

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 FEV₁ 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,

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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 has been devoted to the physiologic roles of angiotensin-1 and angiotensin-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 angiotensin-1 and angiotensin-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 Angiotensin-1 is known to stabilize microvessels and make them leak resistant.¹⁴ The transgenic mice overexpressing angiotensin-1 also exhibited a decrease in vascular leakage.¹⁵ Thus angiotensin-1 has been shown to protect the microvessels against plasma leakage. In contrast, angiotensin-2 is an antagonist of angiotensin-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 angiotensin-2 levels in asthmatic airways.¹⁷ Therefore this study was designed to determine the roles of angiotensin-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 β₂-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 β₂-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 FEV₁ 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 400g 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 angiotensin-1 and angiotensin-2, was diluted with PBS without DTT²⁰ because we had preliminarily detected a detrimental effect of DTT on measurement of angiotensin-1 and angiotensin-2 levels in sputum samples. The supernatant was stored at -70°C for subsequent assay of eosinophil cationic protein (ECP), angiotensin-1, angiotensin-2, and albumin. Angiotensin-1 and angiotensin-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 angiotensin-1 and angiotensin-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 FEV₁ value and was required to be at least 80% of the predicted value of FEV₁ 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 (Ches-tac-25F; Chest Co, Tokyo, Japan) was used for all subjects, and spirometric maneuvers achieved American Thoracic Society acceptability and reproducibility criteria. The best FEV₁ value from each set of measurements was used for analysis. The response to exercise challenge was taken to be the percentage decrease in FEV₁ after exercise and was calculated as follows:

$$\% \text{ Decrease in FEV}_1 = \frac{([\text{Pre-exercise FEV}_1 - \text{Lowest FEV}_1 \text{ after exercise}] / \text{Pre-exercise FEV}_1) \times 100.$$

In addition, bronchoconstrictor response was also assessed as the area under the curve of the percentage decrease in FEV₁ 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 FEV₁ and FEV₁/forced vital capacity values

TABLE I. Clinical characteristics of the study subjects

	Healthy control subjects	Asthmatic patients
No. of subjects (male/female sex)	14 (12/2)	32 (27/5)
Age (y)	31 (23-38)	33 (20-39)
FEV ₁ (% predicted)	103 (90-110)	90 (83-100)*
FEV ₁ /FVC ratio (%)	90 (85-98)	83 (81-89)
PC ₂₀ methacholine (mg/mL)†	>10	7.1 (4.2-9.8)*
Exhaled NO (ppb)	5.8 (3.6-8.5)	6.5 (4.5-8.7)
Sputum		
Eosinophils (%)	0.7 (0-16)	0.8 (0.1-2.5)
ECP (ng/mL)	110 (30-250)	120 (40-260)

Vales are presented as medians (ranges) unless stated otherwise.

FVC, Forced vital capacity.

* $P < .01$ compared with healthy control subjects.

†Geometric mean (log SEM).

(FEV₁ >80% of predicted value; FEV₁/forced vital capacity >80%). Moreover, although they had mild airway hyperreactivity to methacholine, PC₂₀ methacholine values in 8 asthmatic patients were almost within the normal range (>8 mg/mL). Thus all asthmatic patients were well controlled with respect to symptoms and physiologic parameters. In addition, exhaled NO concentrations and the degree of eosinophilic airway inflammation did not significantly differ between asthmatic patients and healthy control subjects. Angiotensin-1 levels in induced sputum 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]; Fig 1). In contrast, angiotensin-2 levels in induced sputum 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]; $P < .001$). The airway microvascular permeability index was also significantly higher in asthmatic patients (asthmatic patients: median, 0.024 [range, 0.012-0.042]; healthy control subjects: median, 0.009 [range, 0.004-0.014]; $P < .001$).

