

Increased Postexercise Lipoxin A4 Levels in Exhaled Breath Condensate in Asthmatic Children With Exercise-Induced Bronchoconstriction

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■ Abstract

Background: Lipoxins could be potential modulators of inflammation in the lungs. To our knowledge, the role of exhaled breath condensate (EBC) lipoxin A4 (LXA4) in asthmatic children with exercise-induced bronchoconstriction (EIB) has not been investigated.

Objective: The aim of our study was to determine the involvement of EBC LXA4 in EIB.

Methods: Forty-five patients aged between 5 and 17 years were included in the study. Patients were divided into 2 groups: asthmatic children with a positive response to exercise (n=17) and asthmatic children with a negative response to exercise (n=28). Levels of LXA4 were determined in EBC before and immediately after the exercise challenge using ELISA.

Results: EBC LXA4 levels were significantly increased immediately after exercise in asthmatic children with a positive response to the exercise challenge ($P=.05$). No significant differences were observed in children with a negative response to exercise ($P>.05$). There was an inverse correlation between LXA4 levels and the percent degree of reduction in forced expiratory volume in the first second (FEV₁%) postexercise in children with a positive exercise challenge ($P=.05$, $r=-0.50$). No significant differences were observed in LXA4 levels between atopic and nonatopic asthmatics ($P>.05$, Mann-Whitney U test).

Conclusions: Levels of EBC LXA4 increased immediately after exercise in asthmatic children with a positive exercise challenge response. We hypothesize that airway LXA4 levels increase to compensate bronchoconstriction and suppress acute inflammation, and that spontaneous bronchodilatation after EIB may be due to LXA4.

Key words: Asthma. Exercise-induced bronchoconstriction. Exhaled breath condensate. Lipoxin A4.

■ Resumen

Introducción: Las lipoxinas pueden actuar potencialmente como inmunomoduladores de la actividad inflamatoria en el pulmón. A nuestro entender, el papel de la lipoxina A4 (LXA4), determinada en condensado de aire exhalado (EBC) en niños asmáticos con broncoconstricción inducida por el ejercicio (BEI) no ha sido previamente investigado.

Objetivo: El objetivo de nuestro estudio fue determinar la implicación de la LXA4 determinada en EBC, en el broncoespasmo inducido por ejercicio.

Métodos: Se incluyeron en el estudio un total de cuarenta y cinco pacientes de edades comprendidas entre 5 y 17 años. Los pacientes se dividieron en dos grupos: niños asmáticos con respuestas positivas (n = 17) y negativas (n = 28) a la provocación bronquial con ejercicio. Los niveles de LXA4 en EBC se determinaron inmediatamente antes y después de la provocación bronquial mediante un método ELISA.

Resultados: Los niveles de LXA4 en EBC aumentaron significativamente tras la provocación con ejercicio en aquellos niños asmáticos con respuestas positivas en la provocación ($p = 0,05$). Sin embargo, no pudimos encontrar ninguna diferencia estadísticamente significativa en pacientes con respuesta negativa al ejercicio ($p > 0,05$). Hubo una correlación inversa entre el incremento de los niveles de LXA4 y el grado de reducción porcentual del volumen espiratorio forzado en un segundo (FEV₁%) en los pacientes con respuesta positiva a la provocación ($p = 0,05$, $r = -0,50$). No se observaron diferencias significativas en los niveles de LXA4 entre asmáticos alérgicos y no alérgicos ($p > 0,05$, prueba de Mann-Whitney).

Conclusiones: Los niveles de EBC LXA4 se incrementan inmediatamente después de la broncoconstricción inducida por el ejercicio en niños asmáticos. Se postula que los niveles de las vías respiratorias aumentan LXA4 para suprimir la inflamación aguda en la vía respiratoria, y podrían ser responsables de la inducción de broncodilatación espontánea que aparece tras el EIB.

Palabras clave: Asma. Broncoconstricción inducida por ejercicio. Condensado de aire exhalado. Lipoxina A4.

