
Relationships of methacholine and adenosine monophosphate responsiveness with serum vascular endothelial growth factor in children with asthma

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Background: Airway hyperresponsiveness, which is a characteristic feature of asthma, is usually measured by means of bronchial challenge with direct or indirect stimuli. Vascular endothelial growth factor (VEGF) increases vascular permeability and angiogenesis, leads to mucosal edema, narrows the airway diameter, and reduces airway flow.

Objective: To examine the relationships between serum VEGF level and airway responsiveness to methacholine and adenosine monophosphate (AMP) in children with asthma.

Methods: Peripheral blood eosinophil counts, serum eosinophil cationic protein (ECP) concentrations, and serum VEGF concentrations were measured in 31 asthmatic children and 26 control subjects. Methacholine and AMP bronchial challenges were performed on children with asthma.

Results: Children with asthma had a significantly higher mean (SD) level of VEGF than controls (361.2 [212.0] vs 102.7 [50.0] pg/mL; $P < .001$). Blood eosinophil counts and serum ECP levels significantly correlated inversely with AMP provocation concentration that caused a decrease in forced expiratory volume in 1 second of 20% (PC₂₀) ($r = -0.474$, $P = .01$; $r = -0.442$, $P = .03$, respectively), but not with methacholine PC₂₀ ($r = -0.228$, $P = .26$; $r = -0.338$, $P = .10$, respectively). Serum VEGF levels significantly correlated with airway responsiveness to AMP ($r = -0.462$; $P = .009$) but not to methacholine ($r = -0.243$; $P = .19$).

Conclusions: Serum VEGF levels were increased in children with asthma and were related to airway responsiveness to AMP but not to methacholine. Increased VEGF levels in asthmatic children may result in increased airway responsiveness by mechanisms related to airway inflammation or increased permeability of airway vasculature.

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INTRODUCTION

Bronchial asthma is physiologically characterized by variable airflow obstruction and airway hyperresponsiveness (AHR).¹ Challenges with histamine and methacholine cause airway narrowing mainly by direct action on receptors of airway smooth muscles, whereas stimuli such as adenosine monophosphate (AMP) are thought to act primarily on other cells, thus initiating processes that indirectly lead to smooth muscle contraction.² Previous studies^{3,4} with asthmatic patients have shown that airway responsiveness to AMP more specifically

reflects underlying airway inflammation than does airway responsiveness to direct stimuli such as methacholine.

Vascular endothelial growth factor (VEGF) is one of the most potent inducers of airway inflammation, and it increases vascular permeability.^{5,6} It also increases angiogenesis, vascular leakage, endothelial cell proliferation, airway smooth muscle hypertrophy, and consequent thickening of the airway wall mucosa, which may lead to narrowing of the airway lumen.⁵ There is evidence to support a key role of VEGF in the reduction of airway flow through the thickened edematous wall by enhanced permeability and inflammation.^{5,6} It has been recognized that the airway mucosa is edematous and contains dilated, congested blood vessels and inflammatory cells even in mild asthma.^{7,8} Thickening of the airway wall and reduced airflow consequently contribute to abnormal airway functions, thus playing an important role in the pathogenesis of asthma.¹ Increased VEGF levels correlate directly with disease activity and inversely with airway caliber and airway responsiveness.^{6,9} However, the relationships between serum VEGF levels and the direct and indirect airway responsiveness of asthmatic children are not known.

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Eosinophilic airway inflammation is another hallmark of asthma.¹⁰ Previous studies^{5,11} have demonstrated that eosinophils are important sources of VEGF, thus contributing to tissue edema formation at sites of allergic inflammation. There is also evidence of a positive relationship between increased VEGF levels and the degree of eosinophilic inflammation in asthmatic patients.^{5,12} The aims of the present study were to investigate the serum VEGF level in children with asthma and to elucidate its relationship with the degree of AHR by conducting direct and indirect bronchial challenges.