On exercise challenge testing, there were no significant differences in temperature or humidity during exercise in all asthmatic patients. The percentage of maximum decrease in FEV₁ with exercise and AUC were 14.0% (range, 7.0% to 30.0%) and 631 minutes (range, 225-1087 minutes), respectively. There was no significant correlation between angiotensin-1 levels and the percentage of maximum decrease in FEV₁ with exercise in asthmatic patients (Fig 2). However, angiotensin-2 levels were significantly correlated with the percentage of maximum decrease in FEV₁ with exercise ($r = 0.53$, $P = .003$). Similarly, angiotensin-2 levels, but not angiotensin-1 levels, were significantly correlated with AUC ($r = 0.62$, $P = .0006$, Fig 3). Moreover, angiotensin-2 levels, but not angiotensin-1 levels, were also significantly correlated with the airway microvascular permeability index ($r = 0.54$, $P = .0028$, Fig 4).

DISCUSSION

In this study we selected clinically stable asthmatic patients who were already receiving inhaled corticosteroid therapy for more than 6 months. Exhaled NO concentrations, percentages of eosinophils, and ECP levels in induced sputum did not differ

significantly between asthmatic patients and healthy control subjects, indicating that airway inflammation in asthmatic patients and healthy control subjects were at similar levels. Moreover, we observed that many of our asthmatic patients had considerably low values in the maximum decrease in FEV₁ after exercise (half of our asthmatic patients had a <15% decrease in FEV₁ after exercise) but that the severity of the EIB could not be completely inhibited, even after inhaled corticosteroid therapy. In this respect levels of angiotensin-2, but not angiotensin-1, after inhaled corticosteroid therapy were significantly higher in asthmatic patients than in healthy control subjects. Moreover, angiotensin-2 levels were correlated with the severity of EIB and the airway microvascular permeability index. Because plasma extravasation and airway edema are part of an inflammatory response, it was reasonable to expect that airway microvascular permeability would be affected by the magnitude of the inflammatory response. For these reasons, we attempted to calculate an index indicative of airway microvascular permeability rather than generalized inflammation of the airway wall. We did not simply evaluate albumin levels in induced sputum but also evaluated a ratio of albumin concentrations in induced sputum and serum, and we suggest this as a reliable index of plasma extravasation. In fact, this index was previously used for the assessment of airway microvascular leakage.²⁴ Our previous study also reported that the severity of EIB and airway microvascular permeability could not be prevented even after long-term, high-dose treatment with inhaled corticosteroids.²⁵ These findings suggest that angiotensin-2 is one of the contributing factors in EIB in asthmatic patients with inhaled corticosteroid therapy.

Expression profiling studies have shown that the main sources of angiotensin-1 and angiotensin-2 are pericytes and endothelial cells, respectively.²⁶ Angiotensin-1 promotes recruitment of pericytes and smooth muscle cells, thereby playing a role in establishing and maintaining the vascular integrity and quiescence. Thus angiotensin-1 is known to stabilize nascent vessels and make them leak resistant, presumably by facilitating communication between pericytes and endothelial cells. On the basis of these findings, the mechanism of leak resistance in microvessels by angiotensin-1 is clear. A second angiotensin, angiotensin-2, antagonizes the effects of angiotensin-1 on Tie-2 receptors and in some contexts acts as a natural inhibitor of angiotensin-1.²⁷ Whereas angiotensin-1 is widely expressed in normal adult tissues, angiotensin-2 is expressed mainly at sites of vascular remodeling, such as chronic inflammation or malignant tumors.²⁸ Angiotensin-1 induces the autophosphorylation of Tie-2, whereas angiotensin-2 is capable of competitively inhibiting this kinase activation. As an antagonist of angiotensin-1, angiotensin-2 competes with angiotensin-1 for binding of Tie-2 and loosens the interactions between endothelial and perivascular supporting cells and extracellular matrix. However, the roles of angiotensin-1 and angiotensin-2 in asthma have remained largely unexplored. Recently, Feltis et al²⁹ have reported that angiotensin-1 levels were increased in the airways of subjects with asthma. We recently found that angiotensin-1 and angiotensin-2 levels in induced sputum were significantly higher in asthmatic patients than in healthy control subjects and that angiotensin-1 and angiotensin-2 play complementary and coordinated roles in regulating airway microvascular permeability.³⁰ In our previous study all asthmatic patients were not treated with inhaled steroids, and angiotensin-1, but not angiotensin-2, levels in these patients were significantly decreased after 12 weeks of inhaled

