

ORIGINAL PAPER

Involvement of 15-lipoxygenase and prostaglandin EP receptors in aspirin-triggered 15-hydroxyeicosatetraenoic acid generation in aspirin-sensitive asthmatics

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Summary

Background The mechanism of aspirin (acetylsalicylic acid: ASA) hypersensitivity in asthmatic patients is related to arachidonic acid metabolism abnormalities, and specific triggering by ASA of 15-hydroxyeicosatetraenoic acid (15-HETE) generation was observed in leucocytes from aspirin-sensitive (AS) but not from aspirin-tolerant (AT) asthmatics.

Objective The aim of this study was to identify the enzymatic pathway involved in ASA-induced 15-HETE generation in AS asthmatics and to assess the regulatory role of prostaglandin EP receptors.

Methods Peripheral blood leucocytes (PBLs) were isolated from AS ($n = 18$) and AT ($n = 20$) asthmatics and challenged with ASA, with and without pre-incubation with caffeic acid (CA) [15-lipoxygenase (15-LO) inhibitor] or prostaglandin receptor non-specific (misoprostol, sulprostone) and specific EP1–4 receptors agonists. Eicosanoids were measured in supernatants using specific immunoassays.

Results Aspirin triggered 15-HETE generation in PBLs of AS asthmatics (mean increase 292%) but not in AT asthmatics and inhibited prostaglandin₂ (PGE₂) generation in both groups of patients to the same degree. Leucocytes from AS patients produced less PGE₂, both before and after ASA incubation. Pre-incubation of PBLs with CA decreased basal 15-HETE production in all patients and completely inhibited ASA-induced 15-HETE generation in AS asthmatics. CA did not change basal PGE₂ production but enhanced induced by ASA inhibition of PGE₂. Non-specific agonists of EP receptors (misoprostol and sulprostone) did not affect basal 15-HETE production but inhibited in a dose-dependent manner the ASA-induced increase of 15-HETE generation in AS asthmatics. On the contrary, in AT asthmatics, pre-incubation of PBLs with misoprostol or sulprostone resulted in a significant increase in 15-HETE generation after addition of ASA (200 μ M). EP1–3 receptor agonists inhibited (range 72–94%) the ASA-induced 15-HETE production significantly.

Conclusion Our study demonstrated that ASA-triggered 15-HETE generation involves the activation of 15-LO and is modulated by prostaglandin EP1–3 receptors. The relevance of these observations to the mechanism of *in vivo* ASA-induced asthmatic attack remains to be established.

Keywords aspirin-induced asthma, ASPITest, 15-HETE, lipoxygenase, prostaglandin E receptors

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Introduction

Ingestion of aspirin (acetylsalicylic acid: ASA) may evoke respiratory symptoms (severe bronchospasm, nasal congestion and rhinorrhoea) in 5–20% of asthmatic patients

and these patients present a typical syndrome, i.e. an 'ASA triad' involving intolerance to ASA and other non-steroidal anti-inflammatory drugs (NSAIDs), bronchial asthma and chronic hyperplastic rhinosinusitis with nasal polyps [1, 2]. ASA-induced asthma attack is not an

immunological phenomenon but is related to the pharmacological activity of ASA and other NSAIDs, namely to prostaglandin biosynthesis inhibition [3]. According to the prostaglandin (or cyclooxygenase) hypothesis, inhibition of cyclooxygenase-1 by NSAIDs, followed by suppression of prostaglandin E₂ (PGE₂) synthesis triggers the activation of inflammatory cells and the subsequent release of inflammatory mediators (e.g. histamine, tryptase, PGD₂, ECP) and increased generation of cysteinyl leukotrienes [4–6]. Decreased spontaneous generation of prostaglandin E was found in nasal polyp epithelial cells, bronchial fibroblasts and (less consistently) in peripheral blood leucocytes (PBLs) from aspirin-sensitive (AS) as compared with aspirin-tolerant (AT) asthmatics [7–11]. Furthermore, the pre-treatment of sensitive patients with PGE₂ or its synthetic analogue misoprostol alleviated the clinical response to ASA provocation, indicating the pivotal role of PGE₂ in the pathomechanism of ASA hypersensitivity [12–14].