METHODS

Study Participants

Thirty-one children aged 7 to 12 years with mild to moderate asthma (asthma group) were enrolled in the present study. All the patients had a history of episodic wheezing, dyspnea, or both and had been diagnosed as having asthma on the basis of airway reversibility (an increase in forced expiratory volume in 1 second [FEV₁] $\geq$ 12% after bronchodilator administration) or a methacholine provocation concentration that caused a decrease in FEV₁ of 20% (PC₂₀) of less than 16 mg/mL. They were followed up at the Pediatric Allergy Clinic of Korea University Anam Hospital. They had been medicated with inhaled short-acting β_2 -agonists on demand to relieve symptoms, with or without controller medications (inhaled corticosteroids [n = 9], leukotriene receptor antagonists [n = 6], or long-acting β_2 -agonist [n = 1]). Patients with a history of near-fatal asthma or major exacerbations necessitating the use of systemic corticosteroids or other respiratory diseases apart from asthma were excluded from the study.

Twenty-six healthy age-matched children (control group) were included for comparison. They were selected from children aged 7 to 12 years who had visited the outpatient clinic for a routine physical checkup and had never experienced wheezing or asthma-like symptoms.

All the participants provided blood samples for the measurement of total eosinophil counts, serum eosinophil cationic protein (ECP) levels, total IgE levels, and VEGF levels and underwent skin prick testing for the evaluation of atopy. Atopy was defined as the presence of at least 1 positive reaction (>3 -mm wheal diameter) to a battery of 13 common aeroallergens. Parents gave written informed consent for their children to participate in the study. The study protocol was approved by the Korea University Anam Hospital ethics committee.

Methacholine and AMP Challenge Tests

Methacholine and AMP challenges were performed at the same time of day 3 days apart in a randomized order. At the time of the challenges, all the patients were free of acute respiratory tract infection or asthma exacerbation for 4 weeks. All the patients were asked to withhold inhaled short-acting β_2 -agonist use for at least 8 hours, and most patients (n = 16) were asked to discontinue use of their controller medications for 7 days before the tests; however, 2 of these patients had difficulty controlling their asthma symptoms

without medications and were asked to stop drug use for at least 3 days before the tests. Methacholine inhalation tests were performed using a modification of the method described by Chai et al,¹³ and AMP challenge tests were performed using a method proposed by the European Respiratory Society.¹⁴ Fresh solutions of methacholine and AMP were prepared in buffered saline solution (pH 7.4) at concentrations ranging from 0.075 to 50 mg/mL for methacholine and from 3.125 to 400 mg/mL for AMP.

Spirometric measurements (FEV₁ and forced vital capacity [FVC]) were made using a computerized spirometer (Micro-spiro-HI 298; Chest Corp, Tokyo, Japan) in accordance with the recommendations of the American Thoracic Society.¹⁵ All the patients were asked to undergo spirometry in a reproducible way (ie, a coefficient of variation of FEV₁ in 3 consecutive flow-volume curves of $<5\%$) and to have an FEV₁ of at least 60% of predicted.¹⁶ Four to 8 tests were required to achieve 3 consecutive curves with a coefficient of variation of less than 5% at baseline FEV₁ measurement in each child. A Rosenthal-French dosimeter (Laboratory for Applied Immunology, Baltimore, Maryland) triggered by a solenoid valve set to remain open for 0.6 second was used to generate an aerosol from a DeVilbiss 646 nebulizer (DeVilbiss Health Care, Somerset, Pennsylvania) with pressurized air at 20 psi. Each patient inhaled 5 inspiratory capacity breaths of buffered saline solution and increasing concentrations of methacholine or AMP at 5-minute intervals. Both FEV₁ and FVC were measured 90 seconds after inhalation at each concentration, and the largest value of triplicate FEV₁ or FVC was used for the analysis. The procedure was terminated when FEV₁ decreased by more than 20% of its postsaline value or when the highest methacholine (50 mg/mL) or AMP (400 mg/mL) concentration was reached. Patients who manifested bronchospasm after bronchial challenge received inhaled short-acting β_2 -agonist, 400 μ g, by metered-dose inhaler. The percentage decline in FEV₁ from the postsaline value was plotted against the log concentration of inhaled methacholine or AMP. Methacholine PC₂₀ and AMP PC₂₀ were calculated by interpolating between the 2 adjacent data points. A censored value of 800 mg/mL for AMP PC₂₀ was recorded in children who did not show a 20% decline in FEV₁ after inhalation of the 400 mg/mL concentration of AMP.

