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Oral Administration of L-Arginine Potentiates Allergen-Induced Airway Inflammation and Expression of Interleukin-5 in Mice

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ABSTRACT

The role of nitric oxide in the airway hyperresponsiveness and inflammation of bronchial asthma has not yet been established. However, L-arginine, the substrate for nitric oxide synthases, reportedly alleviates airway hyperresponsiveness caused by parainfluenza virus and reduces granulocytic inflammation induced by ischemia-reperfusion. We investigated the effects of L-arginine on a murine model of allergic asthma that included airway hyperresponsiveness, eosinophilic inflammation and expression of interleukin (IL)-5 in the lung. The mice received drinking water with or without L-arginine for 9 weeks. Histologic evaluation and cellular profiles in bronchoalveolar lavage fluid showed that p.o. administration of L-arginine (72 μmol/kg/day) significantly enhanced eosinophilic airway inflammation and goblet cell proliferation that were associated with intratracheal instillation of ovalbumin. L-Arginine also increased protein levels of IL-5 and IL-2 in supernatants from the lung exposed to ovalbumin. The number of eosinophils in bronchoalveolar lavage fluid correlated significantly with the expression of IL-5. L-Arginine did not reverse ovalbumin-associated airway hyperresponsiveness to inhaled ACh. These results suggest that p.o. administration of L-arginine aggravates allergen-induced eosinophilic airway inflammation *via* expression of IL-5, and in this model it does not show therapeutic efficacy against airway hyperresponsiveness associated with allergen exposure. Oral administration of L-arginine, the precursor of nitric oxide, may not be an effective intervention in allergic asthma.

Bronchial asthma is characterized by airway hyperresponsiveness and inflammation in which activated eosinophils and lymphocytes are pivotal (Expert Panel Report, 1991). Various lines of evidence have indicated that IL-5 is a central pathophysiologic mediator of bronchial asthma (Robinson *et al.*, 1993; Foster *et al.*, 1996; Takano *et al.*, 1997a). The effects of NO on bronchial asthma are incompletely understood, because NO can exert broad actions on airway hyperresponsiveness and inflammation including cytokine expression, at least in part because three distinct isoforms of NO synthases are present. NO appears to possess bronchodilator properties (Dupuy *et al.*, 1992; Nijkamp *et al.*, 1993; Li and Rand, 1991), and it may afford protection against airway inflammation by inhibiting platelet aggregation and leukocyte adhesion to vascular endothelium (Radomski *et al.*, 1992; Kubes *et al.*, 1991) and by the maintenance of microvascular integrity (Erjefalt *et al.*, 1994). Besides these anti-inflammatory actions, however, NO exerts a variety of pro-inflammatory effects (Bernareggi *et al.*, 1997; Rettori *et al.*, 1992; Salvemini *et al.*, 1995; Lander *et al.*, 1993) that may be deterious in airway inflammation. NO also can react with superoxide to form the highly reactive anion peroxynitrite, which initiates lipid peroxidation and rapidly oxidizes sulfhydryl groups (Radi *et al.*, 1991).

In clinical studies, inhalation of NO by patients with mild asthma significantly reduces bronchospasm induced by methacholine inhalation (Kacmarek *et al.*, 1996), whereas asthmatics apparently exhale a greater amount of NO than healthy volunteers (Kharitonov *et al.*, 1994). Experimentally, an aerosol containing L-arginine, the substrate for NO synthases, prevented airway hyperresponsiveness to histamine caused by intratracheal inoculation of parainfluenza virus in guinea pigs (Folkerts *et al.*, 1995). In addition, systemic administration of L-arginine reduced granulocytic inflammation induced by ischemia-reperfusion (Weyrich *et al.*, 1992). However, the effects on bronchial asthma of L-arginine, the precursor of NO, have not been elucidated.

The present study was undertaken to examine the pathophysiologic effects of L-arginine on a murine model of allergic asthma that involves airway hyperresponsiveness, eosinophilic airway inflammation and expression of IL-5 in the lung.

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ABBREVIATIONS: NO, nitric oxide; IL, interleukin; Rrs, respiratory resistance; BAL, bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assays; PC_{150}, provocative concentration of ACh causing a 50% increase in Rrs.