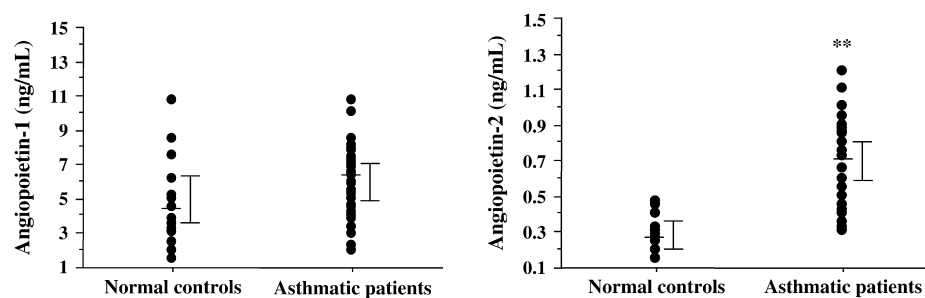


FIG 1. Comparison of angiotensin-converting enzyme (ACE) levels in induced sputum between asthmatic patients and healthy control subjects. Each bar represents the median value. The intervals represent 95% CIs. ** $P < .001$ compared with healthy control subjects.

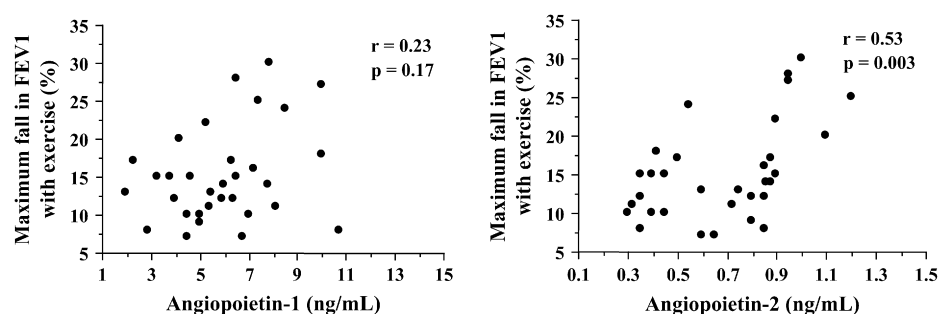


FIG 2. Correlation between angiotensin-converting enzyme (ACE) levels in induced sputum and percentage maximum decrease in FEV_1 with exercise in asthmatic patients.

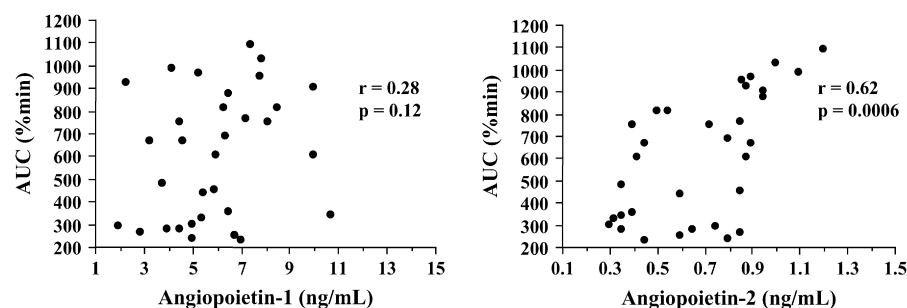


FIG 3. Correlation between angiotensin-converting enzyme (ACE) levels in induced sputum and AUC with exercise in asthmatic patients.

