

Urinary 3-bromotyrosine and 3-chlorotyrosine concentrations in asthmatic patients: lack of increase in 3-bromotyrosine concentration in urine and plasma proteins in aspirin-induced asthma after intravenous aspirin challenge

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Summary

Background Eosinophil peroxidase and myeloperoxidase halogenate tyrosine residues in plasma proteins and generate 3-bromotyrosine (BY) and 3-chlorotyrosine (CY), respectively.

Objectives (1) To estimate urinary concentrations of BY and CY in asthmatic patients. (2) To investigate BY concentration in relation to urinary leukotriene E4 (LTE4) concentration in order to evaluate the activation of eosinophils in patients with aspirin-induced asthma (AIA).

Methods BY and CY were quantified with a gas chromatograph-mass spectrometer using ^{13}C -labelled compounds as internal standards.

Results (1) Activation of eosinophils and neutrophils by immobilized IgG1 induced preferential formation of BY and CY, respectively. (2) A significantly higher concentration of BY was observed in the urine from asthmatic patients than in that from healthy control subjects (45 ± 21.7 vs. 22.6 ± 10.8 ng/mg-creatinine, $P < 0.01$). CY concentration was also elevated in the urine from asthmatic patients (4.4 ± 3.2 vs. 1.5 ± 1.0 ng/mg-creatinine, $P < 0.01$). (3) After intravenous aspirin challenge of aspirin-induced asthmatic patients, the concentration of BY in urine did not significantly change. No significant change was also observed in the ratio of BY concentration to total tyrosine concentration in plasma proteins. In contrast, the concentration of urinary LTE4 significantly increased after the intravenous aspirin challenge.

Conclusion Determination of BY and CY concentrations may be useful for monitoring the activation of eosinophils and neutrophils in asthmatic patients, respectively. After aspirin challenge of AIA patients, the increased concentration of urinary LTE4 did not accompany changes in BY concentration in both urine and plasma proteins. These results may preclude the activation of eosinophils after aspirin challenge in patients with AIA.

Keywords aspirin-induced asthma, 3-bromotyrosine, 3-chlorotyrosine

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Introduction

Eosinophils play a role in the pathogenesis of various inflammatory diseases including asthma. Eosinophils possess a wide range of biological properties that may participate in the pathogenesis of a disease by releasing proteins, eicosanoids, platelet-activating factor and cytokines. Eosinophil peroxidase (EPO) resides in a matrix of cytoplasmic granules and is one of the most abundant proteins of eosinophils. Eosinophils activated by various stimuli generate large amounts of hydrogen peroxide (H_2O_2) and secrete EPO. Halogenated oxidants formed by the EPO– H_2O_2 –halide system are potent cytotoxic oxidants possessing microbicidal and viricidal activities and also contribute to inflammatory injury in host tissues. Although chloride is found *in vivo* at concentrations at least 1000-fold higher than those of other

halide ions, human EPO preferentially oxidizes bromide ions under physiological conditions, and the major product of EPO is hypobromous acid (HOBr) [1]. HOBr is too reactive to measure directly in biological fluids. However, HOBr easily reacts with primary amines to form bromamines, and the intermediates convert tyrosine into 3-bromotyrosine (BY) as a stable end product [2–5]. The presence of BY is considered as a chemical marker of eosinophil activation. On the other hand, myeloperoxidase (MPO), which is stored in neutrophils, monocytes and macrophages, converts H_2O_2 into hypochlorous acid (HOCl) at plasma chloride concentrations, and the reaction of tyrosine with HOCl yields 3-chlorotyrosine (CY) [3, 6, 7]. Thus, detection of CY may provide a means for studying oxidative damage promoted by MPO. BY concentration in proteins in bronchoalveolar lavage fluid (BALF) significantly increased after segmental allergen challenge of asthmatic patients [3]. A significant increase in BY, CY and 3-nitrotyrosine concentrations was also observed in BALF from severely asthmatic patients [8]. In addition, a

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recent study has shown that BY concentration in total protein was significantly elevated in sputa collected from stable asthmatic patients [9]. In addition, EPO and MPO also use nitrite to generate 3-nitrotyrosine [10–13].

Urine has been found to be a useful biological fluid in monitoring the endogenous release of chemical mediators such as arachidonic acid metabolites and histamine by measuring their stable urinary metabolites [14]. In addition to the ease in collecting urine, measurements of metabolites in urine are not confounded by problems involving artificial *ex vivo* formation of metabolites during sampling, which could be a major problem when arachidonic acid metabolites and histamine in plasma were measured. On the other hand, the major drawback of using urine is that a reliable marker of endogenous production of the mediator needs to be identified and urine analysis cannot provide any information on the cellular origin of mediators. It has been reported that leukotriene E4 (LTE4) concentration in the urine of patients with aspirin-induced asthma (AIA) is elevated even in a clinically stable condition and is further elevated after aspirin challenge [15–20]. Although the cellular source of leukotriene C4 (LTC4) release has not been identified, aspirin-induced reactions seem to involve activation of mast cells and/or eosinophils. However, previous studies do not provide information regarding which cells are the target of aspirin to initiate the adverse reaction. If LTC4 is released from eosinophils, it is possible to detect an increase in the concentration of urinary BY.

In this study, we evaluated whether the concentrations of BY and CY increase in urine from asthmatic patients. We also analysed the concentration of BY in relation to urinary LTE4 concentration to assess the contribution of eosinophils to the pathogenesis of AIA.

Materials and methods

Patients

The study group consisted of 12 patients with AIA, 12 patients with aspirin-tolerant asthma (ATA) and 18 healthy control subjects. All patients were in a clinically stable condition. All medications were stopped for at least 12 h prior to the challenge test. Permission to conduct the study was obtained from the National Sagami Hospital Ethics Committee and all the subjects gave their informed consent. Urine was collected in the morning (9:00–11:00 hours).