We have reported previously that nasal polyp epithelial cells and PBLs from AS asthmatics but not AT patients may be triggered *in vitro* with ASA to generate increased amounts of 15-hydroxyeicosatetraenoic acid (15-HETE), providing evidence of another abnormality in arachidonic acid metabolism in these patients [10, 11]. Aspirin-triggered 15-HETE release *in vitro* seems to mimic, to a large extent, clinical reaction to ASA observed *in vivo*: firstly, it is specific for leucocytes from AS asthmatics; secondly, the release of 15-HETE – similar to clinical symptoms – could be triggered by other non-selective cyclooxygenase inhibitors and thirdly, celecoxib (COX-2 selective NSAID), which is well tolerated by sensitive patients, did not affect the 15-HETE *in vitro* release. Accordingly, the measurement of ASA-triggered 15-HETE release in leucocytes (ASPISTest) has been proposed as a specific *in vitro* test to detect ASA sensitivity [15]. However, the contribution of 15-HETE generation to the mechanism of ASA hypersensitivity is far from being understood, e.g. the enzymatic source of ASA-triggered 15-HETE generation has not been determined and the potential modulatory role of prostaglandin E receptors has not been investigated.

In this study, the involvement of the 15-lipoxygenase (15-LO) pathway in ASA-triggered 15-HETE generation was assessed and the modulator role of prostaglandin receptors was investigated by assessing the effects of non-specific (misoprostol, sulprostone) as well as specific prostaglandin EP1–4 receptor agonists on ASA-induced 15-HETE production.

Methods

Patients

The study included 18 AS patients and 20 AT asthmatics recruited from the Medical University Asthma Clinic

Table 1. Characteristics of aspirin-sensitive (AS) and aspirin-tolerant (AT) asthmatics

	AS asthmatics	AT asthmatics	<i>P</i>
No. of patients (<i>n</i>)	18	20	
Sex			
Male	4	4	NS
Female	14	16	
Age			
Mean	49	45	NS
Range	22–67	18–73	
Atopy	12	14	NS
Patients with nasal polyps	15	3	< 0.05
FEV ₁ % (mean)	83	80	NS
Inhaled GCS – daily dose (µg)			
Mean ± SEM	1194 ± 136	906 ± 133	< 0.05
Range	0–2400	0–2400	
Oral GCS (no. of patients)	4	2	NS
Blood eosinophilia (%) (mean)	4.40	3.35	NS

(Table 1). The diagnosis of asthma was made based on a medical history, a clinical examination and a pulmonary function test. Aspirin hypersensitivity was diagnosed based on history and confirmed by a positive result of an inhaled [16], intranasal [17] or oral [18] ASA challenge tests. The atopic status was defined by the presence of a positive skin prick response to at least one of 14 inhaled allergens. Both groups of patients were comparable with respect to sex distribution, atopic status, mean age, respiratory function (FEV₁) and blood eosinophilia. Nasal polyposis was more prevalent in AS asthmatics and the mean doses of inhaled corticosteroids was higher in AS than in AT asthmatics. All patients were in a stable clinical condition and had not received inhaled corticosteroids for 12 h before venipuncture. None of them had obtained anti-leukotriene drugs, antihistaminics and NSAIDs for at least 2 weeks before inclusion. The study was approved by the Medical University Ethics Committee.

Leucocytes preparation and experimental protocol

Venous blood samples were taken from patients and collected into a syringe containing 3.8% sodium citrate. The PBLs were separated by centrifugation (2500/min, Eppendorf, Hamburg, Germany) on Gradisol G (AquaMedica, Lodz, Poland) and washed twice with phosphate-buffered saline (PBS) to minimize platelets contamination. Finally, the cells were counted and resuspended in PBS in separate tubes. The experiments involved incubation of cells (3×10^6 cells/mL) with 200 µM of lysine ASA (Bayer, Wuppertal, Germany) for 60 min after 60 min of stabilization. Before stimulation with ASA, cells were pre-incubated for 60 min with a diluent or with prostaglandin receptor agonists: misoprostol (Cayman Chemical, Ann Arbor, USA), sulprostone (Sigma-Aldrich, St Louis, MO,

USA) or ONO-DI-004 (EP1), ONO-AE1-259-01 (EP2), ONO-AE-248 (EP3), ONO-AE1-329 (EP4) (ONO Pharmaceuticals Company, Osaka, Japan) in increased concentrations (1, 10, 100 and 1000 nM). Caffeic acid (CA) (lipoxygenase inhibitor; Sigma-Aldrich) was used for pre-incubation in the following concentrations: 2, 20, 200 and 2000 nM. After incubation, the cells were centrifuged and supernatants were collected into tubes and finally stored at -70°C .

Eicosanoid measurement

Eicosanoid release was measured by competitive ELISA immunoassay kits: 15-HETE purchased from Assay Designs Inc., Ann Arbor, USA and PGE_2 from Cayman Chemical. The procedure was performed following the original manuals and each measurement was performed in duplicates. The results were expressed in picograms per 1 mL of cell suspension containing three million cells.

