Angiopoietin-2 as a contributing factor of exercise-induced bronchoconstriction in asthmatic patients receiving inhaled corticosteroid therapy

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Background: Airway microcirculation has the potential to contribute to the pathogenesis of exercise-induced bronchoconstriction (EIB) in asthma. Recently, angiopoietin-1 has been found to stabilize microvessels and make them leak resistant, whereas angiopoietin-2 is an antagonist of angiopoietin-1 and enhances microvascular permeability.
Objective: We sought to examine the roles of angiopoietin-2 in EIB in asthmatic patients with inhaled corticosteroid therapy.
Methods: Levels of angiopoietin-1 and angiopoietin-2 in induced sputum were examined in 32 asthmatic patients who were receiving inhaled corticosteroid therapy for more than 6 months at the entry of this study and 14 healthy control subjects. All asthmatic patients performed an exercise test.
Results: The degree of eosinophilic airway inflammation did not differ significantly between asthmatic patients and healthy control subjects. Angiopoietin-1 levels were also similar in the 2 groups (asthmatic patients: median, 6.0 ng/mL [range, 2.0-10.7 ng/mL]; healthy control subjects: median, 4.2 ng/mL [range, 1.5-10.7 ng/mL]). In contrast, angiopoietin-2 levels were significantly higher in asthmatic patients than in healthy control subjects (asthmatic patients: median, 0.74 ng/mL [range, 0.3-1.2 ng/mL]; healthy control subjects: median, 0.26 ng/mL [range, 0.05-0.47 ng/mL]; $P < .001$). There was no significant correlation between angiopoietin-1 levels and the severity of EIB in asthmatic patients. However, angiopoietin-2 levels were significantly correlated with the severity of EIB and airway microvascular permeability index.
Conclusion: Angiopoietin-2 levels were increased in the airways of asthmatic patients with inhaled corticosteroid therapy, and its levels were associated with the severity of EIB.
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Key words: *Exercise, bronchial asthma, airway microcirculation, induced sputum*

Abbreviations used
AUC: Area under the curve of the percentage decrease in FEV_1 plotted against time
BDP: Beclomethasone dipropionate
DTT: Dithiothreitol
ECP: Eosinophil cationic protein
EIB: Exercise-induced bronchoconstriction
NO: Nitric oxide
Tie: Tyrosine kinase with immunoglobulin-like loop and epidermal growth factor homology domain

Exercise-induced bronchoconstriction (EIB) is the term used to describe the increase in airway resistance that follows vigorous exercise in 40% to 90% of asthmatic patients.[1] Despite the wide prevalence and clinical significance of EIB, its mechanism has not been fully elucidated. The importance of vascular phenomena in exercise-induced airway narrowing has previously been suggested.[2,3] The bronchial circulation arises from the aorta and supplies the trachea and extrapulmonary and intrapulmonary airways. In asthmatic patients the airway capillary bed is hypertrophied and hyperplastic.[4] Because of its location and ability to alter its size in the asthmatic state, airway microcirculation could exert an important influence on airway geometry: vascular engorgement, capillary leakage, and edema formation could induce airway narrowing. Many of the inflammatory mediators thought to cause constriction of bronchial smooth muscle can also cause dilatation and leakage of the mucosal and submucosal capillary beds and induce airway wall edema. Small increases in airway wall edema induced by airway inflammation could produce striking changes in airway responsiveness to various stimuli, such as exercise, even when there is only a trivial increase in resting airway muscle tone. Thus mucosal edema might have a profound effect on airway function and can explain the heightened reactivity characteristic of asthma.[5] In a previous study we found that there was a significant correlation between airway microvascular permeability and the severity of EIB.[6] These findings suggest that increased microvascular permeability resulting from microvascular phenomena induces airway wall edema and follows exercise-induced airway narrowing. Therefore EIB is, at least in part, caused by airway microvascular phenomena, such as vascular engorgement and plasma leakage, that could thicken the mucosa and thereby narrow airway diameters, which could in turn amplify the effects of airway smooth muscle contraction.