Introduction

Lipoxins were the first agents to be identified as anti-inflammatory endogenous lipid mediators involved in the resolution of inflammation [1]. Their actions contrast with actions associated with the majority of other lipid mediators, which have been shown to be proinflammatory (eg, leukotrienes). In vitro, lipoxins exert cell type-specific actions: they inhibit granulocyte recruitment and activation, cytokine and chemokine production, and biosynthesis of proinflammatory lipid mediators, stimulate the clearance of apoptotic leukocytes, and block edema formation [1]. Studies from the past 2 decades have implicated the biological activity of lipoxins in asthma and allergic diseases [2]. Sanak et al [3] were the first authors to report defective lipoxin biosynthesis in patients with severe asthma, and later studies in adults revealed that lipoxin generation and receptor expression were decreased in severe asthma compared with mild asthma [4,5]. Lipoxin A4 (LXA4) suppresses airway hyperresponsiveness and pulmonary inflammation through anti-inflammatory receptors, namely ALX/FPR2 receptors, expressed on both leukocytes [6] and airway epithelial cells [7].

In animal models, bioactive, stable LXA4 analogs inhibit airway hyperresponsiveness and allergic inflammation by containing eosinophil trafficking and tissue gathering [8]. In humans, LXA4 is produced during asthmatic responses [9] and has been found to decrease leukotriene C4-induced bronchoconstriction when given to asthmatic individuals via nebulization [10].

Exhaled breath condensate (EBC) collection is a noninvasive method of obtaining samples from respiratory surface lining fluids [11], and measurement of several markers in this condensate can provide information about certain pulmonary diseases, their severity, and response to therapy [11,12]. Both lipoxins and leukotrienes have been isolated from EBC [11-13].

Previous studies by our group on serum levels of LXA4 in exercise-induced bronchoconstriction in asthmatic children [14] and wheezy infants [15] showed decreased serum levels of LXA4 following exercise. However, no data have been published on LXA4 levels in the EBC of asthmatic children with exercise-induced bronchoconstriction. We decided to measure LXA4 levels in the EBC of asthmatic children following exercise in order to determine whether bronchoconstriction is associated with LXA4 production or excessive LXA4 consumption.

Methods

Individuals

This prospective study was performed in the Department of Pediatric Allergy of Erciyes University in Kayseri, Turkey. The study procedures were performed in conformity with a protocol already approved by the institutional review board at the university. Written informed consent was acquired from the parents of all participating children. A total of 45 children between the ages of 5 and 17 years were recruited. These were randomly chosen from among the new patients at

the unit. Asthma was diagnosed according to the published Global Initiative for Asthma guidelines [16]. In an attempt to prevent confounding due to disease severity, only children who strictly fulfilled the criteria for intermittent or persistent mild asthma [16] were enrolled and those with moderate to severe asthma were excluded. Children who had had an upper or lower airway infection or an asthma exacerbation in the 6 weeks prior to the study were excluded. All children underwent a standard exercise challenge, with collection of EBC before and immediately after this challenge. Spirometric measurements, total IgE, and eosinophil counts were obtained and skin patch tests were performed with a series of 23 antigens (15 aeroallergens and 8 food allergens), with appropriate positive and negative controls. The patches were placed on the upper back of the children during the first visit. Reactions with a wheal 3 mm larger than the negative control were considered positive.

Collection of EBC

EBC was collected using a commercial device (R tube, Respiratory Research, Inc.) as previously described [17] and following the guidelines of the American Thoracic Society/European Respiratory Society (ATS/ERS) [18]. The children were instructed to breathe tidally for 10 minutes through the mouthpiece of the R tube with a 1-way valve connected to a condenser. They were also instructed to temporarily stop breathing through the mouthpiece if they needed to swallow saliva or had an urge to cough. The EBC samples were transferred into sterile containers and immediately stored at -70°C until analysis.

Study Measurements

The exercise challenge was undertaken in accordance with ATS/ERS guidelines, with the child on a treadmill at submaximal workload for 6 minutes while breathing dry air [18]. Children were required to not take medications that can influence bronchial challenges (Table 1). Children with asthma increased their exercise effort until their heart rate reached 85% to 90% of the age-predicted maximum (220-age in years) within 1 minute of starting the test; this effort was then maintained for 6 minutes. Spirometry was performed immediately after the challenge (minute 0) and also at 5, 10, 15, and 20 minutes. Exercise-induced bronchoconstriction

Table 1. Drugs Withheld Prior to Commencement of Protocol

Short-acting β -agonists	For 8 h
Medium-acting bronchodilators	For 24 h
Long-acting β_2 -agonists	For 48 h
Leukotriene-receptor antagonists	For 24 h
Sodium cromoglycate	For 8 h
Nedocromil	For 48 h
Antihistamines	For 72 h

was considered to be present if the percent change in postexercise forced expiratory volume in the first second (FEV₁%) was 15% or greater than the pre-exercise value [19]. IgE levels were measured by Uni-Cap in accordance with the manufacturer's instructions (Pharmacia, Kalamazoo). Eosinophil counts were determined using Coulter Counter leukocyte measurements.