Aspirin challenge test

The 12 AIA patients received intravenous aspirin challenge. These patients were divided into two groups. The challenge test was performed at about 9:00 hours to noon as previously reported [20]. Briefly, urine or blood samples were collected at the beginning of the study. After intravenous injection of 1 mL of saline, if FEV_{1.0} did not change by more than 10% from the prechallenge baseline, doubled doses of lysine aspirin (12.5, 25, 50, 100 and 200-mg equivalent of aspirin) were intravenously administered. FEV_{1.0} was recorded every 10 min after the administration and the time interval between administrations of increasing doses was 30 min. The challenge test was stopped when a positive reaction occurred, which

was defined as a decrease in FEV_{1.0} by 20% or more from the baseline. ATA patients did not show a changed respiratory function even after receiving the highest dose of aspirin. Urine samples were collected for the measurement of LTE4, BY and CY during the following periods: 0–3, 3–6, 6–9 and 9–24 h after the onset of bronchoconstriction. Urine samples were collected in polypropylene tubes containing 4-hydroxy-TEMPO and were stored at –35 °C until analysis. The second subgroup of six AIA patients also underwent aspirin challenge, and blood samples were collected 1 and 3 h after the aspirin challenge.

Preparation of internal standards

¹³C₆-labelled BY was prepared by reacting ¹³C₆-tyrosine (L-tyrosine-ring ¹³C₆, ¹³C-99%, Cambridge Isotope Laboratories, Inc., Andover, MA, USA) with bromine water in 50 mM phosphate buffer (pH 7.3). ¹³C₆-BY was isolated by high-performance liquid chromatography (HPLC) using a NOVA-PAK C18 column (Waters, Milford, MA, USA) with a solvent mixture of 10 mM ammonium formate containing 5% methanol (adjusted to pH 3.2 with formic acid). Similarly, ¹³C₆-CY was prepared by reacting ¹³C₆-tyrosine with NaOCl in 1 N HCl. The retention times of BY and CY were about 6 and 5 min under the HPLC conditions used, respectively.

Analysis of BY and tyrosine in biological fluids

Urine: ¹³C₆-BY (50 ng) and ¹³C₆-CY (30 ng) were added to 1 mL of urine as internal standards and the urine was passed through a Bond Elut C18 column (Varian, Harbor City, CA, USA) to remove polar compounds. After addition of 1 mL of 0.2% trifluoroacetic acid, the solution was loaded on an LC18 column (Supelco, Bellefonte, PA, USA) and tyrosine and its derivatives were eluted with 1.6 mL of 25% methanol from the column. Heptafluorobutyl derivatives were prepared according to the method of Frost et al. [21]. After derivatization, the reaction mixture was concentrated under a nitrogen stream, to which 0.2 mL of 0.1 N HCl and 0.6 mL of ethyl acetate were added. Heptafluorobutyl derivatives were extracted into the organic phase and then converted into the corresponding tert-butyldimethylsilyl derivatives [21, 22]. The concentrations of BY and CY were determined using Shimadzu GC-MS QP2010 (Kyoto, Japan) equipped with an SPD-5 capillary column (15 m, 0.25 mm internal diameter, 0.25-µm film thickness, Supelco, Bellefonte, PA, USA) in the negative ion chemical ionization mode with methane as the reagent gas. The ion source and interface temperatures were set at 250 °C. The initial column temperature was maintained at 50 °C for 2 min and then increased to 250 °C at 15 °C/min. BY and CY concentrations were determined by measuring the fragment ions at *m/z* 489.10 for endogenous compounds and *m/z* 495.15 for the internal standards.

Plasma protein Proteins were precipitated from 0.02 mL of plasma using a mixture of water:methanol:water-washed diethyl ether (1:3:7, v/v/v) [23]. After addition of ¹³C₆-tyrosine (25 µg) and ¹³C₆-BY (50 ng) as internal standards, proteins were hydrolysed in 0.5 mL of 4 N methanesulfonic acid containing 1% phenol at 110 °C for 24 h. Tyrosine and BY in the hydrolysate were quantified as described earlier. An aliquot of the eluate from the LC18 column (0.05 mL) was

separated for quantitation of tyrosine. Tyrosine was quantified using the fragment ions at m/z 407.15 and m/z 413.15, which is the corresponding fragment ion derived from $^{13}\text{C}_6$ -tyrosine. The ratio of BY concentration to total tyrosine concentration in plasma proteins has been calculated and used as a measure of bromination of plasma proteins.

Activation of eosinophils and neutrophils by immobilized human IgG1

Eosinophils and neutrophils were isolated from peripheral blood as reported previously [24]. The procedure yielded >99% pure eosinophils. On the other hand, the neutrophil preparation contained a small percentage of eosinophils in four of five cases (0.3–5.2%). Eosinophils and neutrophils were suspended in RPMI-1640 containing fetal calf serum (5%), penicillin, streptomycin and $100\ \mu\text{M}$ NaBr at densities of $2.5 \times 10^6/\text{mL}$ and $7.5 \times 10^6/\text{mL}$, respectively. The suspension (0.2 mL) was placed in a tube (MaxiSoap, Nalge Nunc Int. Rochester, NY, USA), which had been coated with human IgG1 λ (Sigma, St Louis, MO, USA), and cultured in humidified 95% air and 5% CO_2 at 37°C overnight. As a control, the cells were cultured in tubes coated with human albumin instead of human IgG1 λ . After proteins in the supernatant were hydrolysed, tyrosine and its derivatives were quantified as described above.

Quantitation of LTE4 and EDN in urine

LTE4 was quantified by enzyme immunoassay after purification by HPLC as reported previously [25]. EDN was measured using an EDN enzyme immunoassay kit (MBL, Nagoya, Japan) after diluting the urine from most of our patients 50 times with a dilution buffer. The concentrations were expressed as ng per mg of creatinine.

Statistical analyses

Data were expressed as the mean $\pm$ SD unless otherwise specified. A non-parametric statistical test was performed using Friedman's test for time-course experiments. When the test indicated significant differences, the Tukey-type test was used to compare the data. The Mann–Whitney U -test was used to examine differences between asthmatic patients and healthy controls. Spearman's rank correlation test was used to evaluate correlated data. Differences were considered significant for P -values less than 0.05.