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Materials and Methods

Animals and experimental protocol. Male ICR mice 6 to 7 weeks old and weighing 29 to 33 g (Japan Clea Co., Tokyo, Japan) were used in all experiments. The animals were fed a commercial diet (Japan Clea Co.) and housed in a facility maintained at 24°C to 26°C with 55% to 75% humidity and a 14 h/10 h light/dark cycle. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals. All animal studies were approved by our Institutional Review Board.

Mice were divided into four experimental groups: a water-vehicle group, a water-ovalbumin group, an L-arginine-vehicle group, and an L-arginine-ovalbumin group. The mice in the water-vehicle group and the water-ovalbumin group received plain drinking water for a continuous 9-week period, whereas the animals in the L-arginine-vehicle group and the L-arginine-ovalbumin group received drinking water containing L-arginine (Sigma Chemical Co., St. Louis, MO) for the same period. The dose of L-arginine administered was 72 μmol/kg/day. In preliminary studies this dose of L-arginine had no significant effect on water consumption or gain in body weight (data not shown). The normal mouse reportedly drinks about 100 ml of H_2O/kg b.wt. every 24 h (Grisham *et al.*, 1994). In the present study, the mice consumed 139 ml/kg/day of plain water and 144 ml/kg/day of water containing L-arginine (50 μmol in 100 ml of water).

The mice in the water-vehicle group and the L-arginine-vehicle group received intratracheal instillations of 0.1 ml of phosphate-buffered saline (pH 7.4; Nissui Pharmaceuticals, Tokyo, Japan) containing 0.05% Tween 80 (Nacalai Tesque, Kyoto, Japan) once weekly for 9 weeks (10 times), beginning 3 days after initiation of p.o. medication. The mice in the water-ovalbumin group and the L-arginine-ovalbumin group intratracheally received 1 μg of ovalbumin suspended in 0.1 ml of vehicle every 3 weeks for 9 weeks instead of the vehicle alone (4 times for ovalbumin and 6 times for vehicle alone). The intratracheal instillations were performed *via* a polyethylene tube after the animals were anesthetized with 4% halothane (Hoechst Japan, Tokyo, Japan). The animals were studied 24 h after the last intratracheal instillation by evaluation of airway responsiveness, cellular profiles of BAL fluid, lung histology and expression of cytokine proteins in the lung tissue supernatants.

Airway responsiveness. Measurements of pulmonary function were conducted by the method of Sorkness *et al.* (1994) with a minor modification. In brief, mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.) and underwent a tracheostomy with an 18-gauge cannula. Each mouse was mechanically ventilated with a rodent respirator (Model 683; Harvard Apparatus, South Natick, MA) in a plethysmograph box with a pneumotachometer (Model PLYAN-M; Buxco Electronics, Inc., Sharon, CT) at a constant tidal volume (0.2 ml) and at 120 breaths/min. Spontaneous respiration was inhibited by pancuronium bromide (1 mg/kg i.v.). Endotracheal pressure was determined by a differential pressure transducer (Model DP45-28; Buxco) connected to the tracheal cannula. Flow was measured using a pneumotachometer, a differential pressure transducer (Model DP45-14; Buxco) and a preamplifier (Model PREAMP/VAL; Buxco). A continuous measurement of Rrs was computed from the endotracheal pressure and flow using a Pulmonary Mechanics Analyzer (Model-6; Buxco). Endotracheal pressure, flow and Rrs were recorded on a six-channel recorder (Model WR3701-6; Buxco). Four-second averages of Rrs were also recorded on a terminal (Model TI-703; Buxco) *via* a Datalogger (Model LS-12; Buxco). The ACh challenge was administered by inhalation. ACh solutions (0.01–10 mg/ml in saline) were cumulatively inhaled, each concentration being given for 2 min with an interval of 2 min between administrations, by aerosolizing the solution with an ultrasonic nebulizer (NE-U07; Omron, Kyoto, Japan) designed to generate 5-μm aerosol particles.

PC_{150} was estimated in each mouse, calculated from the curve determined by the concentration of ACh and the percent increase in Rrs.

