce the FEV_1 20% rose 53.5% over control ($P = 0.02$). The results of this study demonstrate that NO generated from the airways of asthmatic individuals may play an important role in the pathogenesis of thermally induced asthma.

thermal stimulation; exercise-induced asthma

NITRIC OXIDE (NO) IS AN IMPORTANT component of homeostasis and host defenses (3, 8). It is found in a variety of tissues and is produced from the metabolism of L-arginine by enzymes known collectively as NO synthases (NOS) (3, 8). Both constitutive and inducible isoforms of NOS exist, and the former appears to play the predominant role in releasing NO under physiological conditions (3, 8). In the lung, NO has profound effects on bronchial caliber and vasomotor tone, and NOS is expressed in the airway epithelium, the bronchial and pulmonary endothelium, and the sensory nervous system (3, 8).

In addition to its influences on normal physiology, there is a growing body of information that the concentration of exhaled NO may be a valuable marker of the state of inflammation in the lungs of asthmatic individuals (1, 16). The airways of patients with this illness have increased expression of the inducible isoform of NOS (24), and their expirates contain elevated levels of NO that change in concert with exposure to stimuli that exacerbate or ameliorate the activity of the underlying disease (6, 19, 26). Nonetheless, there has been limited information linking NO to the development of acute exacerbations, and that which is available is disappointingly negative. Typically, the fractional concentration of the exhaled gas after challenges either remains unchanged or falls (7, 14, 15). Most recently, however, it has been shown that expired NO increases in concert with the bronchial narrowing seen with hyperpnea (18). Because this pattern temporally mirrors the heat and water fluxes known to cause the obstruction in this condition (9, 10), and because it differs markedly from the circumstances reported with antigen and other agonists (14, 15, 30), it raises the possibility that NO may be part of the pathogenesis of thermally induced asthma. If this reasoning is correct, then inhibition of NO production should attenuate the airflow limitation that follows airway cooling and rewarming. The present study was undertaken to test this postulate.

METHODS

Ten asthmatic patients [4 men and 6 women, mean age 32 ± 3 (SE) yr] served as our subjects (Table 1). Seven of them participated in an earlier study on NO dynamics (18). Thermally induced asthma was considered present if there were symptoms of airway obstruction associated with a decrease in the 1-s forced expiratory volume (FEV_1) of at least 20% after exertion. None of the participants smoked tobacco products, had symptoms of an upper respiratory tract infection in the 6 wk preceding the study, or used oral corticosteroids.

The subjects continued their regular antiasthma medications during the course of the trial and only stopped them temporarily before challenges. Bronchodilators were withheld for 12 h or more, and long-acting decongestants and leukotriene-modifying agents were not permitted for 5 days before any investigation. Inhaled corticosteroids were continued in patients using them to prevent a destabilization of the disease. The doses of these agents were stable for a minimum of 1 mo before the study was initiated and remained constant throughout it. The institutional review board for human

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Table 1. *Physiological and demographic profiles*

Subject No.	Age, yr	Gender	FEV_1, liters	FEV_1, %predicted	Medications
1	29	M	3.57	84	β_2
2	46	F	1.88	80	β_2, IS
3	28	M	2.78	65	β_2, IS
4	28	F	2.55	89	β_2
5	50	F	1.88	78	β_2, IS
6	29	M	4.01	91	β_2, AL, AH
7	31	F	2.28	79	β_2
8	22	F	3.07	91	β_2, IS
9	27	M	3.89	86	β_2, NS
10	27	F	2.43	90	β_2, IS, AH
Mean $\pm$ SE	32 $\pm$ 3		2.83 $\pm$ 0.26	83 $\pm$ 3	

FEV_1, 1-s forced expiratory volume; M, male; F, female; β_2, β_2-agonist; IS, inhaled steroid; AL, antileukotriene; AH, antihistamine; NS, nasal steroid.

investigation approved the protocol, and all participants gave informed consent.