Results

Quantification of tyrosine and its derivatives

When a calibration graph was constructed by plotting the peak area against the concentration, a linear standard graph was obtained in the concentration range of 1.2–36 ng/mL. The peak area ratio of m/z 489.10 and m/z 495.15 was almost the same as the mixing molar ratio of BY and $^{13}\text{C}_6$ -BY.

To confirm the formation of BY and CY during sample preparation, urine to which $^{13}\text{C}_6$ -tyrosine and NaBr ($100\ \mu\text{M}$) were added in the absence of internal standards was analysed. $^{13}\text{C}_6$ -BY and $^{13}\text{C}_6$ -CY were not generated, suggesting that BY and CY are not formed from tyrosine during sample preparation.

Effect of activation of eosinophils and neutrophils by immobilized human IgG1 on BY and CY formation

Immobilized IgG stimulates degranulation and superoxide production by eosinophils and neutrophils through an Fc γ receptor II (CD32)-mediated mechanism [26–28]. Thus, we examined BY and CY formation by eosinophils and neutrophils after stimulation with immobilized IgG1. As shown in Fig. 1, activation of eosinophils through Fc γ

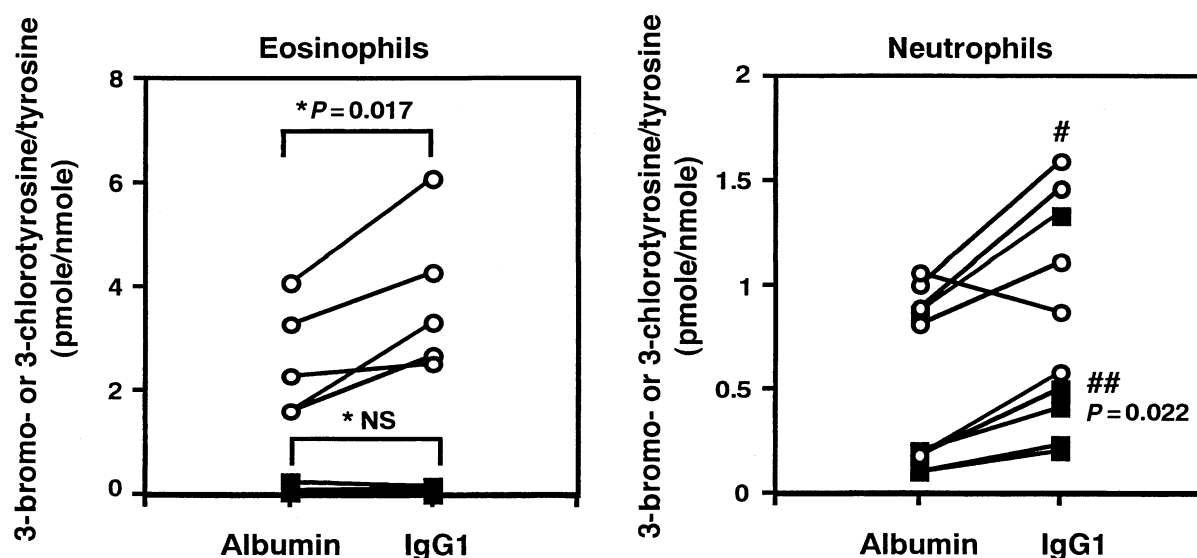


Fig. 1. Generation of 3-bromotyrosine (BY) and 3-chlorotyrosine (CY) by eosinophils (left) and neutrophils (right) through Fc γ receptor activation. Open circle and closed square indicate BY and CY, respectively. *Generation of BY, but not CY, was significantly increased by immobilized IgG ($P = 0.017$). #No statistical significance was found in BY generation by neutrophils. ##CY generation by neutrophils in an IgG1-coated tube was significantly higher than that in an albumin-coated tube ($P = 0.022$).

receptors resulted in a significant increase in the concentration of BY (2.57 ± 1.07 vs. 3.78 ± 1.47 pmol/nmol tyrosine, $P = 0.017$ by a paired two-tailed Student's t test). No significant increase in CY concentration was observed in the supernatant proteins. In contrast, a significant increase in CY concentration was observed in the supernatant proteins when neutrophils were stimulated with immobilized IgG1 (0.29 ± 0.32 vs. 0.53 ± 0.46 pmol/nmol tyrosine, $P = 0.022$). BY concentration also increased in the proteins in four of five patients, albeit to a lesser extent. In one of these patients, whose cell suspensions were not contaminated with eosinophils, no increase in BY concentration was observed. We could not determine whether contaminating eosinophils in a small percentage were responsible for BY generation in neutrophils or whether neutrophils can generate low concentrations of HOBr. Nevertheless, the difference failed to reach statistical significance ($P = 0.08$). These results indicate that BY and CY were preferentially produced by activated eosinophils and neutrophils, respectively.

Characteristics of patients participating in the study

The characteristics of the patients are summarized in Table 1. There were no significant differences in terms of age, gender, duration of asthma, severity and dose of inhaled steroids between AIA and ATA patients. Although IgE concentration was not significantly different between these groups, most of the ATA patients were atopic.

Baseline concentrations of BY, CY and LTE4 in urine

The concentrations of BY in urine from asthmatic patients and control subjects are shown in Fig. 2. Asthmatic patients showed a significantly higher BY concentration than control subjects (45 ± 21.7 vs. 22.6 ± 10.8 , $P < 0.01$). A significant difference was also observed between the two groups even

when data from six AIA patients were excluded from analysis. A significant correlation between BY and EDN concentrations in urine could not be observed in patients with asthma. Neither EDN concentration nor BY concentration showed a significant correlation with the number of peripheral blood eosinophils.

The concentration of CY was about 10-fold lower than that of BY, which was below the sensitivity of the assay, in two patients and one healthy subject. Urinary CY concentration was also significantly different between asthmatic patients and control subjects (4.4 ± 3.2 vs. 1.5 ± 1.0 ng/mg-creatinine, $P < 0.01$). There was no correlation between CY and BY concentrations in urine of patients with asthma.