BAL. In a separate series of animals, the trachea was cannulated after exsanguination by cardiac puncture. The lungs were lavaged three times with 1.2 ml of sterile saline at 37°C used at each lavage. The average volume of BAL fluid retrieved was 90% of the volume of 3.6 ml that was instilled. This amount was similar with each treatment. The lavage fluid was centrifuged at 300 $\times$ *g* for 10 min. The total number of cells was counted in a fresh specimen of fluid stained with Turk's solution. Differential counts were assessed on cytologic preparations. Slides were prepared using a Cytospin (Tomy Seiko, Tokyo, Japan) and stained with Diff-Quik (International Reagents Co., Kobe, Japan). A total of 300 cells in each lavage sample were counted under oil immersion microscopy. After the BAL procedure, the lungs were removed, snap-frozen in liquid nitrogen and stored at −80°C.

Cytokine protein levels in lung tissue supernatants. The frozen lungs were homogenized with 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA (Sigma), 0.1 mM phenylmethanesulphonyl fluoride (Nacalai Tesque), 1 μM pepstatin (Peptide Institute, Osaka, Japan) and 2 μM leupeptin (Peptide Institute). The homogenates were then centrifuged at 105,000 $\times$ *g* for 1 h. The supernatants were stored at −80°C. ELISA were conducted for IL-5 and IL-2 in lung tissue supernatants using matching antibody pairs (Endogen, Cambridge, MA). The following antibody pairs were used for detection of IL-5 and IL-2: TRFK5 and TRFK4 for IL-5 and S4B6 and 5H 4.1.1. for IL-2. The second antibodies were conjugated to horseradish peroxidase. Subtractive readings of 550 nm from the readings at 450 nm were converted to picograms per milliliter using values obtained from standard curves generated with varying concentrations of recombinant IL-5 and IL-2 with limits of detection of 5 pg/ml and 3 pg/ml, respectively.

Histologic evaluation. In a separate series of animals, the lungs were removed after exsanguination and fixed in 10% neutral phosphate-buffered formalin instilled intratracheally at a pressure of 20 cm H_2O for at least 72 h. Slices of each pulmonary lobe 2 to 3 mm thick were embedded in paraffin. Sections 3 μm thick were stained with Diff-Quik for determination of the number of infiltrating eosinophils and neutrophils or with periodic acid-Schiff for quantitation of the goblet cells. Examinations were conducted in blind fashion by two of us. The length of the basement membrane of the airways on each sample slide was measured by videomicrometer (Olympus, Tokyo, Japan). The number of eosinophils and neutrophils around the airways, and the number of goblet cells in the bronchial epithelium, were counted with a micrometer under oil immersion. Results were expressed as the number of cells per millimeter of basement membrane.

Statistical analysis. Data are reported as mean ± S.E. Differences in the numbers of inflammatory cells and goblet cells, airway responsiveness and cytokine proteins among groups were determined using analysis of variance (Statview version 4.0; Abacus Concepts, Inc., Berkeley, CA). If the differences among groups were statistically significant ($P < .05$), Fisher's protected least significant difference test or Scheffé's *F* test was used to distinguish between pairs of groups. The correlations between the number of eosinophils or neutrophils and the protein levels of cytokines were evaluated by Pearson's correlation coefficient (Statview version 4.0; Abacus Concepts, Inc.).

Results

L-Arginine does not affect airway responsiveness. To determine the effects of orally administered L-arginine on airway hyperresponsiveness, we measured Rrs in animals that inhaled ACh. Base-line Rrs was not significantly different among the four experimental groups (data not shown). The value of PC_{150} in the water-ovalbumin group was significantly less than that in the water-vehicle group (table 1, $P < .05$). There was no significant difference in the value of PC_{150}

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TABLE 1

Provocative concentration of ACh causing a 50% increase in respiratory resistance (PC_{150})

Mice drank plain water or water containing L-arginine for 9 weeks. They received intratracheal instillations of ovalbumin or vehicle alone. The animals were studied by evaluation of the Rrs and PC_{150}. Measurements of pulmonary function were conducted by the method of Sorkness *et al.* (1994) with a minor modification. Eight animals were studied in each group.