Measurement of Serum Total IgE Levels, Peripheral Blood Eosinophil Counts, and Serum ECP Levels

Serum total IgE levels were measured using a total IgE immunoradiometric assay kit (Coat-A-Count; Diagnostic Products Co, Los Angeles, California). The number of peripheral blood eosinophils was counted in blood samples containing EDTA using an automated hematology analyzer (Coulter Counter STKS; Beckman Coulter, Fullerton, California). The serum ECP level was measured using a commercially available fluoroimmunoassay kit (UniCAP System FEIA; Pharmacia Diagnostics, Uppsala, Sweden) with a detection limit of less than 2.0 μ g/L. Blood sample collection,

serum preparation, and serum ECP measurement were performed according to the manufacturer's instructions.

Measurement of Serum VEGF Concentration

Venous blood samples from each participant were centrifuged at 1,000g for 10 minutes at 4°C. The samples were stored at -70°C until VEGF concentration was determined using a human VEGF kit (Biosource, Camarillo, California). All the assays were performed in duplicate, and mean values were used for statistical analysis. The lower detection limit was 2 pg/mL.

Statistical Analysis

Data are presented as mean (SD) or geometric mean and a range of 1 SD. The FEV₁ and FVC values are expressed as percentages of predicted values.¹⁶ The values for PC₂₀, serum total IgE levels, serum ECP levels, and blood eosinophil counts were log transformed before statistical analysis. The variables were compared between the 2 groups using the *t* test. Frequencies were examined using the χ^2 test. Correlations between eosinophil markers and methacholine PC₂₀ or AMP PC₂₀ and between VEGF levels and methacholine PC₂₀ or AMP PC₂₀ were examined using the Pearson correlation test. All statistical analyses were performed using a software package (SPSS; SPSS Inc, Chicago, Illinois). *P* < .05 was considered statistically significant.

RESULTS

The clinical characteristics and eosinophilic inflammatory markers of the asthma group (*n* = 31) and the control group (*n* = 26) are given in Table 1. There were no significant differences in age and sex between the 2 groups. The geometric mean (range of 1 SD) peripheral blood eosinophil count in the asthma group (436.5/ μ L [208.9–912.0/ μ L]) was significantly higher than that in the control group (40.7/ μ L [17.8–93.3/ μ L]) (*P* < .05). The geometric mean (range of 1 SD) serum total IgE level was significantly higher in the asthma group vs the control group (371.5 [162.2–851.1] vs 74.1 [24.0–229.1] IU/mL; *P* < .05). The geometric mean (range of 1 SD) serum ECP level was 24.0 (7.24–79.4) μ g/mL in the asthma group and 13.2 (4.9–35.5) μ g/mL in the control group (*P* < .05). The asthma group had a significantly higher mean (SD) VEGF level (361.2 [212.0] pg/mL) than the control group (102.7 [50.0] pg/mL; *P* < .001) (Figure 1).

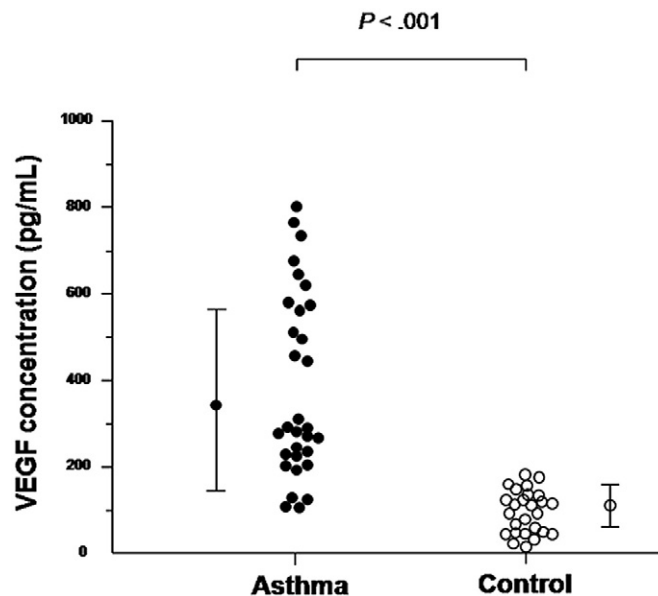


Figure 1. Distribution of serum vascular endothelial growth factor (VEGF) concentrations in children with asthma (*n* = 31) and control subjects (*n* = 26). Vertical bars represent group mean (SD) values.