Urinary LTE4 concentration was significantly higher in asthmatic patients (median, 107 pg/mg-creatinine; range, 26–5816 pg/mg-creatinine) than in control subjects (median, 50.9 pg/mg-creatinine; range, 18.8–77.6 pg/mg-creatinine). However, when the data of six AIA patients were excluded, this difference was not statistically significant, suggesting that the higher concentration of LTE4 in asthmatic patients is not related to their asthmatic status, but rather to the presence of aspirin sensitivity. When examined in AIA patients showing increased urinary LTE4 concentration (> 200 pg/mg-creatinine), a significant correlation could not be observed between the concentrations of urinary BY and LTE4.

No significant difference was observed in the concentration of urinary EDN between AIA patients (1588 ± 1464 ng/mg-creatinine) and ATA patients (865 ± 864 ng/mg-creatinine), although the possibility that the number of AIA patients may have been too small to detect the difference cannot be ruled out.

Changes in urinary BY and LTE4 concentrations after intravenous aspirin challenge

After the aspirin challenge of the first subgroup of six AIA patients, urinary BY concentration did not change significantly in 24 h (Fig. 3). There were considerable interindividual differences in the extent and time course of the increase in urinary LTE4 concentration in the AIA patients. However, a significant increase has been observed in urinary LTE4 concentration during the 3–6 h period in these patients after the onset of bronchoconstriction ($P < 0.01$). The urinary LTE4 concentration increased from 210 pg/mg-creatinine (median; range, 75–5819 pg/mg-creatinine) as the baseline concentration to 6485 pg/mg-creatinine (median; range, 408–33 600 pg/mg-creatinine) in the 3–6 h period, that is, the concentration increased by 12-fold (median; range, 3.4- to 448-fold) of the baseline concentration. There were also no significant changes in CY concentration in AIA patients after the onset of bronchoconstriction (data not shown). Urinary LTE4 and BY concentrations did not increase significantly in ATA patients after aspirin challenge (Fig. 4).

The second subgroup of six AIA patients also received aspirin challenge. The ratio of BY concentration to total tyrosine concentration in plasma proteins did not show any significant change at 1 h and at 3 h after aspirin challenge (Fig. 5). On the other hand, urinary LTE4 concentration increased significantly from the baseline concentration (median, 182 pg/mg-creatinine; range, 31.5–979 pg/mg-creatinine) at the 0–3 h period (median, 957 pg/mg-creatinine; range, 476–8486 pg/mg-creatinine, $P < 0.05$). Overall, we

Table 1. Clinical characteristics of patients

	AIA ($n = 12$)	ATA ($n = 12$)	P -value
Age (years)	53.7 ± 13	53.8 ± 16.7	NS
Male/female	4/8	7/5	NS
Atopy/non-atopy	4/8	10/2	$P < 0.05$
Duration of asthma (years)	18.7 ± 13.4	19.1 ± 17.6	NS
Severity			NS
Mild persistent asthma	0	2	
Moderate persistent asthma	4	4	
Severe persistent asthma	7	4	
FEV _{1.0} (% predicted)	73.8 ± 13.1	86.0 ± 27.6	NS
Blood eosinophils (/mm ³)	759 ± 636	625 ± 365	NS
Serum IgE (IU/mL)*	110 (18–2490)	300 (19–20 200)	NS
Inhaled steroid dose (mg/day)*	1600 (0–1600)	800 (400–2400)	NS
Patients (n) receiving			
prednisolone	1	1	
Dose (mg/day)	4	5	
Patients (n) receiving leukotriene			
receptor antagonist	6	2	
Patients (n) receiving salmeterol	0	2	
Dose (μ g/day)		100	

AIA, aspirin-induced asthma; ATA, aspirin-tolerant asthma; FEV_{1.0}, forced expiratory volume in 1 s; NS, non-significant.

*Median (range).

failed to obtain evidence for enhanced formation of BY in urine and plasma proteins after aspirin challenge of AIA patients. BY generation did not occur in AIA patients who demonstrated increased urinary LTE4 concentration, which indicates that cells other than eosinophils may be responsible for LTC4 production in AIA patients after aspirin challenge.

Discussion

BY and CY concentrations in bronchial lavage fluid [3, 8], sputa [9] and tracheal aspirate [29] were measured, and the results clarified that patients with bronchial asthma or respiratory diseases have high concentrations of BY and

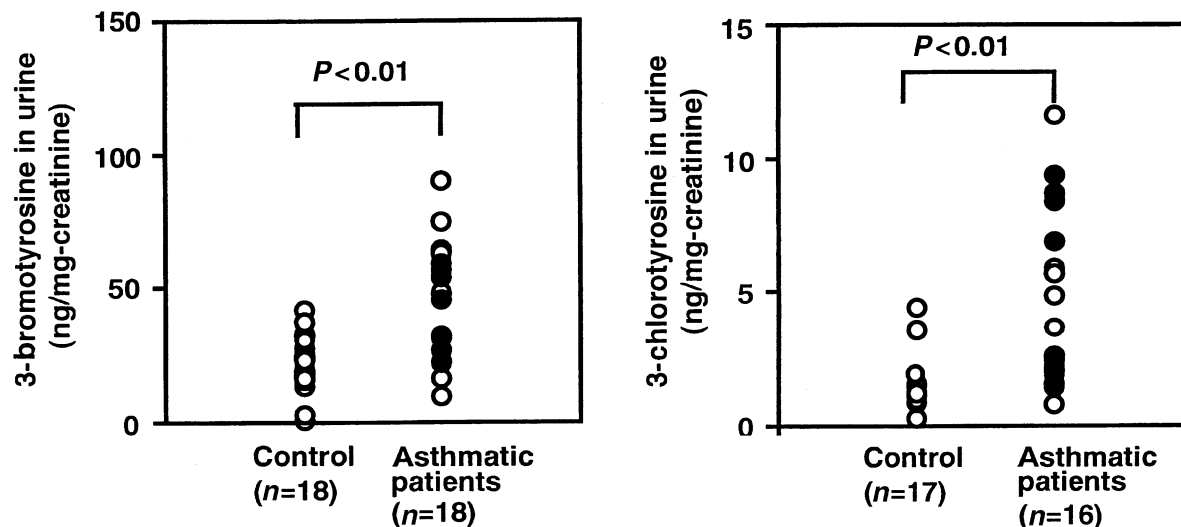


Fig. 2. Concentrations of urinary 3-bromotyrosine (left) and urinary 3-chlorotyrosine (right) in patients with asthma and healthy subjects. Closed symbols indicate the patients with aspirin-induced asthma.