Group	PC_{150} (mg/ml of ACh)
Water-vehicle	0.61 ± 0.08
Water-ovalbumin	0.35 ± 0.07[a]
L-arginine-vehicle	0.55 ± 0.09
L-arginine-ovalbumin	0.49 ± 0.16

[a] P < .05 *vs.* water-vehicle group.

between the water-ovalbumin and L-arginine-ovalbumin groups.

L-Arginine potentiates eosinophilic airway inflammation. The number of eosinophils and neutrophils in BAL fluid showed 15-fold and 4-fold increases, respectively, in the water-ovalbumin group as compared with the water-vehicle group (table 2). Oral administration of L-arginine significantly enhanced ovalbumin-associated increases in number of eosinophils (P < .001 *vs.* the water-vehicle group and the water-ovalbumin group; P < .01 *vs.* the L-arginine-vehicle group) and number of neutrophils (P < .0001 *vs.* the above groups). L-Arginine treatment did not affect findings obtained by the intratracheal administration of the vehicle alone.

The number of eosinophils infiltrating around the airways and goblet cells in the bronchial epithelium showed 9-fold and 4-fold increases, respectively, in the water-ovalbumin group as compared with the water-vehicle group (table 3). Oral administration of L-arginine significantly enhanced ovalbumin-associated increases in number of eosinophils (P < 0.01 *vs.* other groups) and number of goblet cells (P < .05 *vs.* the water-vehicle group and the water-ovalbumin group; P < .01 *vs.* the L-arginine-vehicle group). The number of neutrophils infiltrating around the airways was significantly greater in the L-arginine-ovalbumin group than in the water-vehicle group (P < .05). L-Arginine treatment did not enhance the findings obtained with the vehicle alone.

L-Arginine increases IL-5 expression. The mean values of the protein levels of IL-5 and IL-2 in the lung tissue supernatants were greater in the water-ovalbumin group than in the water-vehicle group, although the differences did not attain statistical significance (table 4). Levels of IL-5 and IL-2 were significantly greater in the L-arginine-ovalbumin group than in the other groups (table 4, P < .01 *vs.* other groups).

A significant correlation was evident between number of eosinophils in BAL fluid in each mouse and protein levels of

TABLE 3

Histologic evaluation of airway inflammation and goblet cell proliferation

Mice were treated as described in the footnote to Table 1. The animals were studied by evaluation of lung histology. Specimens were stained with Diff-Quik for quantitation of infiltrating eosinophils and neutrophils or with the periodic acid-Schiff for quantitation of goblet cells. Results are expressed as numbers of cells per millimeter of basement membrane. Eight animals were studied in each group.

Group	Eosinophils	Neutrophils	Goblet cells
	(number/mm basement membrane)		
Water-vehicle	0.48 ± 0.34	0.16 ± 0.12	0.55 ± 0.37
Water-ovalbumin	4.30 ± 2.36	0.36 ± 0.14	2.16 ± 1.18
L-arginine-vehicle	0.04 ± 0.02	0.03 ± 0.01	0.28 ± 0.16
L-arginine-ovalbumin	18.5 ± 7.48[a]	0.59 ± 0.29[b]	13.0 ± 6.66[c]

[a] P < .01 *vs.* other groups. [b] P < .05 *vs.* water-vehicle group. [c] P < .05 *vs.* water-vehicle group, P < .05 *vs.* water-ovalbumin group, P < .01 *vs.* L-arginine-vehicle group.

TABLE 4

Protein levels of IL-5 and IL-2

Mice were treated as described in the footnote to Table 1. The animals were studied by evaluation of protein levels of interleukin IL-5 and IL-2 in lung tissue supernatants by ELISA. Eight animals were studied in each group.

Group	IL-5	IL-2
	(pg/total lung tissue supernatants)	
Water-vehicle	34.9 ± 1.34	23.4 ± 0.99
Water-ovalbumin	40.6 ± 5.30	25.9 ± 1.35
L-arginine-vehicle	34.2 ± 2.82	25.2 ± 1.22
L-arginine-ovalbumin	295 ± 99.5[a]	43.8 ± 7.51[a]

[a] P < .01 *vs.* other groups.