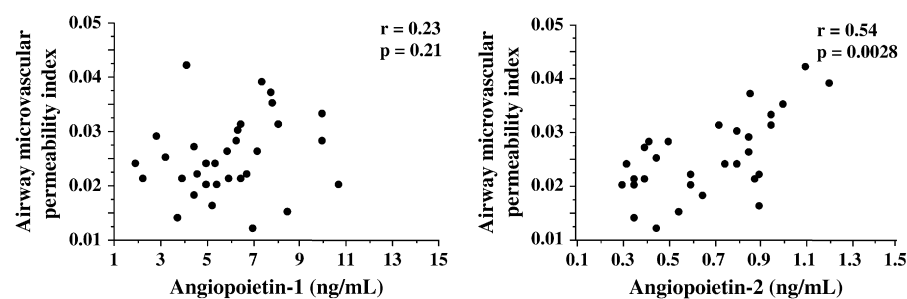


FIG 4. Correlation between angiotensin-converting enzyme (ACE) levels in induced sputum and microvascular permeability index in asthmatic patients.

steroid therapy. Moreover, we found a significant correlation between angiotensin-1 levels and airway microvascular permeability before inhaled steroid therapy but that angiotensin-1 levels after inhaled steroid therapy were not correlated with airway microvascular permeability. Compared with this previous study, we could not observe the significant correlation between angiotensin-1 levels and airway microvascular permeability because all asthmatic patients in this study were treated with inhaled steroids. Thus we think that a significant correlation between angiotensin-1 levels and airway microvascular permeability might be lost by using inhaled steroid therapy.

The effects of angiotensin-1 and angiotensin-2 on EIB have yet to be elucidated. We have already found that excessive production of angiotensin-2 in the airways leads to an increase in airway microvascular permeability and might subsequently correlate with airway obstruction in asthmatic patients.¹⁷ Plasma leakage results from gaps between endothelial cells, as well as from increased vascular surface area in the newly formed and remodeled blood vessels. In addition to the increased baseline leakage, the remodeled vessels in the airway mucosa are abnormally sensitive to various stimuli. Microvascular remodeling in asthma can result not only in more and larger blood vessels in the airway mucosa but also in functionally abnormal blood vessels that exhibit sustained leakage. Angiotensin-2 increases microvascular permeability, so that plasma proteins can leak into the extravascular space, leading to mucosal edema and thereby narrow airway diameters, which could amplify the effects of airway smooth muscle contraction. We determined that excessive expression of angiotensin-2 in asthmatic airways might be associated with the EIB response through increased airway microvascular permeability. In this study the fact that angiotensin-2 contributes to the pathogenesis of EIB has not been fully clarified because the pharmacologic blockade of angiotensin-2 cannot be performed. The temporal and quantitative balance in the expression of angiotensin-1 and angiotensin-2 seems to be key in the regulation of angiogenesis. In this study we found that angiotensin-2 levels were nearly 10 times less than angiotensin-1 levels. As shown for angiogenesis, a balance between angiotensin-1 and angiotensin-2 levels might be more important than the absolute angiotensin-2 level for determining the effects of Tie-2 signaling on pulmonary endothelial cell permeability.^{31,32}

β_2 -Adrenergic receptor agonists are commonly used in the treatment of chronic airway diseases and are known to inhibit the plasma leakage evoked by a variety of stimuli.³³ β_2 -agonists, like salbutamol, might thus prevent EIB not only through their action on bronchial smooth muscle but also by tightening of the endothelium (prevention of endothelial cell contraction) and their capacity to increase the rate of water transport to the airway surface, leading to removal of airway edema fluid.³⁴ Leukotriene receptor antagonists have also been suggested as an efficacious therapy for the treatment of EIB.³⁵ The cysteinyl leukotrienes induce bronchoconstriction, mucus hypersecretion, and mucosal edema and enhance airway hyperreactivity. Therefore it is not surprising that leukotriene receptor antagonists attenuate EIB. Recently, we have determined that montelukast, a selective leukotriene receptor antagonist, decreases airway angiotensin-2 levels in asthmatic patients.³⁰ Therefore leukotriene receptor antagonists would prevent EIB not only through their effects themselves but also by reducing angiotensin-2 levels. Thus the intervention against angiotensin-2 offers a possible new strategy for the treatment of EIB in asthmatic patients.