Statistical analysis

The results were expressed as means $\pm$ SEM, and $P < 0.05$ was considered to be statistically significant. Analyses of differences within the group were performed using Wilcoxon's matched pairs test. The Mann-Whitney U test was used to compare data between two groups of patients and the Kruskal-Wallis test was used for a few unrelated probes. The W. Kendall test was used to assess the dose-dependent effects.

Results

Effect of aspirin on 15-hydroxyeicosatetraenoic acid and prostaglandin E_2 generation by peripheral blood leucocytes

PBLs from AS and AT asthmatic patients generated similar amounts of 15-HETE (1875.8 ± 394.2 and 1146.7 ± 258.2 pg/mL, respectively; NS, $P = 0.137$). In AS asthmatics, ASA in concentrations of 2 and 20 μM did not influence 15-HETE generation significantly (the mean increase was 8% and 40%, respectively; NS) but 200 μM ASA increased 15-HETE from mean 1875.8 ± 394.2 to 6993.5 ± 1530.6 pg/mL ($P < 0.002$) after 60 min (the mean increase was 292%, range from 28% to 932%) (Fig. 1a). In contrast, in AT patients, ASA at 2 and 20 μM did not influence 15-HETE generation and for 200 μM , there was even a tendency to decrease 15-HETE generation (mean 1146.7 ± 258.2 and 821.5 ± 175.8 pg/mL before and after ASA incubation, respectively; NS).

The mean baseline generation of PGE_2 was significantly lower in AS patients (144.9 ± 22.7 pg/mL) than in AT patients (261.1 ± 47.8 pg/mL) ($P = 0.029$) and ASA reduced PGE_2 generation to a similar degree in both groups: in AS patients by 29% (to 96.6 ± 16.1 pg/mL; $P = 0.017$)

and in AT patients ($n = 7$) by 32% (to 170.0 ± 31.2 pg/mL; $P = 0.028$); the mean concentration of PGE_2 after ASA was still lower in AS as compared with AT asthmatics ($P = 0.049$) (Fig. 1b).

There was no significant correlation between PGE_2 and 15-HETE generation by PBLs either before or after ASA in AS and AT asthmatics. The ASA-induced increase in 15-HETE generation did not correlate with a degree of PGE_2 inhibition induced by ASA in AS patients.

Influence of caffeic acid on 15-hydroxyeicosatetraenoic acid generation

In order to identify the enzymatic source of 15-HETE generation, leucocytes were incubated with increasing doses of CA, a specific 15-LO inhibitor. In AS patients, CA in all concentrations decreased baseline 15-HETE levels (by 31%, 33%, 53% and 55% for 2, 20, 200 and 2000 nM concentrations, respectively) (Fig. 2a). Pre-incubation of leucocytes from AS patients with CA inhibited ASA-induced 15-HETE generation in a dose-dependent fashion ($P < 0.00$); the mean decrease was -63% , -82% , -97% and -119% for consecutive CA concentrations. In AT patients, 200 nM concentration of CA significantly

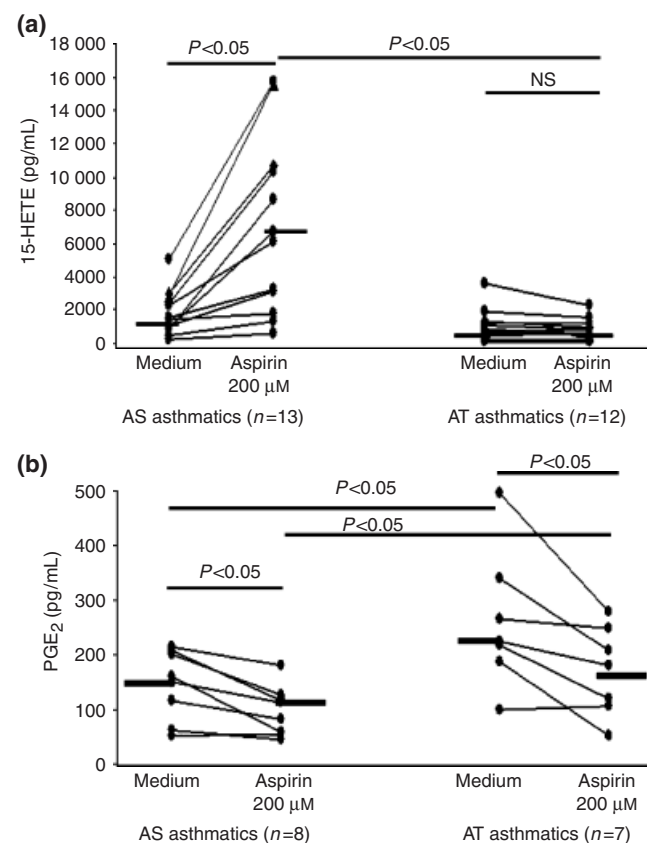


Fig. 1. Effect of aspirin (200 μM) on 15-hydroxyeicosatetraenoic acid (a) and prostaglandin E_2 production (b) in peripheral blood leucocytes from aspirin-sensitive (AS) and aspirin-tolerant (AT) asthmatics.

