Exhaled 15-HETE and thromboxin-B2 are associated with the therapeutic outcome in childhood asthma

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Abstract

Background: Dysregulation of eicosanoids is associated with asthma and a composite of oxylipins, including exhaled LTB4, characterizes childhood asthma. While FeNO has been used as the standard for monitoring steroid responsiveness, the potential utility of eicosanoids in monitoring the therapeutic outcomes remains unclear. We aimed to examine the levels of major eicosanoids representing different metabolic pathways in exhaled breath condensates (EBCs) of children with asthma during exacerbation and after treatment. Methods: Levels of 6 exhaled eicosanoid species in asthmatic children and healthy subjects were evaluated using ELISA. Results: In addition to those previously reported, including LTB4, the levels of exhaled 15-HETE, but not TXB2, showed significant difference between asthmatics (N=318) and healthy controls (N=97), particularly the severe group showed the lowest levels of exhaled 15-HETE. Receiver Operating Characteristic (ROC) analyses revealed similar distinguishing power for the levels of 15-HETE, FEV1 and FeNO, whilethe 15-HETE/LTB4 ratio was significantly lower in subjects with severe asthma ($p<0.01$). Analysis of asthmatics (N=75) during exacerbation and convalescence showed significant improvement in lung function (FEV1; $p<0.001$), but not FeNO, concomitant with significantly increased levels of 15-HETE ($p<0.001$) and reduced levels of TXB2 ($p<0.05$) after therapy, particularly for those who at the top 30% level during exacerbation. Further, decreased LTB4 and LXA4 at convalescence were noted only in those at the top 30 percentile during exacerbation. Conclusion: The exhaled 15-HETE was found to discriminate childhood asthma while decreased levels of exhaled TXB2 and increased levels of 15-HETE were prominent after treatment.

Introduction

Asthma is a chronic inflammatory disorder of the airways and is characterized by airway hyperresponsiveness and reversible airflow obstruction that fluctuates over time. It is also recognized as a heterogeneous disease with varying severity, responsiveness to therapy, and long-term outcome[1]. Eicosanoids are a family of bioactive lipid mediators that regulate a wide variety of inflammatory processes[2]. Eicosanoid species are generated from polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA). AA can be converted into prostaglandins (PGs), leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs)[3] by cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450 epoxygenases (CYP450). 5-LOX-derived LTA_4 can be converted to lipoxins in the presence of 15-LOX, while 15-LOX generates 15-HETE[3]. Lipoxins and 15-HETE have been reported to exert anti-inflammatory activity; for example, LXA_4 and 15-HETE inhibit LTB_4-induced chemotaxis of neutrophils[4-6]. Thromboxane B_2(TXB_2) is non-enzymatically hydrolyzed from

COX-derived TXA_2, a potent bronchial smooth muscle spasmogen[7], is known to reduce T cell secretion of the Th1 cytokine and favor T cell differentiation toward a Th2 cytokine profile[8].

Together, these functionally diverse classes of eicosanoids are thought to play a critical role in maintaining homeostasis and have been an active area of investigation in assessing the mechanism underlying asthma and their potential utility in monitoring disease progression and treatment outcome. Indeed, several studies have suggested their roles as the biomarkers for screening, diagnosis, and, to a limited extent, monitoring the treatment outcome. For example, several independent studies have shown elevated levels of eicosanoids in the EBC of patients with asthma[9,10], but due, perhaps in part, to the limited sample sizes and the heterogeneity of the study patient populations, unified evidence is currently lacking. As the result, knowledge about eicosanoids in disease progression and therapeutic outcome remains incomplete, and their relationship with the disease status has yet to be comprehensively explored and their clinical utility as biomarkers remains to be determined. We have previously reported that in a pediatric study population in Taiwan, the levels of exhaled LTB_4, LTE_4, LXA_4, and PGE_2 in asthmatic children were significantly different from those of healthy controls, and the combination of exhaled LTB_4 and LXA_4, together with FeNO and FEV_1, best characterized childhood asthma[11]. We described herein an analysis of the levels of exhaled eicosanoids differed at the time of acute exacerbation and convalescence and reported that the levels of $TXB_2$and 15-HETE were the most responsive to therapy.