In discussing the mechanism of EIB, there is now fairly strong evidence of mast cell activation, eosinophil activation, and prolonged increased levels of proinflammatory eicosanoids, such as cysteinyl leukotrienes.[7-9] Therefore it is plausible that anti-inflammatory therapy might attenuate the severity of EIB in asthmatic patients. However, our previous study reported that the severity of EIB could not be completely inhibited, even after inhaled corticosteroid therapy.[10] Recently, considerable attention

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has been devoted to the physiologic roles of angiopoietin-1 and angiopoietin-2 as regulatory factors of airway microvascular phenomena. Although there are currently 2 known members of tyrosine kinases with immunoglobulin-like loop and epidermal growth factor homology domain (Tie) receptors (Tie-1 and Tie-2),[11] 2 of the ligands for Tie-2 receptor are angiopoietin-1 and angiopoietin-2,[12] both of which bind to Tie-2 receptors with equal affinity but result in distinct effects. Because Tie-2 mRNA and protein are most abundant in the lung, it appears that the lung is uniquely dependent on Tie-2 signaling.[13] Angiopoietin-1 is known to stabilize microvessels and make them leak resistant.[14] The transgenic mice overexpressing angiopoietin-1 also exhibited a decrease in vascular leakage.[15] Thus angiopoietin-1 has been shown to protect the microvessels against plasma leakage. In contrast, angiopoietin-2 is an antagonist of angiopoietin-1 that competes for Tie-2 receptors and subsequently increases vascular leakage.[16] Recently, we found that inhaled corticosteroid therapy could not induce the decrease in angiopoietin-2 levels in asthmatic airways.[17] Therefore this study was designed to determine the roles of angiopoietin-2 as a contributing factor of EIB in asthmatic patients with inhaled corticosteroid therapy.

METHODS

Subjects

Nonsmoking subjects with clinically stable asthma aged 20 to 40 years, receiving inhaled beclomethasone dipropionate (BDP; 800 μg/d) for more than 6 months, and having the ability to complete an exercise challenge test were recruited to participate in this study. Exclusion criteria included current smokers or exsmokers with more than a 5 pack-year history, clinically significant medical disorders except asthma, concurrent use of certain respiratory medications (long-acting β_2-agonists, leukotriene modifiers, and systemic corticosteroids), and recent asthma exacerbation or respiratory tract infection (within 4 weeks). Inhaled BDP therapy was continued at a constant dose throughout the study, and short-acting β_2-agonists were withdrawn for at least 24 hours before exercise challenge testing. All healthy control subjects were healthy, lifelong nonsmoking volunteers who had no history of lung disease. Methacholine inhalation challenge testing was performed for all study subjects. Exhaled nitric oxide (NO) levels were also measured for all subjects with a chemiluminescence analyzer with a resolution of 1 parts per billion, in accordance with American Thoracic Society standards.[18] All subjects gave their written informed consent for participation in the study, which was approved by the Ethics Committee of Osaka City University, Japan.