Extraction Protocol for LXA4

EBC samples of 100 µL were diluted with 200 µL of methanol to which 1.5 mL of water was added. One-mL samples were then acidified to pH 3.5 with 1 N HCl, vortexed, and subsequently homogenized in ethanol (5 mL/g). After centrifugation of the homogenate, 1 mL of supernatant was diluted with 5 mL of deionized water and the pH was adjusted to 3.5. A C18 Sep-Pak light column was washed with 2 mL of methanol followed by 2 mL of deionized water. The samples were added at a flow rate of 1 mL per minute. The column was washed with formate, which was evaporated under a stream of nitrogen gas. The residue was resuspended in 1 mL of diluted extraction buffer.

LXA4 Measurements

Quantitative detection of LXA4 was performed using the Lipoxin A4 ELISA kit (Eagle Bioscience Inc, Catalog Number: LA439-K01) according to the manufacturer's instructions; the lower and upper detection limits are 0.02 and 2.0 ng/mL, respectively. We added 50 µL of sample diluent to the corresponding wells on the microplate in duplicate. Then, 50 µL of diluted LXA4-HRP conjugate was added to each well and these were incubated at room temperature for 1 hour. After incubation, the microwell strips were washed 3 times with 300 µL of diluted wash buffer per well. Following this step, 150 µL of TMB substrate solution was added to all the wells and incubated at room temperature for 30 minutes for color development. Next, 50 µL of stop solution (1N HCl) was added to all wells and the plate was

read on an ELISA reader at 450 nm. A standard curve was prepared from 7 human LXA4 standard dilutions and human LXA4 sample concentrations were determined.

LXA4 was detected in the EBC of all patients. In the group with a positive exercise challenge, pre-exercise levels ranged between 55 and 200 pg/mL, while postexercise levels ranged between 68 and 210 pg/mL. In the group with a negative exercise challenge, the levels ranged between 41 and 190 pg/mL pre-exercise and 25 and 220 pg/mL post-exercise (Table 3).

Statistical Analyses

Statistical analysis was performed using version SPSS 15 for Windows. LXA4 levels in EBC were compared using the Mann-Whitney U or the Wilcoxon test, depending upon the distribution of the data. Correlation analysis was performed with the Spearman tests. A *P* value of .05 or less was considered significant.

Results

Patient Characteristics

Forty-five children with asthma were screened for exercise response. Seventeen had a positive response, while 28 had a negative response. No significant differences were detected between these 2 groups for age, sex, IgE levels, eosinophil numbers, atopic status, or FEV₁% (*P*>.05, Table 2).

EBC LXA4 Levels

We found no statistically significant differences in pre-exercise LXA4 levels between the groups (*P*>.05, Mann-Whitney U test, Figure 1). Significant increases were observed for EBC LXA4 levels immediately after the exercise challenge in asthmatic children with a positive exercise response (*P*=.05, Wilcoxon, Table 3, Figure 1), but not in those with a negative response (*P*>.05, Wilcoxon, Figure 1). No significant

Table 2. Patient Characteristics

	Asthmatic Patients With Positive Exercise Response (n=17)	Asthmatic Patients With Negative Exercise Response (n=28)	<i>P</i> Value
Age, (range), y ^a	11 (5-16.5)	10 (6-17)	>.05 ^b
Sex			>.05 ^c
Boys, No. (%)	9 (52.9)	16 (57.1)	
Girls, No. (%)	8 (47.1)	12 (42.9)	
FEV ₁ % predicted ^a	114.6 (83.7-163.9)	115.2 (86.8-205)	>.05 ^b
Maximum % fall after exercise ^a	18 (15-50)	7 (1-13)	<.05 ^b
Skin test positivity, %	52.9	50	>.05 ^c
Eosinophil count ^a	200 (30-1630)	175 (40-1360)	>.05 ^b
IgE, kU/L ^a	53.8 (4.93-568)	70.5 (2.1-1193)	>.05 ^b

Abbreviation: FEV₁%, percent change in forced expiratory volume in the first second.

^aMedian (interquartile range, 27-75 percentile).

^bMann-Whitney U test.

^cχ² test.