Isocapnic hyperventilation of frigid air was used as a surrogate for exercise, and stimulus-response relationships were generated using standard techniques (9, 10). Minute ventilation ($\dot{V}E$) was progressively increased while the subjects inhaled through a heat exchanger (9, 10). Hyperpnea started at 20 l/min and progressed in 20 l/min intervals until the FEV_1 fell the desired amount. Each bout of hyperventilation lasted 4 min, and recovery took place in room air. The water content of the inspirate during hyperpnea was <1 mg H_2O/l, which for the purposes of this study was considered zero. The expired air was directed away from the heat exchanger into a reservoir balloon that was being constantly evacuated at a known rate through a calibrated rotameter. The subjects were coached to keep the balloon filled, and in so doing, their $\dot{V}E$ could be controlled at any desired value. The level of ventilation was then verified directly with a dry-gas meter. End-tidal P_{CO_2} concentrations were monitored with a Nellcor N-1000 analyzer (Mallinckrodt, Kansas City, KS), and sufficient CO_2 was added to the inspiratory port of the exchanger during hyperpnea to maintain end-tidal P_{CO_2} at eucapnic levels. The provocation was stopped when FEV_1 decreased ≥20% from the prechallenge values.

Maximum forced exhalations were performed in triplicate using a waterless spirometer before and 5 min after cessation of each bout of hyperpnea. The curves with the largest FEV_1 were chosen for analysis. The level of ventilation required to produce a 20% decrement in lung function ($P\dot{V}E_{20}$) was obtained by linear interpolation.

NO production was inhibited by the inhalation of N^G-monomethyl-L-arginine (L-NMMA) (11, 19). The compound was supplied as a dry powder by Sigma Chemical (St. Louis, MO) and reconstituted immediately before use. One milligram of L-NMMA in a volume of 5 ml of normal saline was administered by a DeVilbiss nebulizer over 15–20 min. Blood pressure (BP) and pulse rates (PR) were monitored before, during, and after nebulization. The FEV_1 was also recorded before and after the drug.

The concentrations of nitric oxide in the exhaled air (F_ENO) were measured in eight subjects using previously reported techniques (2, 18). The subjects exhaled against a fixed resistance of 5 cmH_2O built into the mouthpiece to exclude nasal NO (2). NO concentration was recorded with a chemiluminescence analyzer designed for physiological measurements (CLD 77 AM, ECO Physics, Ann Arbor, MI). The system was calibrated with NO-free gas and a standard NO concentration of 15 parts per million (Praxair, Bethlehem,

PA) daily before use. The detection limit was 1.1 ± 0.2 parts per billion (ppb) (coefficient of variation <1%). Ambient NO levels were recorded at the start and end of each experiment. During the studies, samples were drawn continuously from the expired port of the mouthpiece at a rate of 300 ml/min during resting tidal breathing and hyperventilation. The output of the analyzer was fed into a time-based recorder (Omega Engineering, Stamford, CT) for on-line display. The 100% response time of the instrument complex (analyzer, sample tubing, and recorder) to a square wave of NO was 320 ms.

The investigation was performed in a randomized, single-blinded, placebo-controlled fashion. On one occasion, baseline measurements of FEV_1, BP, PR, and ambient levels of NO were recorded before the administration of 5 ml of nebulized isotonic saline. Thirty minutes later, spirometry and the hemodynamic variables were repeated and resting values for F_ENO were obtained. The frigid-air challenge then commenced. The second study was identical to the first in all particulars except that L-NMMA was the aerosolized agent.

The data were analyzed by paired *t*-tests and a two-factor repeated-measures analysis of variance. All statistical tests were two-sided, and *P* values < 0.05 were considered significant.

RESULTS

The prechallenge FEV_1 was 83 ± 3% of predicted (2.83 ± 0.26 liters) (Table 1). All of the subjects used β_2-agonists, and five took inhaled steroids. One each used an antileukotriene drug and a nasal steroid, and two took specific H_2 antihistamines. The BP and PR were similar before each arm of the trial and were unaffected by saline or L-NMMA (mean BP presaline 114/77 mmHg, postsaline 115/75 mmHg, *P* = 0.79; pre-L-NMMA 113/75 mmHg, post-L-NMMA 115/76 mmHg, *P* = 0.82; mean PR presaline 81 beats/min, postsaline 78 beats/min, *P* = 0.36; pre-L-NMMA 78 beats/min, post-L-NMMA 80 beats/min, *P* = 0.63).