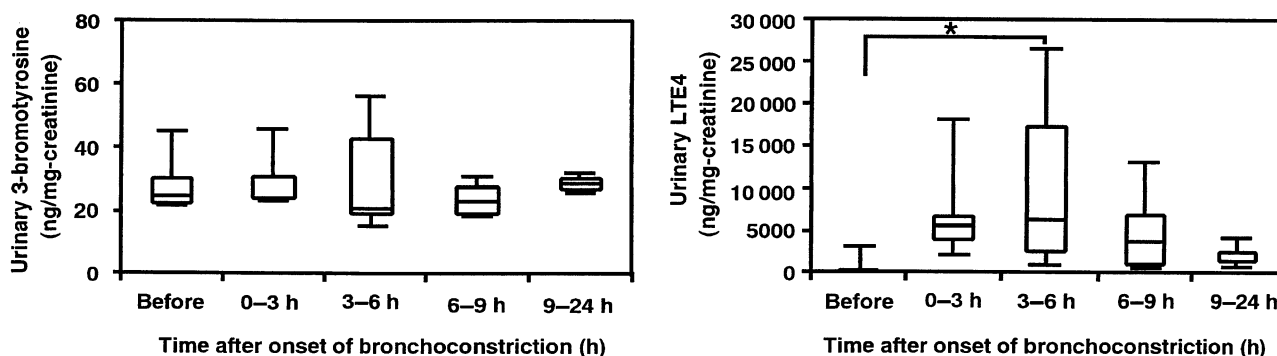


Fig. 3. Changes in urinary 3-bromotyrosine (left) and leukotriene E4 (LTE4) (right) concentrations in patients with aspirin-induced asthma after intravenous aspirin challenge. Data are presented as box plots displaying medians and interquartile ranges. In the box plots, the lower boundary indicates the 25th percentile. The line within the box indicates the 50th percentile (median) and the upper boundary of the box indicates the 75th percentile. Error bars above and below the box indicate the 90th and 10th percentiles, respectively. The results were analysed using Friedman's test with a *post hoc* test. *Significantly different from the baseline concentration (P < 0.01).

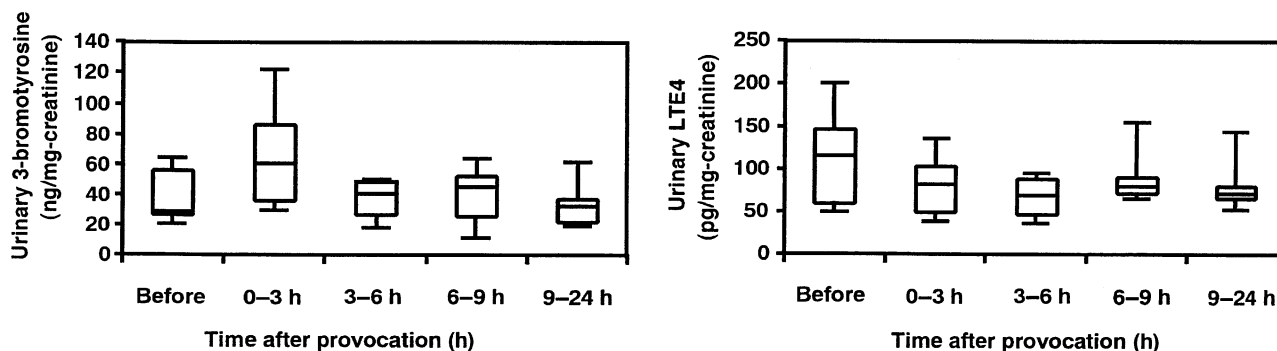


Fig. 4. Changes in urinary 3-bromotyrosine (left) and leukotriene E4 (LTE4) (right) concentrations in patients with aspirin-tolerant asthma after intravenous aspirin challenge.

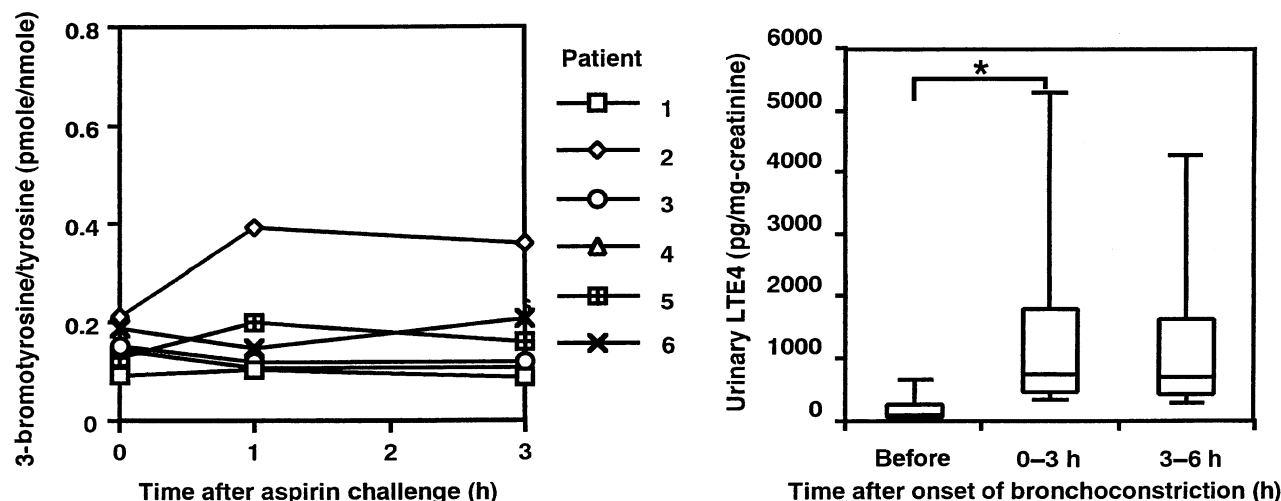


Fig. 5. Changes in the ratio of 3-bromotyrosine concentration to total tyrosine concentration in plasma proteins (left) and urinary leukotriene E4 (LTE4) concentration (right) after aspirin challenge in patients with aspirin-induced asthma. * $P < 0.05$ compared with the baseline concentration.