Table 3. Exhaled Breath Condensate (EBC) Lipoxin A4 Levels (LXA4)

	Pre-Exercise EBC LXA4 Levels, pg/mL	Postexercise EBC LXA4 Levels, pg/mL	<i>P</i> Values
Asthmatic children with a positive exercise response (n=17)	109 (55-200)	131 (68-210)	.05 ^b
Asthmatic children with a negative exercise response (n=28)	118 (41-190)	126 (25-220)	.879 ^b

^aLevels are expressed as median (range).

^bWilcoxon test.

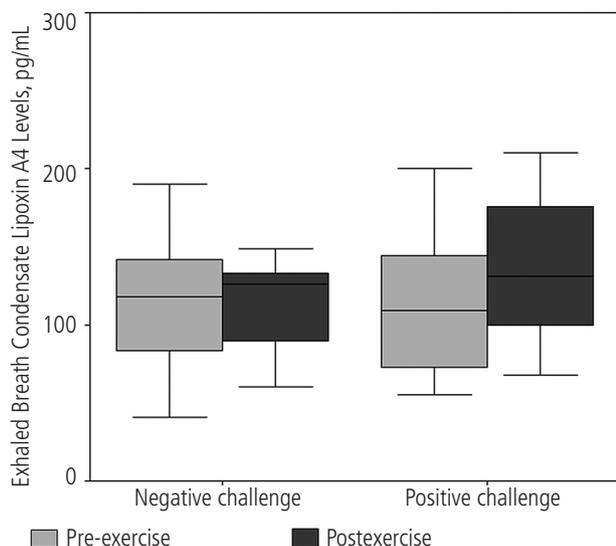


Figure 1. Increase in exhaled breath condensate lipoxin A4 levels immediately after the exercise challenge in asthmatic children with a positive challenge.

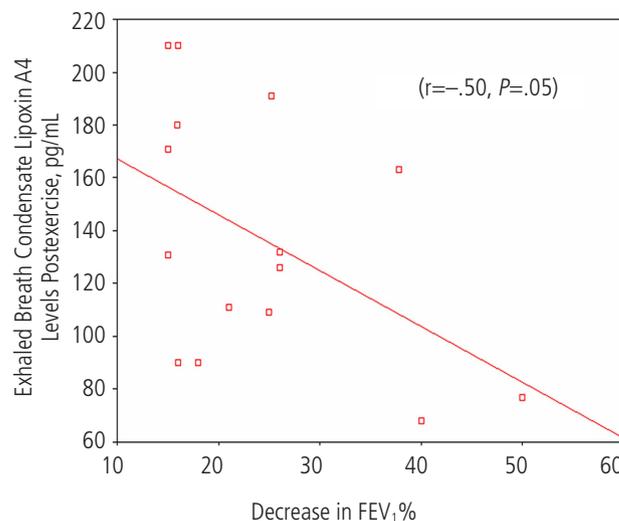


Figure 2. Correlation between lipoxin A4 levels and decrease in percent change in forced expiratory volume in the first second (FEV₁%) after the exercise challenge.

differences was observed for LXA4 levels between atopic and nonatopic asthmatics ($P > .05$, Mann-Whitney U test).

Correlation Analyses

In the correlation analyses, no correlations were detected between pre-exercise EBC LXA4 levels and eosinophil counts, total IgE levels, or atopic status ($P > .05$, Spearman). We did, however, find an inverse correlation between LXA4 levels and the degree of reduction in postexercise FEV₁% in children with a positive response to exercise ($P = .05$, $r = -0.50$, Figure 2).

Discussion

To best of our knowledge, this is the first study to investigate the role of EBC LXA4 in the bronchoconstrictor response observed in asthma. Our results show increased LXA4 levels immediately after exercise in children with a positive response to the exercise challenge and an inverse correlation between LXA4 levels and the degree of reduction in postexercise FEV₁% in the same group of children.

Products of arachidonate 15-lipoxygenases types I and II exert both helpful effects (eg, stereoselective signal counterregulation) and potentially harmful effects [20]. As numerous studies have shown, 15-lipoxygenase expression is increased in many conditions, including human bronchotracheal epithelial cell differentiation [21] and aspirin-sensitive asthma [22].