Materials and Methods

Study subject

A total of 393 bronchial asthmatic children aged between 5 and 12 years, consisting of 318 stable asthmatics and 75 acute asthma attack sufferers (47 males and 28 females), were recruited from the pediatric clinics of the Chang Gung Memorial Hospital, Taiwan, as a part of the ongoing PATCH study (Prediction of Allergies in Taiwanese Children). A total of 97 (59 males and 38 females) age-matched healthy subjects were enrolled from an elementary school in Taoyuan City, Taiwan. The diagnosis and classification of the clinical severity of asthma followed the published guidelines[12]. Asthma severity was categorized as mild (intermittent), moderate and severe, based on the previously described criteria[11]. An acute asthma attack was defined as a patient with dyspnea symptoms and audible expiratory wheeze accompanied by a 20% reduction in FEV_1. Levels of EBC eicosanoids, FEV_1, and FeNO were measured during acute asthma attack episodes and at two weeks after the acute asthma attacks. For the management of acute asthma exacerbation, all of the asthmatic children received terbutaline inhalation and oral prednisolone 1 mg/kg/day for 3 days. In the two weeks prior to EBC collection, none of the patients took medication containing antipyretics or anti-platelet agents. The healthy, non-asthmatic, and non-allergic subjects served as normal controls. This study was approved by the Humane Research Committee of the Chang Gung Memorial Hospital and informed consents were obtained from patients' parents or guardians prior to the start of the study. Additional phenotypes included BMI, serum IgE levels, FEV_1, and FeNO measured as previously described[13].

Exhaled breath condensate (EBC) collection and exhaled nitric oxide measurement

EBC was collected by Turbo-Deccs system (Medivac, Parma, Italy). Approximately 1 ml of EBC was collected and immediately stored at -80 for further analysis. Following EBC collection (after 30 minutes of rest), fractional exhaled nitric oxide (FeNO) was measured according to published standards by using NIOX MINO Airway Inflammation Monitor (Aerocrine, Solna, Sweden).

Lung function tests, methacholine challenge tests and analysis of eicosanoids

The lung function tests were performed with the spirometer-Lungest 1000 (MES, Krakow, Poland) according to ERS/ATS[14]standards. These children had not received any anti-asthmatic medication, including oral steroids, for at least two days.

Utilizing established solid phase extraction approach for the collection and purification of eicosanoids in EBCs, a panel of 6 eicosanoid species derived from arachidonic acids, representing products from major enzymatic pathways, was selected for initial discovery phase of the study population consisting of 60 asthmatics

and 20 healthy controls, who were randomly selected from among the study populations. Eicosanoids were extracted from EBCs[15] and measured with the respective enzyme immunoassay kit (Cayman Chemical, Ann Arbor, Michigan and Neogen, Lexington, Ky) as described previously[11].

Statistical analysis

The significance of differences between the asthmatic and healthy children in their categorical variables was estimated by theχ^2 test and continuous variables (for example, age, BMI, Δ%FEV_1) by the t test or ANOVA analyses. Receiver operating characteristic (ROC) curve with analysis of differences in the area under curves (AUC) was used to estimate the diagnostic accuracy. Furthermore, asthmatic subjects with repeated data were further divided into three groups according to the levels of eicosanoid species during exacerbation, i.e. the top 30%, middle 40%, and bottom 30%. A two-way mixed-design analysis of variance (i.e. split-plot ANOVA) was performed for analyzing the effect of stratified eicosanoid levels (top 30% vs. middle 40% vs. bottom 30%) and phases (active exacerbation vs. convalescence).

Results

Analysis of exhaled eicosanoid species for differentiating asthma from normal controls

In the discovery phase of the study, in addition to those previously reported[11], including LTB_4, the level of 15-HETE, but not TXB_2, in EBCs of subjects with asthma (N=60) was significantly lower than that noted in the control group (N=20) (Data not shown). To confirm the validity of these eicosanoid species in differentiating asthma patients from normal subjects, a total of 415 children were included in the validation phase, which consisted of 318 stable asthmatic and 97 healthy subjects. The demographics of these asthmatic children and healthy children are summarized in Table 1. Significant differences were noted for age, gender, serum total IgE, FEV_1 and FeNO between the subjects in the asthmatic and the control groups (all had $p<0.001$ except for age and gender with $p<0.05$ and $p<0.01$, respectively; Table 1). No significant difference was found for BMI. In the expanded case-control design, the levels of exhaled 15-HETE were significantly lower for asthmatic subjects than for healthy subjects ($p<0.0001$; Table 2), while the level of TXB_2 was similar between the two groups. Correlation analysis revealed that in asthmatic children, there was a significant positive correlation between the levels of TXB_2 and those of LTB_4 and PGE_2 (Supplementary Figs. S1A and S1B) in the exhaled condensate. Moreover, among the asthmatic subjects, negative correlations were found for TXB_2 and FEV_1, and also for 15-HETE and LTB_4, ($r=-0.13$, $p<0.05$; $r=-0.11$, $p<0.05$, respectively; Supplemental Figs. S1C and S1D).