Sputum induction and processing

Sputum induction was performed as previously described.[19] Spirometry was performed before inhalation of 200 μg of salbutamol administered through a metered-dose inhaler. All subjects were instructed to wash their mouths thoroughly with water. They then inhaled 3% saline at room temperature nebulized by an ultrasonic nebulizer (NE-U12; Omron Co, Tokyo, Japan) at maximum output. They were encouraged to cough deeply after 3-minute intervals thereafter. After sputum induction, spirometry was repeated. If FEV_1 decreased, the subjects were required to wait until it returned to baseline value. The volume of sputum samples was measured, and the sample was divided into 2 portions. One portion was diluted with PBS containing dithiothreitol (DTT; final concentration of 1 mmol/L; WAKO Pure Chemical Industries Ltd, Osaka, Japan) and then centrifuged at 400*g* for 10 minutes, and the cell pellet was resuspended. The slides were prepared with a cytospin (Cytospin 3; Shandon, Tokyo, Japan) and stained with May-Grunwald-Giemsa stain for differential cell counts. The other portion of the sputum sample, for assay of angiopoietin-1 and angiopoietin-2, was diluted with PBS without DTT[20] because we had preliminarily detected a detrimental effect of DTT on measurement of angiopoietin-1 and angiopoietin-2 levels in sputum samples. The supernatant was stored at −70°C for subsequent assay of eosinophil cationic protein (ECP), angiopoietin-1, angiopoietin-2, and albumin. Angiopoietin-1 and angiopoietin-2 concentrations were measured by means of quantitative sandwich enzyme immunoassays (Quantikine; R&D Systems, Inc, Minneapolis, Minn). Samples were analyzed in triplicate. ECP concentrations were measured with a radioimmunoassay kit (Pharmacia Diagnostics, Uppsala, Sweden). The limits of detection for angiopoietin-1 and angiopoietin-2 were 62.5 and 8.29 pg/mL, respectively. Albumin concentrations were measured by means of laser nephelometry, and then we calculated the airway vascular permeability index (the ratio of albumin concentrations in induced sputum and serum).[21] All subjects produced an adequate specimen of sputum; a sample was considered adequate if the subject was able to expectorate at least 2 mL of sputum and if on differential cell counting the slides contained less than 10% squamous cells.

Exercise challenge testing

Three days after sputum induction, exercise tests were performed in all asthmatic patients at approximately 1 PM to eliminate the effects of diurnal variation on an electrically driven treadmill (Q55xt, Series 90; Quinton Instrument Co, Seattle, Wash) for 6 minutes with a fixed workload adjusted to increase the cardiac frequency to 90% of the maximum predicted value for the age of the patient.[22] Single-lead electrocardiography and pulse oximetry (502-US; CSI, Tokyo, Japan) were monitored continuously. Pre-exercise spirometry was performed 5 minutes before exercise. The best of 3 attempts was considered the pre-exercise FEV_1 value and was required to be at least 80% of the predicted value of FEV_1 for the exercise challenge to be performed. Spirometry was performed immediately after exercise (0 minutes) and at 3, 5, 10, 15, 20, 25, 30, 45, and 60 minutes later. A standard spirometer (Chestac–25F; Chest Co, Tokyo, Japan) was used for all subjects, and spirometric maneuvers achieved American Thoracic Society acceptability and reproducibility criteria. The best FEV_1 value from each set of measurements was used for analysis. The response to exercise challenge was taken to be the percentage decrease in FEV_1 after exercise and was calculated as follows:

$$\%\ Decrease\ in\ FEV_1 = ([\mathrm{Pre}-exercise\ FEV_1 - Lowest\ FEV_1\ after\ exercise]/\mathrm{Pre}-exercise\ FEV_1) \times 100.$$

In addition, bronchoconstrictor response was also assessed as the area under the curve of the percentage decrease in FEV_1 plotted against time for 60 minutes (AUC). The AUC was calculated by using trapezoidal integration, as previously described.[23]

Statistical analysis

All data were expressed as medians (ranges). The Mann-Whitney *U* test was used for intergroup comparisons. The significance of correlation was evaluated by determining Spearman rank correlation coefficients. A *P* value of less than .05 was considered significant.

RESULTS

Thirty-two asthmatic patients and 14 age-matched healthy control subjects were included in this study (Table I). Because all asthmatic patients were receiving inhaled BDP therapy for more than 6 months, they were clinically stable and complained of neither wheezing nor dyspnea at rest. Although the results of baseline pulmonary function were significantly lower in asthmatic patients than in healthy control subjects, all asthmatic patients exhibited relatively normal FEV_1 and FEV1/forced vital capacity values (FEV_1 >80% of predicted value; FEV_1/forced vital capacity >80%). Moreover, although they had mild airway hyperreactivity to methacholine, PC_{20} methacholine values in 8 asthmatic patients were almost within the normal range (>8 mg/mL). Thus