In summary, a significant correlation was observed between concentrations of angiotensin-2 and the degree of airway microvascular permeability. Therefore it is plausible that the capillary leakage and airway mucosal edema formation mediated by angiotensin-2 might contribute to the airway narrowing after exercise. Thus we determined that angiotensin-2 levels are increased in the airways of asthmatic patients with inhaled corticosteroid therapy and correlated with the severity of EIB and airway microvascular permeability. These findings suggest that angiotensin-2 overexpression might contribute to the pathophysiology of EIB by making the vessels more susceptible to vascular leakage.

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Clinical implications: Increased levels of angiotensin-2 can contribute to the severity of EIB by making the airway vessels more susceptible to vascular leakage.

REFERENCES

1. Cabral AL, Conceicao GM, Fonseca-Guedes CH, Martins MA. Exercise-induced bronchospasm in children: effects of asthma severity. *Am J Respir Crit Care Med* 1999;159:1819-23.
2. McFadden ER Jr. Hypothesis: exercise-induced asthma as a vascular phenomenon. *Lancet* 1990;335:880-2.
3. McFadden ER Jr, Gilbert IA. Exercise-induced asthma. *N Engl J Med* 1994;330:1362-7.
4. Bousquet J, Jeffery PK, Busse WW, Johnston M, Vignola AM. Asthma: from bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000;161:1720-45.
5. Kanazawa H, Nomura S, Yoshikawa J. Role of microvascular permeability on physiologic differences in asthma and eosinophilic bronchitis. *Am J Respir Crit Care Med* 2004;169:1125-30.
6. Kanazawa H, Asai K, Hirata K, Yoshikawa J. Vascular involvement in exercise-induced airway narrowing in patients with bronchial asthma. *Chest* 2002;122:166-70.
7. Hallstrand TS, Moody MW, Wurfel MW, Schwartz LB, Hengerson WR Jr, Aitken ML. Inflammatory basis of exercise-induced bronchoconstriction. *Am J Respir Crit Care Med* 2004;172:679-86.
8. Yoshikawa T, Shoji S, Fujii T, Kanazawa H, Kudoh S, Hirata K, et al. Severity of exercise-induced bronchoconstriction is related to airway eosinophilic inflammation in patients with asthma. *Eur Respir J* 1998;12:879-84.
9. Hallstrand TS, Moody MW, Aitken ML, Hengerson WR Jr. Airway immunopathology of asthma with exercise-induced bronchoconstriction. *J Allergy Clin Immunol* 2005;116:586-93.
10. Kanazawa H, Hirata K, Yoshikawa J. Role of endogenous nitric oxide in exercise-induced airway narrowing in patients with bronchial asthma. *J Allergy Clin Immunol* 2000;106:1081-7.
11. Peters KG, Kontos CD, Lin PC, Wong AL, Rao P, Huang L, et al. Functional significance of Tie2 signaling in the adult vasculature. *Recent Prog Horm Res* 2004;59:51-71.
12. Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, et al. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 1995;376:70-4.
13. Wong AL, Haroon ZA, Werner S, Dewhirst MW, Greenberg CS, Peters KG. Tie 2 expression and phosphorylation in angiogenic and quiescent adult tissues. *Circ Res* 1997;81:567-74.
14. Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, et al. Angiotensin-1 protects the adult vasculature against plasma leakage. *Nat Med* 2000;6:460-3.
15. Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, et al. Leakage-resistant blood vessels in mice transgenically overexpressing angiotensin-1. *Science* 1999;286:2511-4.
16. Rudolfsson SH, Johansson A, Franckl L, Wikstrom P, Bergh A. Localized expression of angiotensin 1 and 2 may explain unique characteristics of the rat testicular microvasculature. *Biol Reprod* 2003;69:1231-7.