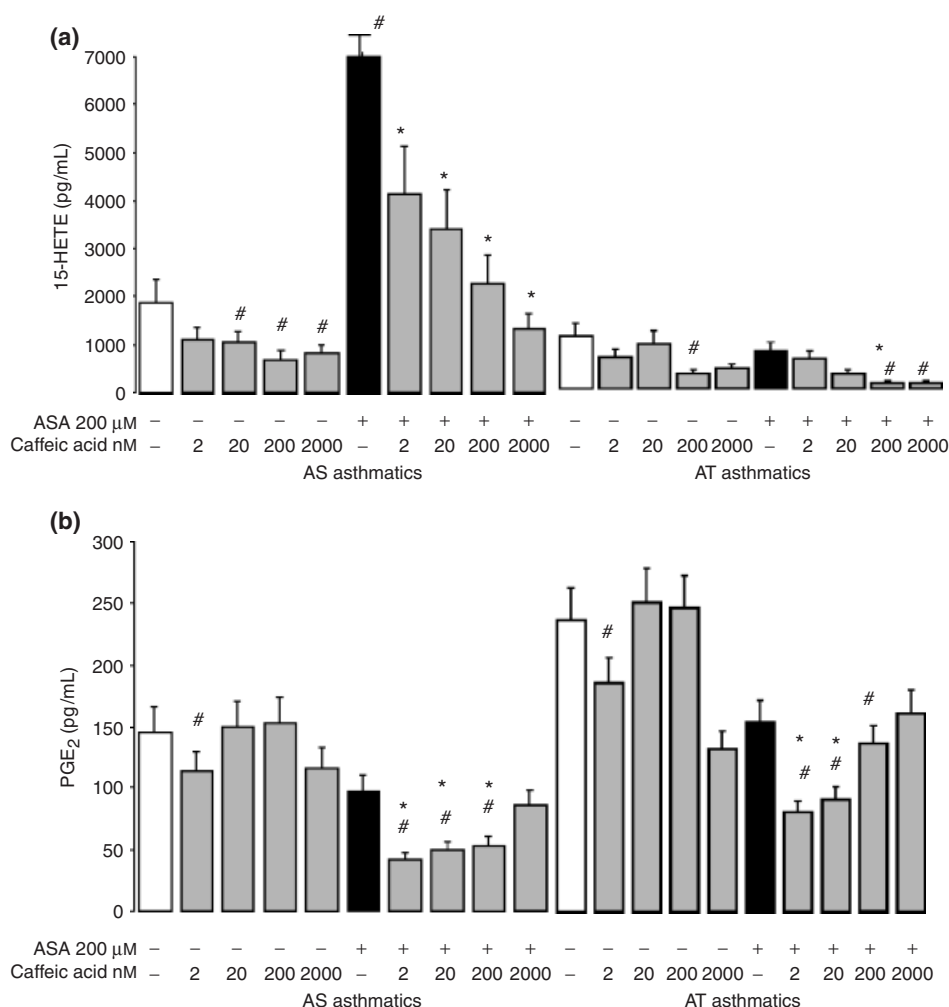


Fig. 2. Effect of caffeic acid (CA) on 15-hydroxyeicosatetraenoic acid (15-HETE) (a) and prostaglandin E₂ (PGE₂) generation (b) after aspirin (ASA) in peripheral blood leucocytes from aspirin-sensitive (AS) asthmatics ($n=10$) and aspirin-tolerant (AT) asthmatics ($n=8$). Cells were incubated with medium, ASA and CA, alone or pre-incubated for 60 min with four different concentrations of CA, followed by 200 μ M of ASA. * $P<0.05$ significantly different from cells stimulated with ASA alone; # $P<0.05$ significantly different from cells incubated with medium alone.

decreased baseline 15-HETE generation by a mean of 67%; however, when 200 μ M of ASA was added to leucocytes pre-incubated with 200 and 2000 nM of CA, almost complete depression of 15-HETE generation was observed.