CY. To investigate the clinical significance of BY concentration as a marker of eosinophil activation, we first measured urinary BY concentration in asthmatic patients and compared it with that in healthy control subjects. To date, there have been no studies regarding the measurement of urinary BY or CY concentration. Urinary BY concentration in the asthmatic group was significantly higher than that in the control group (Fig. 2). Since urine samples were used in this experiment, it is impossible to identify the cellular origin of BY. In addition, there has been no information about urinary metabolite of BY. However, our result provides compelling evidence that a large amount of HOBr is produced in asthmatic patients, thereby, tissue damage and oxidative injury associated with eosinophil activation may occur. On the other hand, CY is considered to be a marker of the activation of cells including MPO. According to previous reports, CY concentration in bronchial lavage fluid increased following an allergen challenge test [3], and a high concentration of CY was also observed in bronchial lavage fluid obtained from severely asthmatic patients [8]. In contrast, no significant difference in the concentration of CY in sputa between asthmatic patients and controls was observed [9]. In our current study, urinary CY concentration in the asthmatic group was significantly higher than that in the control group (Fig. 2). This result may suggest the involvement of neutrophils and monocytes/macrophages in the pathogenesis of bronchial asthma. There have been many reports on the involvement of neutrophils in bronchial asthma. Neutrophil influx and activation and a high concentration of MPO were observed in the sputa of asthmatic patients [30–34]. Neutrophils obtained from asthmatic patients showed an increased release of MPO when stimulated with a bacterial peptide, and the concentration of MPO released was associated with lung function [35, 36]. In addition, neutrophils from asthmatic patients have been shown to generate a larger amount of superoxide anions than those from control subjects [37]. These results support the idea that neutrophils are involved in airway injury in asthma. However, it is unclear to what extent the activation of neutrophils affects the deterioration of the pulmonary function of asthmatic patients.

A significant correlation between BY concentration and EPO concentration in sputa was observed [9]; however, in the current study, no significant correlation was observed between urinary BY concentration and urinary EDN concentration, which is another marker of eosinophil degranulation. EDN is released from eosinophil granules together with eosinophil cationic proteins and EPO. The molecular weight of EDN is 18–19 kDa, which means that EDN is excreted in the urine more easily than EPO, which has a molecular weight of 66 kDa. Namely, EDN easily permeates through the glomerulus. Therefore, EDN is used as an eosinophil degranulation marker in urine [38]. Concentrations of mediators released at local sites in airways are easily detected in sputa. This is why BY concentration correlates with EPO concentration. Our data showed that the concentration of BY might be regulated in a manner different from that of EDN in urine. EDN is released from eosinophils not only by their degranulation but also by their degradation. However, BY is generated by the interaction between hydrogen peroxide and EPO; therefore, the activation of eosinophils is definitely necessary. Such a difference in the production mechanism may account for the low correlation between EDN concentration and BY concentration in urine. Alternatively, BY and EDN are eliminated from the systemic circulation and enter the urine at various rates.

The overproduction of cysteinyl-LT is considered to be an indication of AIA. However, cells that produce a large amount of LTC4 have not yet been clarified. 5-Lipoxygenase, the enzyme required for the initiation of LT production, is expressed predominantly by cells of myeloid origin. In contrast, enzymes involved in the second step of LT biosynthesis, LTC4 synthase and LTA4 hydrolase, are more broadly expressed in various cell types. LTC4 is considered to be released mostly from mast cells, basophils and eosinophils, as well as from monocytes/macrophages at a lesser concentration. In our previous study, the concentration of 9 α , 11 β prostaglandin F2 (9 α , 11 β -PGF2), which is a metabolite of prostaglandin D2 (PGD2), increased with increasing LTE4 concentration, and methylhistamine concentration slightly increased in some of the AIA patients [20]. These results

suggest the activation of mast cells following the administration of aspirin [39]. PGD2 was reported to be produced not only by mast cells but also by macrophages/monocytes [14], T cells [40] and fibroblasts [41]. In addition, recently, a divergent pathway from isoprostane, which is produced non-enzymatically, to PGD2 via epimerization has been discovered [42]. PGD2 and its metabolites have been used as markers of mast cell activation [43]. Recently, an increase in blood tryptase concentration following the aspirin challenge in AIA patients has been reported [44]. This result also strongly suggests the activation of mast cells. On the other hand, eosinophils are known to produce a large amount of cysteinyl-LT, and an increased number of infiltrating eosinophils in the lungs of AIA patients was also reported [45]. Therefore, we cannot exclude the possibility of the involvement of eosinophils in the reaction following aspirin administration in AIA patients. How eosinophils participate in the induction of AIA remains to be elucidated. If indeed eosinophils participate in aspirin-induced bronchoconstriction and cysteinyl-LT is, at least in part, released from eosinophils, it is possible to detect an increase in BY concentration in biological fluids. We, therefore, measured BY concentration before and after aspirin administration in the first AIA subgroup and the results were compared with those observed in ATA patients after aspirin administration. After aspirin was administered to AIA patients, urinary LTE4 concentration increased by 3- to 448-fold of the baseline concentration; in contrast, urinary BY concentration did not show any significant changes (Fig. 3).

In our laboratory, aspirin was intravenously administered to subjects in order to test their aspirin sensitivity. We then investigated whether aspirin activates eosinophils in peripheral blood and whether tyrosine residues in plasma proteins are brominated in the second subgroup of AIA patients. As shown in Fig. 5, even at the 1-h time-point after aspirin challenge, the ratio of brominated tyrosine concentration to total tyrosine concentration in plasma proteins did not show any significant changes compared with the ratio obtained before the test. Namely, although AIA patients have demonstrated an increased concentration of LTE4 in urine, this increase has not been associated with a change in the concentrations of both urinary free BY and brominated tyrosine in plasma proteins. The ratio of brominated tyrosine residue concentration to total tyrosine concentration was 0.152 ± 0.04 pmol/nmol in plasma proteins from the patients with AIA. This ratio is similar to that in BALF that was collected from mildly asthmatic patients [3].