IL-5 in the lung tissue supernatants in the same mouse (table 5, r = 0.869, P < .0001). The number of neutrophils in BAL fluid correlated positively with protein levels of IL-5 and IL-2 in lung tissue supernatants (r = 0.581 and P = .0004 for IL-5, r = 0.574 and P = .0005 for IL-2).

Discussion

The present study demonstrated that p.o. administration of L-arginine significantly enhanced the eosinophilic airway inflammation and goblet cell proliferation associated with intratracheal instillation of allergen. The number of eosinophils in BAL fluid correlated significantly with the levels of IL-5 in lung tissue supernatants. The L-arginine treatment did not reverse allergen-associated airway hyperresponsiveness to inhaled ACh.

Inhaling NO reportedly can reverse methacholine-induced bronchoconstriction in guinea pigs (Dupuy *et al.*, 1992), and inhibitors of NO synthesis have been demonstrated to induce airway hyperresponsiveness to histamine both *in vitro* and *in vivo* (Nijkamp *et al.*, 1993). In addition, NO is recognized as a neurotransmitter of inhibitory nonadrenergic noncholin-

TABLE 2

Cellular profile in BAL fluid

Mice were treated as described in the footnote to Table 1. The animals were studied by evaluation of the BAL fluid. The total number of cells was counted in a fresh specimen of fluid stained with Turk's solution. Differential cell counts were assessed on cytologic preparations stained with Diff-Quik. Eight animals were studied in each group.

Group	Total Cells	Macrophages	Eosinophils	Neutrophils
	($\times 10^4$/total BAL fluid)			
Water-vehicle	34.8 ± 3.54	34.1 ± 3.64	0.007 ± 0.007	0.71 ± 0.28
Water-ovalbumin	45.6 ± 7.37	42.5 ± 6.45	0.106 ± 0.047	3.01 ± 1.21
L-arginine-vehicle	43.1 ± 1.17	41.8 ± 1.15	0	1.34 ± 0.51
L-arginine-ovalbumin	78.2 ± 9.68[a]	50.2 ± 5.59[b]	11.4 ± 4.19[c]	16.6 ± 3.82[d]

[a] P < .0001 *vs.* water-vehicle group, P < .01 *vs.* water-ovalbumin group, P < .001 *vs.* L-arginine-vehicle group. [b] P < .05 *vs.* water-vehicle group. [c] P < .001 *vs.* water-vehicle group, P < .001 *vs.* water-ovalbumin group, P < .01 *vs.* L-arginine-vehicle group. [d] P < .0001 *vs.* other groups.

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TABLE 5

Correlation coefficients between number of granulocytes in BAL fluid and expression of cytokine proteins

The correlations between number of eosinophils or neutrophils in BAL fluid in each mouse and levels of cytokine proteins in lung tissue supernatants in the same mouse were evaluated by Pearson's correlation efficient; $n = 32$.

	IL-5 in Lung	P Value	IL-2 in Lung	P Value
Eosinophils in BAL fluid	0.869	<0.0001	0.258	0.1631
Neutrophils in BAL fluid	0.581	0.0004	0.574	0.0005

ergic nerves that induces relaxation in airways (Li and Rand, 1991). These effects of NO appear to be protective against airway hyperresponsiveness, a pathophysiologic hallmark of bronchial asthma. Inhalation of NO by patients with bronchial asthma has been shown to reduce bronchospasm induced by methacholine (Kacmarek *et al.*, 1996). Furthermore, L-arginine, the precursor of NO, prevents airway hyperresponsiveness associated with intratracheal inoculation of parainfluenza virus in guinea pigs (Folkerts *et al.*, 1995). Inhalation of NO gas has limited applicability to clinical use because special apparatus is required, so therapeutic intervention using L-arginine, if effective, would be more practical. In the present study, however, p.o. administration of L-arginine failed to reverse allergen-associated airway hyperresponsiveness and instead aggravated eosinophilic airway inflammation and goblet cell proliferation associated with allergen exposure. These results make it doubt that p.o. administration of L-arginine would be an effective intervention in allergic asthma.