There were no significant differences for the baseline values for FEV_1 between saline and L-NMMA (*P* = 0.41), and neither treatment had any particular influence (presaline FEV_1 2.83 ± 0.26 liters, postsaline FEV_1 2.79 ± 0.25 liters, *P* = 0.18; pre-L-NMMA FEV_1 2.90 ± 0.29 liters, post-L-NMMA FEV_1 2.91 ± 0.34 liters, *P* = 0.87). Similarly, there were no differences between the mean temperatures of the inspired air between challenges (saline −19 ± 2°C, L-NMMA −19 ± 2°C; *P* = 0.82).

The individual changes in lung function that followed the thermal provocations are presented in Fig. 1. After saline, hyperpnea produced a stimulus-response reduction in FEV_1 to levels ≥20% in each subject (maximum decrement in FEV_1 31.5 ± 2.2%, *P* < 0.001; $\dot{V}E$ 43 ± 8 l/min, *P* < 0.001). Blockade of NOS attenuated the obstructive response and shifted the curves to the right in 9 of the 10 subjects. After L-NMMA, the mean FEV_1 fell 23.4% less at the maximum $\dot{V}E$ (L-NMMA ΔFEV_1 24.0 ± 1.3%, *P* = 0.002; $\dot{V}E$ 65 ± 11 l/min, *P* < 0.001), and $P\dot{V}E_{20}$ rose 53.5% over control (saline $P\dot{V}E_{20}$ 36.2 ± 6.8 l/min, L-NMMA $P\dot{V}E_{20}$ 55.4 ± 8.4 l/min, *P* = 0.02) (Fig. 2).

The average ambient concentration of NO ranged between 16 and 18 ppb, and there were no significant
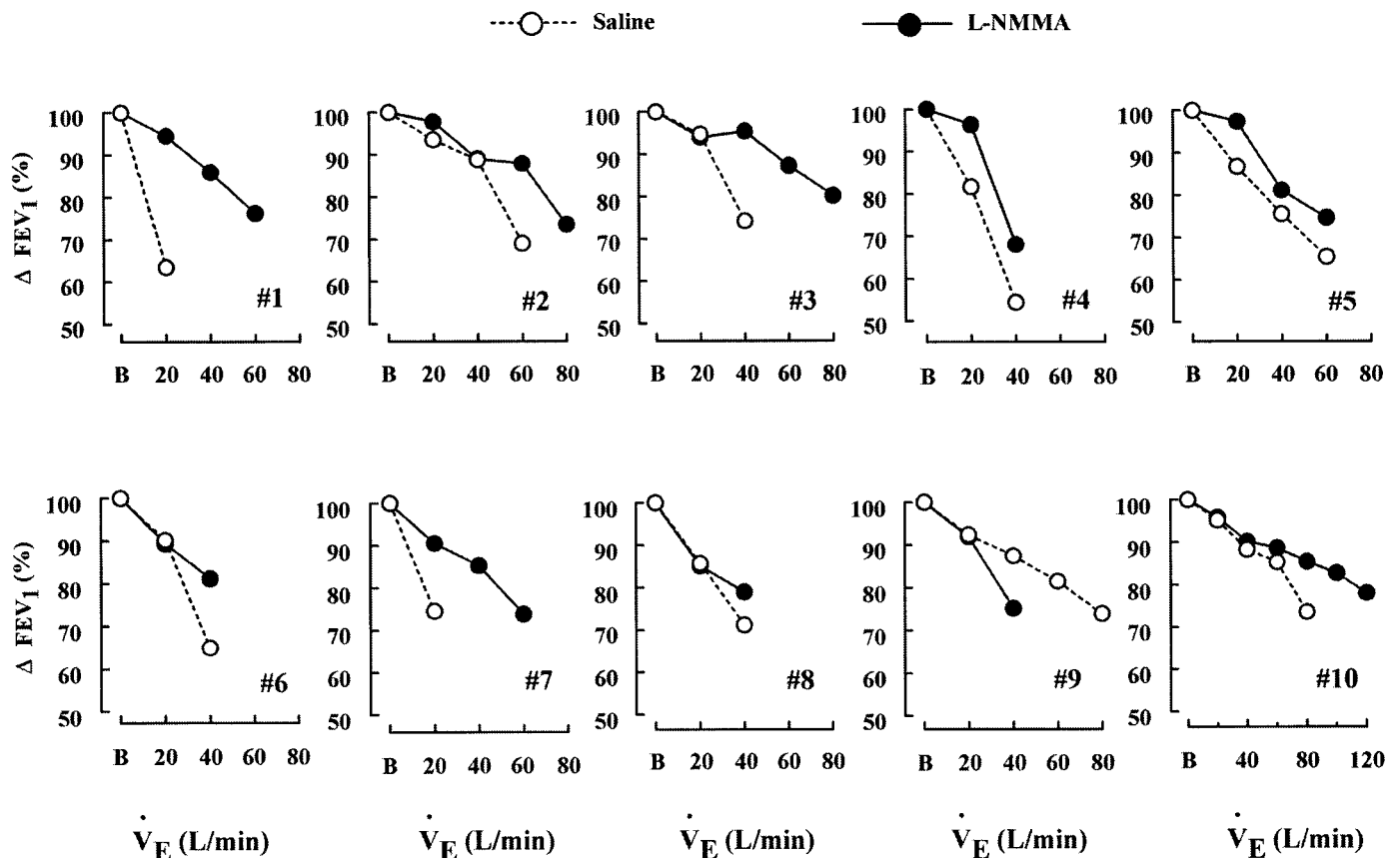
Fig. 1. Individual stimulus-response relationships to isocapnic hyperventilation after saline and N^G-monomethyl-L-arginine (L-NMMA). ΔFEV_1%, percent decrement in 1-s forced expiratory volume (FEV_1) from baseline (B). $\dot{V}E$, minute ventilation. $\circ$, Effects of saline; $\bullet$, post-L-NMMA data.