We may have to consider the possibility that urinary BY concentration does not show changes that are sufficiently large to reflect the activation of eosinophils. However, in our preliminary study, the urinary BY concentration in patients with Churg Strauss syndrome in the active phase was 197.1 ng/mg-creatinine (median), and the LTE4 concentration was 506.4 pg/mg-creatinine (median). Thus, both the LTE4 and BY concentrations were higher than those in healthy controls. As described above, when aspirin was administered to AIA patients, LTE4 was excreted in the urine at a concentration 10-fold that in urine from patients with Churg Strauss syndrome; nevertheless, BY concentration in the AIA patients did not show any significant increase. Based on these results, it is unlikely that changes in the concentration of

urinary BY were not sufficiently large to detect the activation of eosinophils.

Collectively, our results demonstrated that (1) there were significantly high urinary BY and CY concentrations in patients with bronchial asthma, and (2) although urinary LTE4 concentration in AIA patients after administration of aspirin significantly increased, increases in BY concentrations in urine and plasma proteins were not confirmed. These results suggest that eosinophils are not directly activated by aspirin administration in AIA patients. Further studies need to focus on identifying the specific mechanism underlying the increased generation of cysteinyl-LT in patients with AIA.

References

- 1 Weiss SJ, Test ST, Eckmann CM, Roos D, Regiani S. Brominating oxidants generated by human eosinophils. *Science* 1986; 234:200–3.
- 2 Heinecke JW. Eosinophil-dependent bromination in the pathogenesis of asthma. *J Clin Invest* 2000; 105:1331–2.
- 3 Wu W, Samoszuk MK, Comhair SAA et al. Eosinophils generate brominating oxidants in allergen-induced asthma. *J Clin Invest* 2000; 105:1455–63.
- 4 Wu W, Chen Y, d'Avignon A, Hazen SL. 3-Bromotyrosine and 3, 5-dibromotyrosine are major products of protein oxidation by eosinophil peroxidase: potential markers for eosinophil-dependent tissue injury *in vivo*. *Biochemistry* 1999; 38:3538–48.
- 5 Moqbel E, Bagu J, Musat-Marcu S et al. Nuclear magnetic resonance: an effective tool for the analysis and quantification of *in vivo* mediator release from human eosinophils and neutrophils in induced sputum. *J Allergy Clin Immunol* 2001; 107:S143.
- 6 Hazen SL, Heinecke JW. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. *J Clin Invest* 1997; 99:2075–81.
- 7 Hazen SL, Hsu FF, Mueller DM, Crowley JR, Heinecke JW. Human neutrophils employ chloride gas as an oxidant during phagocytosis. *J Clin Invest* 1996; 98:1283–9.
- 8 MacPherson JC, Comhair SAA, Erzurum SC et al. Eosinophils are a major source of nitric oxide-derived oxidants in severe asthma: characterization of pathways available to eosinophils for generating reactive nitrogen species. *J Immunol* 2001; 166:5763–72.
- 9 Aldridge RE, Chan T, van Dalen CJ et al. Eosinophil peroxidase produces hypobromous acid in the airways of stable asthmatics. *Free Radic Biol Med* 2002; 33:847–56.
- 10 Wu W, Chen Y, Hazen SL. Eosinophil peroxidase nitrates protein tyrosyl residues: implication for oxidative damage by nitrating intermediates in eosinophilic inflammation disorders. *J Biol Chem* 1999; 274:25933–44.
- 11 Kaminsky DA, Mitchell J, Carroll N, James A, Soultanakis R, Janssen Y. Nitrotyrosine formation in the airways and lung parenchyma of patients with asthma. *J Allergy Clin Immunol* 1999; 104:747–54.
- 12 Duguet A, Iijima H, Eum SY, Hamid Q, Eidelman DH. Eosinophil peroxidase mediates protein nitration in allergic airway inflammation in mice. *Am J Respir Crit Care Med* 2001; 164:1119–26.
- 13 Brennan ML, Wu W, Fu X et al. A tale of two controversies: i) defining the role of peroxidases in nitrotyrosine formation *in vivo* using eosinophil peroxidase and myeloperoxidase deficient mice; and ii) defining the nature of peroxidase-generated reactive nitrogen species. *J Biol Chem* 2002; 277:17415–27.
- 14 Dworski R, Sheller JR. Urinary mediators and asthma. *Clin Exp Allergy* 1998; 28:1309–12.
- 15 Christie PE, Tagari P, Ford-Hutchinson AW et al. Urinary leukotriene E4 concentrations increase after aspirin challenge