Table 2 summarizes the results of methacholine and AMP challenge tests in the asthma group. There was no significant difference in baseline FEV₁ before methacholine and AMP challenges (90.6% [8.7%] of predicted vs 89.1% [7.9%] of predicted). Twenty-nine patients (93.5%) had a methacholine PC₂₀ less than 16 mg/mL, the cutoff point for AHR to methacholine. On the other hand, 27 patients (87.1%) exhibited AHR to AMP (PC₂₀ <200 mg/mL). The geometric means (range of 1 SD) of methacholine PC₂₀ and AMP PC₂₀ were 2.45 (0.71–8.51) mg/mL and 64.6 (18.2–229.1) mg/mL, respectively. Individual values for the 2 challenges and the correlations between them are shown in Figure 2. Methacholine PC₂₀ and AMP PC₂₀ correlated significantly in the asthma group.

There was an inverse relationship between blood eosinophil counts and serum ECP levels and AMP PC₂₀ (*r* = -0.474, *P* = .01; *r* = -0.442, *P* = .03, respectively) but not methacholine PC₂₀ (*r* = -0.228, *P* = .26; *r* = -0.338, *P* = .12).

Table 1. Clinical Characteristics and Inflammatory Markers of the Participants

	Asthma group (<i>n</i> = 31)	Control group (<i>n</i> = 26)
Age, mean (SD), y	9.5 (2.2)	9.3 (2.1)
Sex, M/F, No.	20/11	18/8
Atopy, +/- , No.	26/5	5/21
Total IgE, IU/mL ^a	371.5 (162.2–851.1)	74.1 (24.0–229.1)
Blood eosinophil count, / μ L ^a	436.5 (208.9–912.0)	40.7 (17.8–93.3)
Eosinophil cationic protein, μ g/mL ^a	24.0 (7.24–79.4)	13.2 (4.9–35.5)
VEGF, mean (SD), pg/mL	361.2 (212.0)	102.7 (50.0)

Abbreviation: VEGF, vascular endothelial growth factor.

^a Geometric mean (range of 1 SD).

Table 2. Results of Bronchial Challenge Tests in Asthmatic Children

	Methacholine challenge	AMP challenge
Baseline FEV ₁ , mean (SD), % predicted	90.6 (8.7)	89.1 (7.9)
Baseline FVC, mean (SD), % predicted	92.9 (8.4)	92.3 (7.7)
Baseline FEV ₁ /FVC, mean (SD), %	82.9 (6.8)	81.9 (6.2)
PC ₂₀ , mg/mL ^a	2.45 (0.71–8.51)	64.6 (18.2–229.1)

Abbreviations: AMP, adenosine monophosphate; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; PC₂₀, provocation concentration that caused a decrease in FEV₁ of 20%.

^a Geometric mean (range of 1 SD).

.10, respectively). A significant correlation was found between serum VEGF levels and peripheral blood eosinophil counts ($r = 0.531$; $P = .02$) and serum ECP concentrations ($r = 0.473$; $P = .03$). Serum VEGF levels significantly correlated with AMP PC₂₀ ($r = -0.462$; $P = .009$) (Fig 3) but not with methacholine PC₂₀ ($r = -0.243$; $P = .19$) (Fig 4).

DISCUSSION

In the present study, we investigated the relationship between serum VEGF concentration and airway responsiveness to methacholine and AMP in the asthma group. Airway responsiveness to AMP but not to methacholine was found to be statistically significantly inversely related to serum VEGF concentrations. To our knowledge, this is the first study to compare methacholine and AMP responsiveness regarding their relationships with VEGF concentrations in children with asthma. These findings suggest that increased VEGF levels in asthmatic children may result in increased airway responsiveness by mechanisms related to airway inflammation or increased permeability of airway vasculature.

Increased VEGF levels in induced sputum⁵ bronchoalveolar lavage (BAL) fluid,¹⁷ and VEGF-positive cells in bronchial biopsy samples⁶ have been found in patients with asthma compared with controls. The results of previous studies^{5,6} have indicated that VEGF is a potent stimulator of airway inflammation and airway physiologic abnormalities. VEGF increases vascular permeability so that plasma proteins can leak into the extravascular space, leading to mucosal edema and, thereby, narrowing of airway diameters, which can amplify the effects of airway smooth muscle contraction.¹⁸ Capillary leakage and airway mucosal edema induced by VEGF may contribute to airway narrowing in asthmatic patients.^{5,9}

In the present study, there was a significant link between VEGF levels and eosinophilic inflammation that was not as strong as that reported by Abdel-Rahman et al.¹² The cellular source of VEGF is peripheral eosinophils^{11,19} and eosinophils in the BAL fluid,²⁰ and many types of cells in the airway other than eosinophils were reported to produce VEGF, eg, epithelial cells, mast cells, myofibroblasts, and smooth muscle cells.⁵ The present results show that serum VEGF levels significantly correlate with peripheral blood eosinophil counts or ECP levels.