Preliminary results regarding lipoxin generation and the role of 15-lipoxygenase in lipoxin biosynthesis [20] suggested that 15-lipoxygenase overexpression could suppress the acute inflammatory response as a main factor in coordinating in vivo lipoxin expression, while other studies have demonstrated that lipoxin production in acute inflammation is associated with resolution [23,24]. In our study, postexercise increases in LXA4 in patients with a positive exercise response suggest that lipoxin levels may increase in order to suppress acute inflammation. Although lipoxin is generated in the inflamed asthmatic lung, insufficient production and/or function of lipoxin has been associated with the more severe phenotype of asthma [5,13,25,26]. It has also been suggested that permanent airway inflammation in asthma may be due to

insufficient production of lipoxin [27]. Hasan et al [13], for example, reported lower EBC LXA4 levels in patients with status asthmaticus compared with controls. In a study by Kazani et al [12], however, EBC LXA4 levels were more than 10 times higher in asthmatic patients than in healthy controls. The authors also showed a correlation between low lipoxin levels and decreased lung function. In addition, they found that of 3 groups of asthmatic patients, those with severe asthma had the lowest mean EBC LXA4 level. Our results show an inverse correlation between LXA4 levels and the degree of reduction in postexercise FEV₁% in children with a positive exercise challenge.

In a study published in 2000, the effects of lipoxin on airway responsiveness and inflammation were analyzed using a lipoxin analog [28], and in an earlier study, Christie et al [29] demonstrated that LXA4 significantly decreased leukotriene C₄-induced airway obstruction in asthmatic individuals. Intravenous application of an LXA4 analog in ovalbumin-sensitized mice has been found to significantly decrease methacholine-induced bronchoconstriction and lung infiltration by eosinophils and lymphocytes. Furthermore, concentrations of type 2 helper cytokines and proinflammatory lipid mediators after the challenge were significantly lower in LXA4 analog-treated mice, suggesting that LXA4 mimetics may significantly inhibit allergic pulmonary inflammation [8].

In a study of children with asthma, Wu et al [30] showed a positive correlation between blood LXA4 levels and peak expiratory flow (PEF) and FEV₁, but a negative correlation between blood LTC₄ levels and PEF or predicted FEV₁. An inverse relationship was also observed between 15-LO, its derivatives LXA₄ and 5-LO, and their derivatives LTB₄ and LTC₄ with decreasing severity of asthma (from severe to mild) [30]. These findings suggest insufficient LXA₄ production and excess leukotriene production. Our results showed an inverse correlation between LXA₄ levels and the degree of reduction in postexercise FEV₁% in asthmatic children with a positive response to exercise.

Inflammatory mediator release can be observed during strenuous exercise [31], and it is known that this type of exercise can induce lipoxin biosynthesis and further metabolism in healthy volunteers. In a study by Gangemi et al [32], a significant increase in LXA₄ urinary excretion was observed immediately after strenuous exercise in 9 healthy volunteers [32]. Lipoxins are locally acting autacoids formed by cell stimulation and inactivated by metabolism. LXA₄ can form very rapidly, and production has been observed within 10 seconds of angioplasty [33]. Most LXA₄ (>60%) is metabolized by peripheral blood monocytes within 30 seconds [34]. Increased lipoxin biosynthesis during exercise may have relevant pathophysiological effects. As our results show that LXA₄ levels in EBC were increased immediately after exercise in children with positive exercise challenge results, we believe that the production of LXA₄ in the course of physical exercise may suppress the action of exercise-induced proinflammatory mediators.

Our finding that LXA₄ levels in EBC were increased immediately after exercise in children with a positive response to exercise contrast with previous findings by our group [14]. We thought that the development of exercise-induced

bronchoconstriction in asthmatic children might be associated with a reduced capability of endogenous lipoxin biosynthesis, but the results of the present study show that airway LXA₄ levels are different from plasma levels.

This study has several limitations. First, it includes a small number of children with exercise-induced bronchoconstriction from a single institution. Second, it did not include a control group. Because of these limitations, our study results may not be applicable to other institutions or other clinical settings.

Lipoxins are known to play an important role in the resolution of the inflammatory response. Their production in the course of physical exercise may suppress the action of exercise-induced proinflammatory mediators, and it is possible that LXA₄ levels may increase in order to compensate inflammation and bronchoconstriction.

In conclusion, we found increased EBC LXA₄ levels immediately after exercise in asthmatic children with a positive exercise challenge. We hypothesize that airway LXA₄ levels increase to compensate bronchoconstriction and suppress acute inflammation. Spontaneous bronchodilatation after exercise-induced bronchoconstriction may be due to LXA₄. Lipoxin mimetics and its components could lead to new treatment approaches for exercise-induced bronchoconstriction in asthma.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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