When the asthmatic population was stratified into different severity groups (Table 2), it was noted that in comparison to the mild group, the moderate group was characterized with lower levels of exhaled 15-HETE, and the severe group exhibited even lower levels. The difference in 15-HETE levels between healthy subjects and all three asthmatic severity groups was significant, but no significant difference was found between groups for TXB_2. Further, as 15-HETE is known to exert inhibitory effect on 5-LOX–derived pro-inflammatory leukotrienes, the ratios of exhaled 15-HETE/LTB_4 were calculated, and the results showed that the ratio of 15-HETE/LTB_4 was significantly lower in subjects with severe asthma ($p<0.01$; Table 2). We then utilized the data of Table 2 to generate the ROC curves and calculated the AUC values for each eicosanoid species. Figure 1 shows the ROC curves and the AUC values of the analyzed eicosanoids in differentiating asthma from healthy controls. Results showed a similar discriminating power for exhaled 15-HETE, FEV_1 and FeNO (Fig. 1).

Assessment of the relationship between the levels of exhaled eicosanoids, FEV_1, and FeNO with steroid responsiveness

To assess whether the levels of exhaled eicosanoids varied during exacerbation and after convalescence, the levels of the exhaled eicosanoids, FeNO and FEV_1 in asthmatic children (N=75; Supplementary Table S1) at acute exacerbation and convalescence stages were measured. As shown in Fig. 2, while the level of FeNO was not at variance between these two stages (Fig. 2A), there was a significant enhancement in the level of FEV_1 (Fig. 2B; $p<0.001$), and 15-HETE (Fig. 2C, $p<0.001$), and a significant reduction in the TXB_2 (Fig.

2D, $p<0.05$) level, while, as a group, the levels of LTB_4, LTE_4, LXA_4 and PGE_2 did not reveal significant difference (Supplemental Fig. S2) before and after prednisone treatment. Furthermore, when the respective levels of each eicosanoid species were stratified into those at the 30th percentile, significant changes were particularly noted for those with higher initial levels of both 15-HETE and TXB_2 at the exacerbation phase (Fig. 3); also, significant decreases for exhaled LTB_4, LTE_4, LXA_4, and PGE_2were noted (all with $p<0.001$; Supplemental Fig. S3) for those at the top 30 percentile.

Discussion

In a study population consisting of 318 children with asthma, lower levels of 15-HETE were found. ROC analysis of individual parameters demonstrated similar levels of the sensitivity and specificity between exhaled 15-HETE and two commonly used parameters in monitoring asthma, FEV_1 and FeNO[16]. Further, positive correlations were found between the levels of TXB_2 and those of LTB_4 and PGE_2 in asthmatic children. Also, among the asthmatic subjects, negative correlations were found for TXB_2 and FEV_1, and for 15-HETE and LTB_4. Among those parameters analyzed, reduced levels of TXB_2, but increased levels of 15-HETE, were noted after 3 days of oral prednisolone treatment, concomitant with the improvement of lung function in asthmatic children. When the asthmatic population was stratified into different severity groups, it was noted that the ratio of 15-HETE/LTB_4 was significantly lower in subjects with severe asthma. Furthermore, when we investigated changes in the levels of 15-HETE and TXB_2 during exacerbation and convalescence in subjects according to the top 30%, middle 40%, and bottom 30% (as determined at the exacerbation levels), it was found that higher initial exacerbation would have responded well to prednisone treatment. These results, collectively, suggest their potential utility as a new set of lipid markers for monitoring asthma and its therapeutic outcome.

The family of eicosanoids is the most prevalent lipid mediators providing both pro-inflammatory signals and terminating the inflammatory process. Eicosanoid profiling in the exhaled breath condensate is complementary to the cellular phenotyping of asthmatic inflammation[17]. Our findings revealed that the levels of 15-HETE were significantly reduced in the EBCs of asthmatic subjects as compared to that of healthy controls, but was increased after treatment. Kowal et al also reported that 15-HETE in asthma patients was significantly lower than in healthy subjects[15]. Song et al demonstrated that 15-HETE regulated MUC5AC expression via modulating MMP-9, MEK/ERK/Sp-1, and PPARγ/PTEN/ Akt signaling pathways in PMA-treated respiratory epithelial cells[18]. Also, high 12/15-LOX activity and 15-HETE levels have been suggested to be indicative of pro-inflammatory responses in asthma[19,20]. Besides the anti-inflammatory effects, 15-HETE has been shown to be an endogenous ligand for PPARγ (peroxisome proliferator-activated receptor gamma), which has anti-inflammatory effects such as regulating inflammatory cytokines[21,22], neutrophil migration and mucin secretion[18]. For instance, the PPARγ agonist rosiglitazone has been shown to display bronchodilator effects in a group of patients with glucocorticoids-resistant asthma[23]. The reduction of 15-HETE may, therefore, suggest its close relationship with asthma and warrant further investigation.