Eosinophils in peripheral blood increase in number and have been shown to be associated with the severity of symptoms and other markers of disease activity in asthmatic patients.²¹ Increased blood eosinophil counts and serum ECP levels, one of the eosinophil-activating markers, have been reported to be closely related to eosinophilic airway inflammation as evaluated with sputum samples or BAL fluid.^{22,23} Thus, it has been suggested that peripheral blood eosinophils and serum ECP are indirect markers of airway inflammation in asthma. Clinical studies^{3,4} with asthmatic patients have shown that AHR to AMP reflects underlying eosinophilic inflammation more acutely than does AHR to methacholine. In the present study, we did not measure any direct markers of airway inflammation in sputum or BAL fluid. Nevertheless, our observation of a significant correlation between blood eosinophil counts or serum ECP levels and AMP responsiveness but not methacholine responsiveness suggests that AHR to AMP may be more closely associated with airway inflammation than is AHR to methacholine.

In the present study, we found a significant inverse relationship between serum VEGF level and AMP PC₂₀ but not methacholine PC₂₀. Many studies^{24,25} have suggested that mucosal edema induced by VEGF has a profound effect on airway function and can explain heightened airway reactivity. In the present study, increased VEGF levels in asthmatic children positively correlated with airway responsiveness to AMP and with the degree of eosinophilic inflammation.

Several studies have shown that there is a significant inverse relationship between VEGF and AHR to methacholine. Kanazawa et al⁹ demonstrated a significant inverse relationship between VEGF levels in induced sputum and methacholine PC₂₀. They suggested that VEGF levels are associated with the degree of AHR and that a key element in the development of AHR is the interaction between airway microcirculation and VEGF. Hoshino et al⁶ also demonstrated a significant inverse relationship in the number of cells expressing VEGF messenger RNA with AHR to methacholine in adult asthmatic patients. It is possible that mucosal edema induced by VEGF has a profound effect on airway function and explains heightened airway reactivity. On the other hand, such a relationship was not found between VEGF in BAL fluid and methacholine PC₂₀ in adult asthmatic patients.¹⁹ We found an insignificant trend between serum VEGF concentrations and methacholine PC₂₀. This discrepancy may be

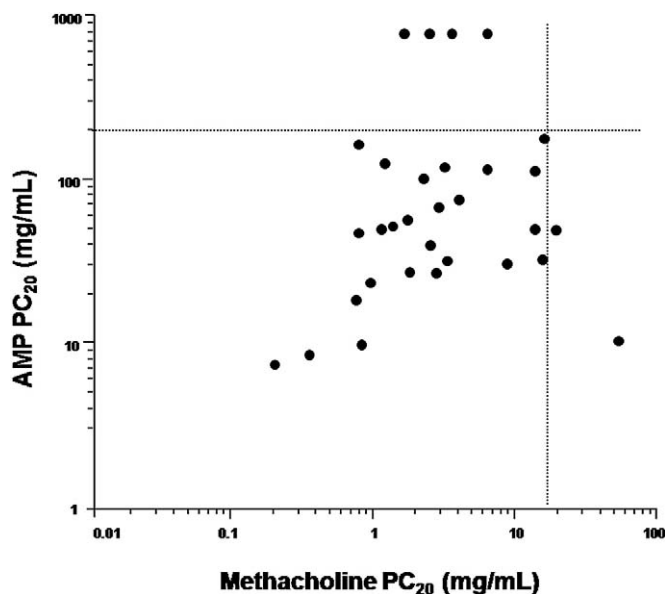


Figure 2. Scatterplot of adenosine monophosphate (AMP) provocation concentration that caused a decrease in forced expiratory volume in 1 second of 20% (PC₂₀) against methacholine PC₂₀ in children with asthma. The horizontal line represents the PC₂₀ limit of 200 mg/mL for AMP, and the vertical line represents the PC₂₀ limit of 16 mg/mL for methacholine.