Incubation of leucocytes with CA alone did not change PGE₂ generation in AS or AT patients, indicating the specificity of its inhibitory activity for 15-LO (Fig. 2b). However, pre-incubation of leucocytes with 2–200 nM of CA significantly enhanced induced by ASA decrease in PGE₂ production in both AS and AT asthmatics.

Influence of non-specific prostaglandin EP receptor agonists (misoprostol and sulproston) on 15-hydroxyeicosatetraenoic acid generation

Three concentrations of misoprostol (10, 100 and 1000 nM) did not change 15-HETE generation as compared with the baseline in AT or AS asthmatics. However, the lowest

concentration of misoprostol (1 nM) significantly decreased baseline 15-HETE levels in both groups (the mean decrease was 42% and 30% for AT and AS patients, respectively) (Fig. 3a). Pre-incubation of leucocytes from AS patients with misoprostol decreased ASA-induced 15-HETE production in a dose-dependent fashion, with maximal inhibition observed for the lowest concentration of misoprostol (the mean inhibition was 105%, 104%, 85% and 61% for 1, 10, 100 and 1000 nM of misoprostol, respectively). In AT patients, ASA alone did not influence 15-HETE production but pre-incubation with misoprostol before addition of ASA resulted in an increase in 15-HETE (mean increases 158%, 135% and 208% for 10, 100 and 1000 nM, respectively), and the increase for 1000 nM of misoprostol was significant ($P=0.03$). The absolute amounts of 15-HETE generated by PBLs after pre-incubation with misoprostol and ASA stimulation were not different between AS and AT patients.