Moreover, as 15-HETE may exert their anti-inflammatory effect through inhibiting 5-LOX–derived pro-inflammatory leukotrienes[24], we also calculated the ratio of exhaled 15-HETE:LTB_4 and found significantly lower in subjects with severe asthma. The mean 15-HETE:LTB_4 ratio was 79% lower in patients with severe asthma when compared with that in patients with moderate asthma ($P<0.01$). These findings suggest that 15-HETE biosynthetic capacity might be defective in patients with severe asthma and thus contribute to the perpetuation of airway inflammation in these patients. Moreover, TXA_2 is a lipid mediator and a bronchoconstrictor contributing to the pathophysiology of asthma[7], while $TXB_2$is a stable metabolite of TXA_2. The reduction of TXB_2 levels might be indicative of steroid's effect and a marker responsive to the intervention.

While, consistent with a previous report[25], but not the others[13,26,27], we did not find difference in the level of exhaled TXB_2 (and its metabolite, 11-dihydro-TXB_2; data not shown) between asthmatic and healthy children, but the level of TXB_2 showed significant reduction after 3 days of oral prednisolone treatment. Further, Dworski et al. found that prednisone was able to reduce the synthesis of eicosanoids, including TXB_2 level, in macrophage-rich BAL-fluid cells from 14 atopic asthmatic volunteers at baseline and after

allergen instillation[28]. It is also worth noting that in double-blind, placebo-controlled trials, the thromboxane receptor antagonist, seratrodast, and the thromboxane synthase inhibitor, ozagrel, were proven efficacious in the treatment of patients with asthma[29]. However, the effect of TXA_2 inhibitors in asthma has not been widely used because no statistically significant difference was observed, but it has been suggested that it might be a good disease marker of asthma only in a certain ethnic group[30]. One explanation for these conflicting results could be phenotypically different in the study population. Nevertheless, while the level of TXB_2 may be dependent on the stage of asthma and its severity, the reduction in TXB_2 after therapy appears to be consistent. Further independent studies are needed to confirm these results. The finding that the level of exhaled TXB_2 was significantly reduced during convalescence is significant in and of itself, providing a basis for further exploring its clinical utility in monitoring the therapeutic outcome in place of FeNO.

Furthermore, it is worth noting that LTB_4, LTE_4, PGE_2 and LXA_4 also showed reduction in patients with the respective levels at the 30% percentile, and, in fact, only in those who had higher levels of exhaled eicosanoids. This could be related to the stages of asthma progression during exacerbation, and to the phenotypic heterogeneity of asthma in the study population in terms of its etiology and pathogenic mechanism. Further investigation into this possibility is clearly required. In conclusion, these results provided insight into the measurements of exhaled eicosanoid profiles, and showed that there was a significant difference between the levels of TXB_2 and 15-HETE during acute asthma exacerbation and convalescence. Additional prospective studies are necessary to evaluate the utility of the proposed discriminator diagnosis and monitoring of childhood asthma.

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Impact statement: The exhaled 15-HETE was found to discriminate childhood asthma while decreased levels of exhaled $TXB_2$and increased levels of 15-HETE were prominent after treatment.

Conflict of interest : The authors declare that they have no conflict of interests

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Table 1. Demographics of study subjects.

Parameter	Asthmatic(N=318)	Healthy(N=97)	P value
Age	8.58±0.16	8.93±0.2	P<0.05
Gender (boy/girl)	205/113	59/38	P<0.01
BMI category, N (%)			
Underweight	11 (3.5%)	7 (7.2%)	
Normal	203 (63.8%)	56 (57.7%)	
Overweight	52 (16.4%)	20 (20.6%)	
Obesity	52 (16.4%)	14 (14.4%)	
Asthma severity, N (%)			
Mild	227 (71.4%)	ND	
Moderate	62 (19.5%)	ND	
Severe	29 (9.1%)	ND	
Serum total IgE	561.1±39.5	68.2±5.1	P<0.001
FEV 1%	72.3±1.0	85.2±1.2	P<0.001
FeNO,ppb	23.5±1.2	10.3±0.7	P<0.001
PC_{20}	8.6±0.6	ND	
ECP(ng/L)	30.4±10.9	ND	

Table 2. Levels of 15-HETE and TXB_2 in subjects in the validation phase and asthmatic subjects stratified by severity.