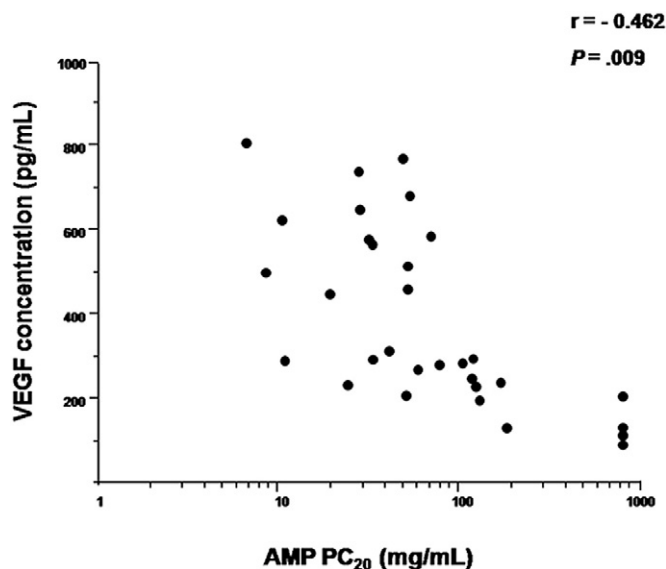


Figure 3. Relationships between serum vascular endothelial growth factor (VEGF) concentrations and adenosine monophosphate (AMP) provocation concentration that caused a decrease in forced expiratory volume in 1 second of 20% (PC₂₀) in children with asthma.

explained by the use of serum rather than sputum or by bronchial biopsy specimen and the different status of asthma. In the present study, we excluded patients with a history of near-fatal asthma or major exacerbations necessitating the use of systemic corticosteroids. The exclusion of these patients

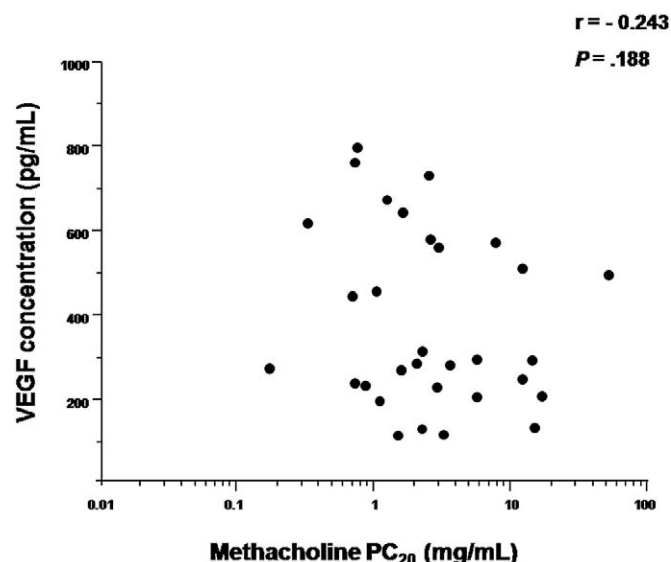


Figure 4. Relationships between serum vascular endothelial growth factor (VEGF) concentrations and methacholine provocation concentration that caused a decrease in forced expiratory volume in 1 second of 20% (PC₂₀) in children with asthma.

from the present study may have reduced the range of bronchial responsiveness values and the level of VEGF. An increase in the range of these variables might be expected to strengthen the observed association of methacholine responsiveness with serum VEGF concentration. In addition, these results may be confounded by the administration of asthma controller medications. Eight-week inhaled corticosteroid therapy⁵ and 4-week therapy with leukotriene modifiers²⁶ can reduce sputum VEGF levels in asthmatic individuals. It is possible that these drugs also have similar effects on serum VEGF levels. However, it would not be expected to affect the 2 challenges differently; and previous studies did not focus on whether VEGF level correlated with AHR to AMP.

In conclusion, elevated serum VEGF concentrations significantly correlated with AMP responsiveness but not with methacholine responsiveness. Level of VEGF may be associated with the pathogenesis of childhood asthma through increased permeability and airway inflammation and may be better reflected by airway responsiveness to AMP rather than to methacholine. These findings indicate that methacholine challenge is a better physiologic marker of smooth muscle dysfunction as opposed to inflammation.

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