differences between the saline and L-NMMA trials ($P = 0.82$). The $F_{E_{NO}}$ values at the ventilatory challenges associated with the threshold changes in the FEV_1 after saline and L-NMMA are shown in Fig. 3. In the
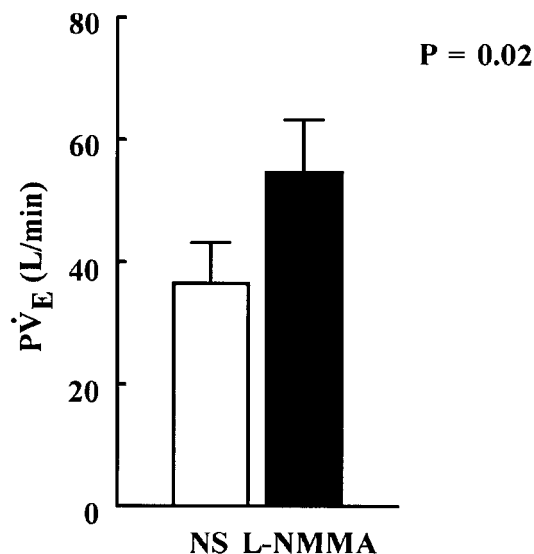

Fig. 2. Comparison of the provocative ventilations ($P\dot{V}E$) required to reduce the FEV_1 20% after saline [normal saline (NS)] and L-NMMA. Bars are mean values, and vertical lines above the bars are 1 SE.

saline arm, resting $\dot{V}E$ averaged 11 ± 2 l/min, rose significantly to 43 ± 8 l/min during the challenge ($P < 0.003$), and then fell to 14 ± 2 l/min during the first minute after hyperpnea ceased ($P < 0.008$ compared with hyperpnea and $P = 0.14$ compared with baseline). The corresponding values for the L-NMMA arm were 11 ± 2, 65 ± 11, and 15 ± 2 l/min, respectively, and the same pattern of significance held as in the control situation. $\dot{V}E$ during hyperpnea was significantly different between trials (Fig. 3, *inset*), but there were no differences in the resting and recovery data between saline and L-NMMA presented above (resting $P = 0.87$; recovery $P = 0.38$). The pretreatment concentrations for $F_{E_{NO}}$ were similar (saline $F_{E_{NO}}$ 23 ± 6 ppb, L-NMMA $F_{E_{NO}}$ 18 ± 4 ppb; $P = 0.51$), and neither agent had any effect on the resting concentrations (baseline saline $F_{E_{NO}}$ 20 ± 5 ppb, L-NMMA 14 ± 2 ppb, pre-post comparison saline $P = 0.69$, L-NMMA $P = 0.24$; postdrug saline vs. L-NMMA $P = 0.34$; 0 time, Fig. 3). After saline, hyperpnea was associated with a significant reduction in $F_{E_{NO}}$ at all points ($P = 0.003$), which then rapidly rose during recovery ($P = 0.008$). By the fifth minute posthyperpnea, $F_{E_{NO}}$ exceeded baseline significantly ($P = 0.03$). As can be seen from Fig. 3, the administration of L-NMMA did not alter NO dynamics qualitatively but did so quantitatively. The exhaled concentrations during hyperpnea and recovery post-