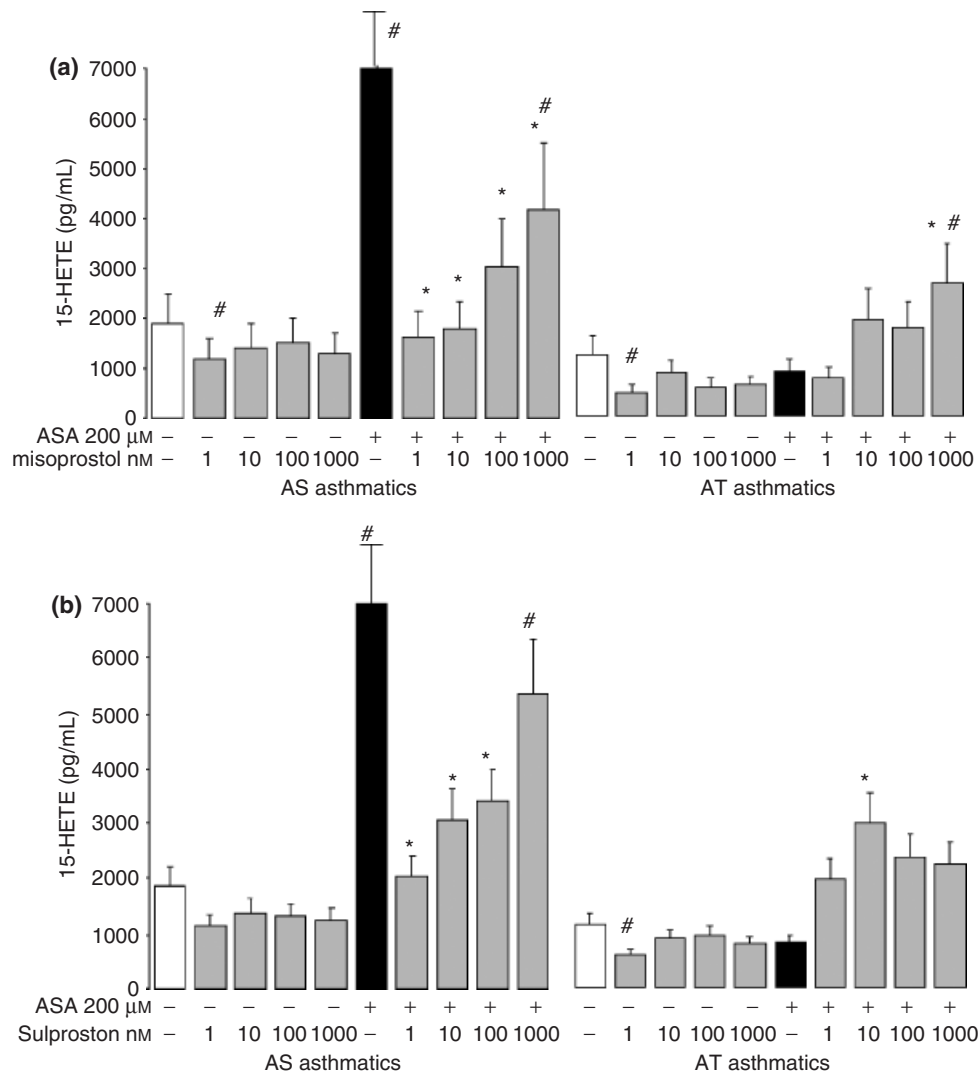


Fig. 3. Effect of misoprostol (a) and sulprostone (b) on 15-hydroxyeicosatetraenoic acid (15-HETE) generation after aspirin (ASA) in peripheral blood leucocytes from aspirin-sensitive (AS) asthmatics ($n=9$) and aspirin-tolerant (AT) asthmatics ($n=7$). Cells were incubated with medium, ASA and misoprostol/sulprostone alone or pre-incubated with four different concentrations of misoprostol/sulprostone, followed by 200 μ M of ASA. * $P<0.05$ significantly different from cells stimulated with ASA alone; # $P<0.05$ significantly different from cells incubated with medium alone.

The effects of sulprostone (EP1/EP3 receptor agonist) on 15-HETE generation in PBLs from AS and AT asthmatics were similar to those observed after misoprostol. In AS patients, sulprostone inhibited ASA-induced 15-HETE production and in AT subjects, pre-incubation with sulprostone resulted in the stimulation of 15-HETE generation after addition of ASA (Fig. 3b).

Influence of specific prostaglandin EP receptor agonists on aspirin-induced 15-hydroxyeicosatetraenoic acid generation

None of the specific prostaglandin EP1–4 receptors' agonists tested in concentrations 1–1000 nM influenced baseline 15-HETE generation in AS patients (Fig. 4). In this series of experiments ($n=4-6$), ASA (200 μ M) increased

15-HETE generation from baseline 4922 ± 1997 to 8069 ± 3060 pg/mL (mean 75% increase) and from baseline 5670 ± 2013 to 8759 ± 3115 pg/mL (mean 69% increase).

Pre-incubation of PBLs with an EP1 receptor-specific agonist (ONO-DI-004) significantly decreased ASA-induced 15-HETE generation to 5639 ± 3034.5 pg/mL ($P<0.05$) and 5626 ± 2813 pg/mL ($P<0.05$) for 1 and 100 nM concentrations, respectively (the mean inhibition was 92% and 94%). The EP2 receptor-specific agonist (ONO-AE1-259-01) decreased ASA-induced 15-HETE generation from 8069 ± 3060 to 5518 ± 2265 pg/mL ($P<0.05$), 4884 ± 1793 pg/mL ($P<0.05$) and 5321 ± 2578 pg/mL ($P<0.05$) for 1, 100 and 1000 nM, respectively. As compared with ASA-induced 15-HETE generation, the mean inhibitions were 72%, 66% and 73% for 1, 100 and 1000 nM, respectively. Pre-incubation of PBLs