- in aspirin-sensitive asthmatic subjects. *Am Rev Respir Dis* 1991; 143:1025–9.
- 16 Christie PE, Tagari P, Ford-Hutchinson AW et al. Urinary leukotriene E4 after lysine–aspirin inhalation in asthmatic subjects. *Am Rev Respir Dis* 1992; 146:1531–4.
 - 17 Kumlin M, Dahlén B, Björck T, Zetterström O, Granström E, Dahlén SE. Urinary excretion of leukotriene E4 and 11-dehydrothromboxane B2 in response to bronchial provocations with allergen, aspirin, leukotriene D4 and histamine in asthmatics. *Am Rev Respir Dis* 1992; 146:96–103.
 - 18 Knapp HR, Sladek K, Fitzgerald GA. Increased excretion of leukotriene E4 during aspirin-induced asthma. *J Lab Clin Med* 1992; 119:48–51.
 - 19 Oosaki R, Mizushima Y, Mita H, Shida T, Akiyama K, Kobayashi M. Urinary leukotriene E4 and 11-dehydrothromboxane B2 in patients with aspirin-sensitive asthma. *Allergy* 1997; 52:470–3.
 - 20 Mita H, Endoh S, Kudoh M et al. Possible involvement of mast cell activation in aspirin provocation of aspirin-induced asthma. *Allergy* 2001; 56:1061–7.
 - 21 Frost MT, Halliwall B, Moore KP. Analysis of free and protein-bound nitrotyrosine in human plasma by a gas chromatography/mass spectrometry method that avoids nitration artifacts. *Biochem J* 2000; 345:453–8.
 - 22 Gaut JP, Byun J, Tran HD, Heinecke JW. Artifact-free quantification of free 3-chlorotyrosine, bromotyrosine, and 3-nitrotyrosine in human plasma by electron capture-negative chemical ionization gas chromatography-mass spectrometry and liquid chromatography-electrospray ionization tandem mass spectrometry. *Anal Biochem* 2002; 300:252–9.
 - 23 Hazen SL, Hsu FF, Gaut JP, Crowley JR, Heinecke JW. Modification of proteins and lipids by myeloperoxidase. *Methods Enzymol* 1999; 300:88–104.
 - 24 Mita H, Hasegawa M, Saito H, Akiyama K. Levels of cysteinyl leukotriene receptor mRNA in human peripheral leukocytes: significantly higher expression of cysteinyl leukotriene receptor 2 mRNA in eosinophils. *Clin Exp Allergy* 2001; 31:1714–23.
 - 25 Mita H, Oosaki R, Mizushima Y, Kobayashi M, Akiyama K. Efficient method for the quantitation of urinary leukotriene E4: extraction using an Empore C18 disk cartridge. *J Chromatogr B* 1997; 692:461–6.
 - 26 Kaneko M, Horie S, Kato M, Gleich GJ, Kita H. A crucial role for β_2 integrin in the activation of eosinophils stimulated by IgG. *J Immunol* 1995; 155:2631–41.
 - 27 Kaneko M, Swanson MC, Gleich GJ, Kita H. Allergen-specific IgG1 and IgG3 through Fc γ R2 induce eosinophil degranulation. *J Clin Invest* 1995; 95:2813–21.
 - 28 Motegi Y, Kita H. Interaction with secretory component stimulates effector functions of human eosinophils but not of neutrophils. *J Immunol* 1998; 161:4340–6.
 - 29 Buss IH, Senthilmohan R, Darlow BA, Mogridge N, Kettle AJ, Winterbourn CC. 3-Chlorotyrosine as a marker of protein damage by myeloperoxidase in tracheal aspirates from preterm infants: association with adverse respiratory outcome. *Pediatr Res* 2003; 53:455–62.
 - 30 Gibson PG, Simpson JL, Saltos N. Heterogeneity of airway inflammation in persistent asthma: evidence of neutrophilic inflammation and increased sputum interleukin 8. *Chest* 2001; 119:1299–300.
 - 31 Barbato A, Panizzolo C, Gheno M et al. Bronchoalveolar lavage in asthmatic children: evidence of neutrophil activation in mild-to-moderate persistent asthma. *Pediatr Allergy Immunol* 2001; 12:73–7.
 - 32 Norzila MZ, Fakes K, Henry RL, Simpson J, Gibson PG. Interleukin 8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma. *Am J Rep Crit Care Med* 2000; 161:769–74.
 - 33 Louis R, Lau LC, Bron AO, Roldaan AC, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. *Am J Respir Crit Care Med* 2000; 161:9–16.
 - 34 Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 1999; 160:1532–9.
 - 35 Little SA, MacLeod KJ, Chalmers GW, Love JG, McSharry C, Thomson NC. Association of forced expiratory volume with disease duration and sputum neutrophils in chronic asthma. *Am J Med* 2002; 112:446–52.
 - 36 Monteseirin J, Bonilla I, Camacho J, Condo J, Sobrino F. Elevated secretion of myeloperoxidase by neutrophils from asthmatic patients: the effect of immunotherapy. *J Allergy Clin Immunol* 2001; 107:623–6.
 - 37 Teramoto S, Shu CY, Ouchi Y, Fukuchi Y. Increased spontaneous production and generation of superoxide anion by blood neutrophils in patients with asthma. *J Asthma* 1996; 33:149–55.
 - 38 Cottin V, Deviller P, Tardy F, Cordier JF. Urinary eosinophil-derived neurotoxin/protein X: a simple method for assessing eosinophil degranulation *in vivo*. *J Allergy Clin Immunol* 1998; 101:116–23.
 - 39 Fisher AR, Rosenberg MA, Lilly CM et al. Direct evidence for a role of mast cells in the nasal response to aspirin in aspirin-sensitive asthma. *J Allergy Clin Immunol* 1994; 94:1046–56.
 - 40 Tanaka K, Ogawa K, Sugamura K, Nakamura M, Takano S, Nagata K. Differential production of prostaglandin D2 by human helper T cell subsets. *J Immunol* 2000; 164:2277–80.
 - 41 Pierzchalska M, Szabó Z, Sanak M, Soja J, Szczeklik A. Deficient prostaglandin E2 production by bronchial fibroblasts in asthmatic patients, with special reference to aspirin-induced asthma. *J Allergy Clin Immunol* 2003; 111:1041–8.
 - 42 Gao L, Zackert WE, Hasford JJ et al. Formation of prostaglandin E2 and prostaglandin D2 via the isoprostane pathway: a mechanism for the generation of bioactive prostaglandins independent of the cyclooxygenase. *J Biol Chem* 2003; 278:28479–89.
 - 43 O'Sullivan S, Dahlén B, Dahlén SE, Kumlin M. Increased urinary excretion of the prostaglandin D2 metabolite 9 α , 11 β -prostaglandin F2 after aspirin challenge supports mast cell activation in aspirin-induced airway obstruction. *J Allergy Clin Immunol* 1996; 98:421–32.
 - 44 Bochenek G, Nagraba K, Nizankowska E, Szczeklik A. A controlled study of 9 α , 11 β -PGF2 (a prostaglandin D2 metabolite) in plasma and urine of patients with bronchial asthma and healthy controls after aspirin challenge. *J Allergy Clin Immunol* 2003; 111:743–9.
 - 45 Cowburn AS, Saldek K, Soja J et al. Overexpression of leukotriene C4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J Clin Invest* 1998; 101:834–46.