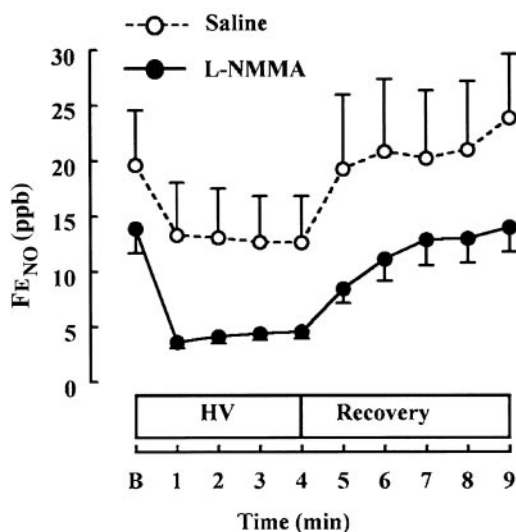|	Saline	L-NMMA	P
$\dot{V}_E$ (L/min)	43 ± 8	65 ± 11	0.01
$\Sigma \dot{V}_{NO}$ (nL/min)	26,153	14,094	0.0002

Fig. 3. Comparison of nitric oxide measurements [fractional concentration of nitric oxide (F_{ENO}) in parts per billion (ppb)] during and after saline and L-NMMA at the points where the FEV_1 fell 20%. Data points are mean values, and vertical lines above and below points are 1 SE. ○, Effects of saline; ● effects of L-NMMA. The time point B presents postinhalation data at the start of 4 min of hyperventilation (HV rectangle). The data in the 5-min recovery period are shown in the second rectangle labeled recovery. *Inset*, comparisons between $\dot{V}_E$ and the total amount of nitric oxide produced ($\dot{V}_{NO}$) in each challenge. The *P* values are derived from L-NMMA-saline comparisons.

L-NMMA were significantly reduced from *minute 1* onward (L-NMMA vs. saline, $F = 26$, $P = 0.00001$; time vs. treatment $F = 2.6$, $P = 0.008$). Unlike saline, however, the F_{ENO} in the fifth minute of recovery did not exceed the baseline ($P = 0.90$). The total concentration of NO exhaled during the experiment (i.e., $\Sigma[F_{ENO} \times \dot{V}_E$ *minute 1* to *minute 9*]) was significantly less post L-NMMA ($P = 0.0002$; Fig. 3, *inset*).

DISCUSSION

The results of the present study demonstrate that inhibition of NO synthesis attenuates the bronchial narrowing produced by cooling and rewarming in asthmatic subjects. After L-NMMA, the stimulus-response curves to isocapnic hyperventilation of frigid air shifted to the right and the $P\dot{V}_{E20}$ rose 53.5% over control. Because NOS blockade lowers F_{ENO} concentrations but has no influence on prechallenge airway tone, it appears that the generation and release of NO during hyperpnea plays an important role in thermally induced asthma.

The prophylaxis offered by L-NMMA against airway cooling and rewarming appears unique and stands in stark contrast to the effects of NOS inhibition on other asthma precipitants. The traditional view of NO is that small amounts reduce airway tone by relaxing airway smooth muscle (3, 8) and its inhibition by the L-arginine analogs, L-NMMA and N^G-nitro-L-arginine methyl ester, typically results in enhancement of bronchoconstriction when given to patients, rather than attenuation. Ricciardolo et al. (23) observed L-NMMA to increase airway reactivity and amplify the changes in lung function associated with methacholine and bradykinin in asthmatic individuals with mild disease, whereas Taylor and associates (27) noted a similar effect with L-NAME on histamine and adenosine 5′-monophosphate. If, however, the generation of NO suddenly rises, as at the end of hyperpnea, it may actually facilitate the development of bronchial narrowing. This postulate fits well with the observations of Sapienza and colleagues (24), who reported that increasing the substrate for NOS by having normal and asthmatic subjects inhale L-arginine and L-alanine increased the formation of endogenous NO in the airways and caused the FEV_1 to fall in the asthmatic individuals.