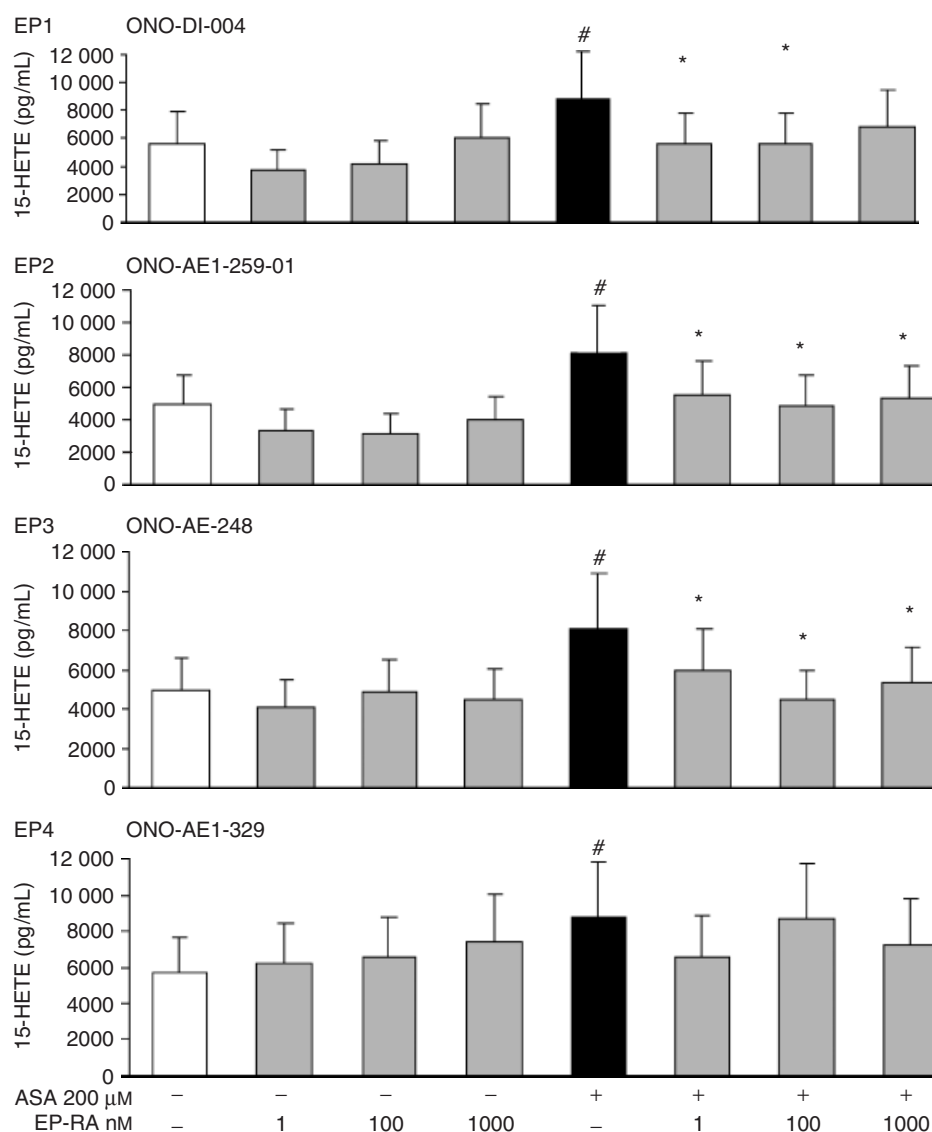


Fig. 4. Effect of peripheral blood leucocytes pre-incubation with specific EP1, EP2, EP3 and EP4 receptor agonists on aspirin (ASA)-triggered 15-hydroxyeicosatetraenoic acid (15-HETE) generation in aspirin-sensitive (AS) asthmatics ($n = 5$). * $P < 0.05$ significantly different from ASA-induced generation; # $P < 0.05$ significantly different from medium.

from AS patients with specific EP3 receptor agonist (ONO-AE-248) inhibited almost completely ASA-induced increase in 15-HETE generation from 8069 ± 3060 to 5966 ± 2198 pg/mL, 4405 ± 1956 and 5302.3 ± 2632 pg/mL for 1, 100 and 1000 nM ($P < 0.05$) (71%, 87% and 75% inhibition, respectively). Following pre-incubation with EP4 receptor-specific agonist ONO-AE1-329, only non-significant decreases in ASA-induced 15-HETE production were observed at 1, 100 and 1000 nM concentrations of the agonist (58%, 34% and 51% inhibition, respectively).

Discussion

In this study, we confirmed earlier observations that inhibition of cyclooxygenase by ASA is associated with

increased generation of 15-HETE by PBLs from AS asthmatics. Specific triggering by ASA of 15-HETE generation was also observed previously in nasal polyp epithelial cells [10] and in nasal polyp explants [19] from AS patients, suggesting a generalized rather than a local defect in arachidonic acid metabolism. Although arachidonic acid can be metabolized to 15-HETE on different enzymatic pathways – 15-LO, cyclooxygenase-1, cyclooxygenase-2 or even by cytochrome P-450 – the enzymatic source of ASA-induced 15-HETE release in sensitive asthmatics is not known [20, 21]. The enzymatic pathway involved in 15-HETE production can be identified based on different stereoisomers of the product: 15-LO metabolizes AA to 15(S)-HETE with configuration (S) at C15, as opposed to 15(R)-HETE produced by ASA-treated COX-2.

Cytochrome P-450 may be the source of both S and R isoforms. Based on the observation that the antibody used to detect 15-HETE in the ELISA assay was highly specific for 15(S)-HETE, we suggested that 15-LOX could be the source of an ASA-triggered metabolite. In this study, we could demonstrate that CA, a relatively specific inhibitor of 15-LOXb, significantly decreased baseline 15-HETE generation and suppressed ASA-triggered 15-HETE production in PBLs from AS asthmatics in a dose-dependent fashion pointing to 15-LOXb as the source of 15-HETE. In low concentrations, CA is considered to be a quite specific 15-LOX inhibitor ($K_i = 0.8 \mu\text{M}$), although other enzymes 5-LOX, 12-LOX and COX may also be inhibited with its higher concentrations [22]. In our study, CA did not affect PGE₂ generation by PBLs in either AS or AT patients, confirming the specificity of the compound for 15-LO inhibition. However, CA produced a significant enhancement of ASA-induced inhibition of PGE₂, indicating its synergistic effect with ASA on the cyclooxygenase pathway.