L-Arginine is the substrate for the three distinct isoforms of NO synthases. Activity of the endothelial and neuronal constitutive forms is regulated in response to intracellular calcium/calmodulin concentrations, and that of the inducible calcium-independent form is regulated at the transcriptional level in response to stimuli such as pro-inflammatory cytokines (Nathan and Xie, 1994). The bronchodilator and anti-inflammatory properties of NO appear to be exhibited by NO produced constitutively from NO synthases in the vascular endothelium, airway epithelium and inhibitory nonadrenergic noncholinergic nerves. NO produced from endothelial NO synthase reportedly inhibits platelet aggregation and leukocyte adhesion to vascular endothelium and maintains microvascular integrity (Radomski *et al.*, 1992; Kubes *et al.*, 1991; Erjefalt *et al.*, 1994). Nijkamp and his colleagues have reported 1) that inhibition of NO synthesis in the guinea pig respiratory tract resulted in a marked increase in airway hyperresponsiveness to histamine and 2) that removal of the airway epithelium also induced airway hyperresponsiveness, which was not further increased by incubation with an inhibitor of NO synthesis (Nijkamp *et al.*, 1993).

In contrast, NO can exert a variety of pro-inflammatory effects. NO induces pathologic vasodilation and enhances plasma leakage in the trachea (Bernareggi *et al.*, 1997). NO increases production of pro-inflammatory prostaglandins both *in vitro* (Rettori *et al.*, 1992) and *in vivo* (Salvemini *et al.*, 1995). In addition, NO induces NF-κB binding activity and secretion of pro-inflammatory cytokines (Lander *et al.*, 1993). Our model suggests that such deterious effects of NO during the process of airway inflammation are likely to overcome its potentially protective effects against bronchial asthma as a bronchodilator, a neurotransmitter promoting relaxation and a regulator of the microvascular integrity and circulation. Excess NO generated by inducible NO synthase has been implicated in the pathogenesis of various inflammatory diseases (McCartney-Francis *et al.*, 1993; Miller *et al.*, 1995; Takano *et al.*, 1997b). Expression of inducible NO synthase has been demonstrated in the epithelial layer of biopsy specimens taken from asthmatic patients (Hamid *et al.*, 1993), and asthmatics were found to exhale a greater amount of NO than healthy volunteers (Kharitonov *et al.*, 1994). Furthermore, expression of inducible NO synthase is decreased strikingly by the corticoid inhalants commonly used in the treatment of inflammatory airway diseases such as bronchial asthma (Guo *et al.*, 1995).

In our study, p.o. administration of L-arginine produced an increase in allergen-associated expression of IL-2, which correlated positively with the infiltration of neutrophils. In addition, L-arginine treatment markedly increased expression of IL-5 in the lung tissue supernatants associated with allergen exposure. Expression of IL-5 correlated significantly with infiltration of eosinophils. As far as we know, the present experiment represents the first demonstration of enhancing effects of L-arginine on production of IL-5 *in vivo*. Because IL-5 is recognized as a key mediator in bronchial asthma, affecting eosinophilic inflammation and airway hyperresponsiveness (Robinson *et al.*, 1993; Foster *et al.*, 1996; Takano *et al.*, 1997a), the deterious effects of L-arginine on our model are likely to involve the increased expression of IL-5. NO-generating compounds have been reported to induce NF-κB binding activity and subsequent production of tumor necrosis factor α, a pro-inflammatory cytokine (Lander *et al.*, 1993). Future experiments should examine whether L-arginine or NO activates transcriptional factors other than NF-κB in the presence or absence of allergen.

To confirm the effects of L-arginine on NO production at the inflammatory site, we measured nitrate and nitrite in BAL supernatants. Unfortunately, the amount of NO produced in the four experimental groups was below the detection limit for the method of Schmidt and co-workers (1988). In the present study, airway inflammation with goblet cell proliferation was not induced by p.o. administration of L-arginine alone (L-arginine-vehicle group).

In conclusion, p.o. administration of L-arginine aggravated eosinophilic airway inflammation associated with allergen exposure *via* expression of IL-5, and L-arginine treatment did not show therapeutic efficacy on airway hyperresponsiveness associated with allergen. Oral administration of L-arginine, the precursor of NO, does not appear to show promise as an intervention in allergic asthma.

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