Evidence is accumulating that suggests that NO is an integral biochemical component in the conditioning of inspired air (4, 12, 13, 18), and it is possible that asthmatic patients have a defect at some level in the process. Whenever ventilation rises, airway temperatures fall (9, 10, 22) and NO production increases (4, 12, 13). Simultaneously, the blood supply to the heat-exchanging regions mounts (12, 17), presumably to provide energy for the process and to prevent thermal damage (9). In asthmatic individuals, this last phase appears critically important in the pathogenesis of the obstruction. When the vascular response is augmented, it not only greatly amplifies bronchial narrowing in asthmatic individuals but also causes normal subjects to develop airflow limitation de novo (21).

As in other studies in both normal and asthmatic people (16, 30), our data demonstrate that inhibition of NO synthesis reduces exhaled NO. As shown in Fig. 3, the F_{ENO} and the calculated total amount exhaled over the experiment were significantly less after L-NMMA. Even so, the obstructive response was merely blunted and not eliminated, suggesting that NO is only part of the reaction sequence in thermally induced asthma. It is known that the cysteinyl leukotrienes are involved in the pathogenesis of this condition (5), and given the similar physiological activities of both mediators, it is conceivable that they may interact positively to aggravate the underlying chronic inflammation in the bronchial circulation (29). As yet, there are no data in humans on this subject, but studies in sensitized guinea pigs indicate that endogenous NO acts to increase leukotriene-induced airway microvascular leakage (20). Obviously, if this were to occur in asthma, physiologically significant bronchial narrowing could ensue.

The anatomic source of the NO is unknown, but all evidence suggests that it derives from elements within the conducting airways rather than the pulmonary vasculature. Because hyperventilation, per se, does not

increase cardiac output or pulmonary blood flow (22), the contributions from this source in our experiment remained constant. In contrast, because the increased airflow heightened the diffusion gradient from the epithelium to the bronchial lumen, excretion from the tracheobronchial tree rose as ventilation increased (28). In addition, L-NMMA limited the development of obstruction without any impact on systemic hemodynamics. If significant absorption from the airway into the bloodstream with recirculation to the tracheobronchial tree had occurred, we would have expected a change in BP and/or PR.

We appreciate that the results after NOS inhibition were not homogeneous. We do not believe that this pattern is due to extraneous factors, such as the experimental design, or to an ineffectualness of L-NMMA. Isocapnic hyperventilation was employed as a surrogate for exercise because it produces the identical thermal events physical exertion as exercise, allows stimulus-response curves to be generated, and is less wearing on the subjects (9, 10). All of the participants had documented exercise-induced asthma, and their responses to cold-air hyperpnea were characteristic. Furthermore, their medication requirements were also typical, and although the drugs they took such as inhaled corticosteroids may have reduced their airway inflammation and so altered the prechallenge NO levels before the study commenced (19, 25, 26), this is unlikely to have made much difference to the overall findings. The treatment they received during the investigation remained constant, and the saline and L-NMMA arms were carried out in identical fashion within days of each other. For example, there were no significant differences between the responses of the five subjects taking and the five not taking inhaled steroids (saline study, users ΔFEV_1 31 ± 2%, nonusers 33 ± 4%, P = 0.93; L-NMMA arm users 23 ± 2%, nonusers 25 ± 2%, P = 0.91). As expected, the fractional expired concentrations of NO were lower in the three individuals who used these agents, and in whom we have NO data, but there was no apparent influence of the drugs on the changes in FEV_1 after saline or L-NMMA. We understand that the sample size is too small to be conclusive, but the trend shows little impact. Seven of them participated in an earlier investigation, and the pattern of NO kinetics seen herein within the control challenge exactly matches earlier findings (18) and lies within reported variations (25). The $F_{E_{NO}}$ fell with hyperpnea and overshot baseline values as bronchial narrowing developed. This series of events continued to take place months after the original study. Finally, the dose of L-NMMA we employed has been used effectively in the past to inhibition NOS synthesis (16, 23), and, clearly, it worked here as well. Even given the limitations of the measurements techniques we employed (18), NO concentrations, irrespective of how expressed, decreased postdrug.

In summary, our data demonstrate that inhibition of NO synthesis attenuates exercise-induced asthma, further supporting the concept that NO generated from the airway during hyperpnea may play an important role in this condition.

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