The mechanism of ASA-induced bronchoconstriction has been related to cyclooxygenase-1 inhibition and a special modulatory role for PGE₂ in this reaction was suggested [2]. Accordingly, depressed generation of PGE₂ in nasal polyp [23], nasal polyps epithelial cells [11] and bronchial fibroblasts from AS patients [24] were reported, indicating PGE₂ deficiency at the level of target organs. However, deficient production of PGE₂ in PBLs from AS asthmatics was reported only in some studies [8, 25] while others could not find any differences between AS and AT leucocytes [9, 10, 24, 26]. In this study, we found significantly lower production of PGE₂ in PBLs from AS patients; however, ASA inhibited PGE₂ production to similar degrees in both groups, suggesting that cyclooxygenase was equally sensitive to ASA in AS and AT asthmatics.

Further evidence for the important role of PGE₂ in the mechanisms of ASA sensitivity arises from clinical studies demonstrating that inhaled PGE₂ and synthetic PGE₂ stable analogues were capable of protecting against ASA-induced bronchoconstriction [12, 13]. Similarly, oral pre-treatment with misoprostol, a synthetic PGE₂ analogue, prevented or alleviated symptoms precipitated by oral ASA in AS patients [14]. The mechanism of protection by PGE₂ of an ASA-triggered asthmatic reaction is not known but seemed to be independent of the bronchodilator response to PGE₂, suggesting a potential effect on the activation of inflammatory cells. In one study, LTC₄ generation triggered by ASA in PBLs from AS patients was inhibited by pre-treatment with prostaglandin E [8]. We decided to use ASA-triggered 15-HETE release as an *in vitro* model of ASA hypersensitivity to study the involvement of prostaglandin EP receptors in the modulation of this reaction. PGE₂ acts via four G-protein coupled receptors (named EP1–4) that differ in tissue distribution,

ligand-binding affinity, coupling to intracellular signalling pathways and function [27, 28]. In this study, two non-selective EP receptor agonists, misoprostol (specific for EP2/EP3) and sulproston (specific for EP1/EP3), inhibited both basal and ASA-triggered 15-HETE generations to a similar degree. Using the range of misoprostol and sulproston concentrations compatible with other studies in which the drug demonstrated biological activity, we showed that the effects of both compounds were dose-dependent, with the strongest inhibition observed with the lowest concentration (1 nM). Such a 'reversed' dose dependency, although unexpected, has been observed previously by Babakhin et al. [29], who showed stronger inhibitory effects of smaller than higher concentrations of misoprostol on histamine release by basophils. However, we cannot exclude that the inverse effect seen at higher concentrations may represent a second superimposed activity of the agonist that appears at higher concentrations. When EP agonists were added together with ASA, in AT patients, they increased the generation of 15-HETE.

All four EP1–4 receptors are expressed in the lower and upper airways and have been implicated in the modulation of smooth muscle tone and allergic inflammation in asthmatic and rhinosinusitis patients; however, it is not known which receptors modulate an ASA-induced asthmatic reaction *in vivo* [30, 31]. More recently, Ying et al. [32] demonstrated that the expression of PGE₂ receptors is significantly reduced on nasal mucosal inflammatory cells from AS patients, suggesting that abnormality of this receptor expression and/or signalling could potentate the relative deficiency of PGE₂, facilitating activation of inflammatory cells in these patients. In order to further characterize receptors involved in the modulation of 15-HETE production, PBLs were incubated with specific EP1–4 receptor agonists [33]. Although none of the specific EP receptor agonists changed basal 15-HETE production, ASA-induced 15-HETE generation was almost completely suppressed by prostaglandin EP2- and EP3-specific receptor agonists in all concentrations tested and in two lower doses by the EP1 receptor agonist, suggesting a major role for EP1–3 receptors in the regulation of 15-HETE production and possibly in the pathogenesis of ASA sensitivity. Similar concerted actions of different EP receptors have been described in other systems, e.g. in human keratinocytes, EP2 and EP3 receptor agonists acted in concert to suppress TNF- α -induced CCL27 production [34].

In conclusion, our study demonstrated that ASA-triggered 15-HETE generation involves activation of the 15-LO pathway of AA metabolism and is modulated by prostaglandin EP1–3 receptors, although the relevance of this information to the mechanism of an *in vivo* ASA-induced asthmatic attack remains to be established.

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