	N	15-HETE[a] mean±S.E.	P value	15-HETE/LTB_4[a] ratio	P value	TXB_2 mean±S.E.	P value
Healthy	97	209.7 ± 22.93		144.63 ± 17.93		8.13 ± 1.92	
Asthma[b]	318	72.71 ± 7.67	<0.0001	35.17 ± 4.13	<0.01	8.16 ± 1.06	NS

	N	15-HETE[a] mean±S.E.	P value	15-HETE/LTB_4[a] ratio	P value	TXB_2 mean±S.E.	P value
Mild	227	76.19 ± 9.03	<0.0001	38.92 ± 5.04	<0.01	8.21 ± 1.25	NS
Moderate	62	71.02 ± 17.4	<0.0001	34.88 ± 7.06	<0.01	8.60 ± 2.4	NS
Severe	29	49.50 ± 25.44	<0.0001	6.92 ± 2.58	<0.01	6.81 ± 3.5	NS

[a] Asthma group or each of its severity groups versus healthy control group.

[b] Among the subgroups of asthma, there are no significant differences from each other.

NS, not significant.

Figure legends

Table 1. Demographics of study subjects.

Table 2. Levels of 15-HETE and TXB_2 in subjects in the validation phase and asthmatic subjects stratified by severity

Figure 1. ROC analysis of exhaled 15-HETE, TXB_2, FeNO and Δ%FEV1.

Figure 2. Changes in the levels of FeNO, $FEV_1$and eicosanoid species during exacerbation and convalescence. The levels of (A) FeNO, (B) FEV_1, (C) 15-HETE and (D) TXB_2 during acute exacerbation (AE) and after treatment (C, convalescence) in a total of 75 children with asthma. Each line represents each individual sample. Note: All p-values were adjusted for multiple testing by Holm methods.

Figure 3. Changes in the levels of TXB_2 and 15-HETE during exacerbation and convalescence in subjects according to the top 30%, middle 40%, and bottom 30% (as determined at the exacerbation levels). FA denotes the between-subjects main effect of stratified eicosanoid levels during exacerbation; FB denotes the within-subjects main effect of phasic change; FAXB denotes the interaction of FA and FB variables.

Supplemental Figure legends

Supplemental Table S1. Demographic comparison of acute asthmatics.

Supplemental Figure S1. Correlation analysis of TXB_2 with (A) FEV_1, (B) LTB_4, (C) PGE_2 and of (D) 15-HETE and LTB_4.

Supplemental Figure S2. Levels of (A) LTB_4, (B) LTE_4, (C) Lipoxin A_4 , and (D) PGE_2 in EBCs of asthmatic children at acute exacerbation and convalescence stages.

Supplemental Figure S3. Levels of LTB_4 (A), LTE_4 (B), Lipoxin A_4 (C), and PGE_2 (D) during acute exacerbation (AE) and two weeks after oral prednisolone treatment (C, convalescence) in asthmatic subjects with the respective eicosanoid levels at the 30th percentile at exacerbation. Each line represents each individual sample. Note: All p-values are adjusted for multiple testing by Holm methods.

Figure 1.

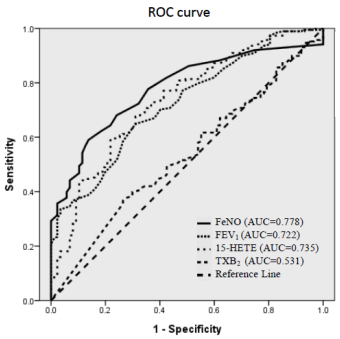

Figure 2.

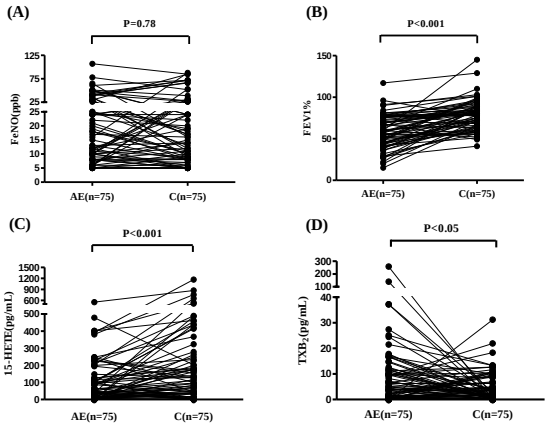

Figure 3.