17. Nomura S, Kanazawa H, Hirata K, Iwao H, Yoshikawa J. Relationship between vascular endothelial growth factor and angiopoietin-2 in asthmatics before and after inhaled beclomethasone therapy. *J Asthma* 2005;42:141-6.
18. Recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide in adults and children. *Am J Respir Crit Care Med* 1999;160:2104-17.
19. Kanazawa H, Shoji S, Yamada M, Fujii T, Kawaguchi T, Kudoh S, et al. Increased levels of nitric oxide derivatives in induced sputum in patients with asthma. *J Allergy Clin Immunol* 1997;99:624-9.
20. Kanazawa H, Nomura S, Hirata K, Yoshikawa J. Effect of inhaled beclomethasone dipropionate on peroxynitrite inhibitory activity in induced sputum from asthmatic patients. *Chest* 2003;124:1755-61.
21. Kanazawa H, Yoshikawa J. A case-control study of bronchial asthma associated with ulcerative colitis: role of airway microvascular permeability. *Clin Exp Allergy* 2005;35:1432-6.
22. Crapo RO, Casaburi R, Coates AL, Enright PL, Hankinson JL, Irvin CG, et al. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am J Respir Crit Care Med* 2000;161:309-29.
23. Leff JA, Busse WW, Pearlman D, Bronsky EA, Kemp J, Hendeles L, et al. Montelukast, a leukotriene-receptor antagonist, for the treatment of mild asthma and exercise-induced bronchoconstriction. *N Engl J Med* 1998;339:147-52.
24. Van Rensen ELJ, Hiemstra PS, Rabe KF, Sterk PJ. Assessment of microvascular leakage via sputum induction. The role of substance P and neurokinin A in patients with asthma. *Am J Respir Crit Care Med* 2002;165:1275-9.
25. Subbarao P, Duong M, Adelroth E, Otis J, Obminski G, Inman M, et al. Effect of ciclesonide dose and duration of therapy on exercise-induced bronchoconstriction in patients with asthma. *J Allergy Clin Immunol* 2006;117:1008-13.
26. Gale NW, Thurston G, Hackett SF. Angiopoietin 2 is required for postnatal angiogenesis and lymphatic patterning, and only the later role is rescued by angiopoietin 1. *Dev Cell* 2002;3:411-23.
27. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 1997;277:55-60.
28. Zagzag D, Amirnovin R, Greco MA, Yee H, Holash J, Wiegand SJ, et al. Vascular apoptosis and involution in gliomas precede neovascularization: a novel concept for glioma growth and angiogenesis. *Lab Invest* 2000;80:837-49.
29. Feltis BN, Wignarajah D, Zheng L, Ward C, Reid D, Harding R, et al. Increased vascular endothelial growth factor and receptors. Relationship to angiogenesis in asthma. *Am J Respir Crit Care Med* 2006;173:1201-7.
30. Kanazawa H, Nomura S, Asai K. Roles of angiopoietin-1 and angiopoietin-2 on airway microvascular permeability in asthmatic patients. *Chest* 2007;131:1035-41.
31. Parikh S, Mammoto T, Schultz A, Yuan HT, Christiani D, et al. Excess circulating angiopoietin-2 may contribute to pulmonary vascular leak in sepsis in humans. *PLoS Med* 2006;3:e46.
32. Hashimoto T, Pittet J-F. Angiopoietin-2: modulator of vascular permeability in acute lung injury? *PLoS Med* 2006;3:e113.
33. Anderson SD, Brannan JD. Long-acting β_2 -adrenoceptor agonists and exercise-induced asthma. Lessons to guide us in the future. *Pediatr Drugs* 2004;6:1-15.
34. Baluk P, McDonald DM. The β_2 -adrenergic receptor agonist formoterol reduces microvascular leakage by inhibiting endothelial gap formation. *Am J Physiol Lung Cell Mol Physiol* 1994;266:L461-8.
35. Villaran C, O'Neill SJ, Helbling A, van Noord JA, Lee TH, Chuchalin AG, et al. Montelukast versus salmeterol in patients with asthma and exercise-induced bronchoconstriction. Montelukast/Salmeterol Exercise Study Group. *J Allergy Clin Immunol* 1999;104:547-53.