Urinary excretion of lipid mediators in response to repeated eucapnic voluntary hyperpnea in asthmatic subjects

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Bood JR, Sundblad BM, Delin I, Sjödin M, Larsson K, Anderson SD, Wheelock CE, Dahlén SE, Dahlén B. Urinary excretion of lipid mediators in response to repeated eucapnic voluntary hyperpnea in asthmatic subjects. J Appl Physiol 119: 272–279, 2015. First published June 25, 2015; doi:10.1152/japplphysiol.00301.2015.—Exercise-induced bronchoconstriction displays refractoriness manifested as a decreased response to repeated exercise challenge within hours. The refractoriness may be attenuated by inhibition of the biosynthesis of prostaglandins (PG). The aim of the study was to determine which PGs and other lipid mediators are excreted during the refractory period. First, 16 subjects with mild stable asthma performed two repeated 4-min challenges with eucapnic voluntary hyperpnea (EVH) 1 and 3 h apart. There was a similar degree of refractoriness in both protocols (~15% protection). The 1-h interval was too short to study mediator excretion because the urinary levels did not return to baseline before the second challenge. With the 3-h protocol, there was increased urinary excretion of cysteinyl-leukotrienes and metabolites of the mast cell product PGD2 after both challenges. Next, another eight subjects performed two 6-min challenges with EVH 3 h apart, which produced a greater bronchoconstrictor response than the 4-min protocol (30.0 ± 5.4 vs. 17.7 ± 1.5%; P = 0.0029) and a greater degree of refractoriness (~30%). Analysis by ultra-performance liquid chromatography triple quadrupole mass spectrometry confirmed excretion of the bronchoconstrictor cysteinyl-leukotrienes and PGD2 during both challenges. In addition, there was increased excretion of the bronchoprotective PGE2, and also of the main metabolite of PGI2. This is the first report of excretion of PGE2 and PGI2 during the refractory period to EVH challenge, suggesting that they may mediate the refractoriness. Maintained excretion of PGD2 and leukotriene E4 following the repeat challenge argues against mast cell mediator depletion as the mechanism of refractoriness.

exercise-induced bronchoconstriction; refractoriness; leukotrienes; prostaglandins; asthma

EXERCISE IS A COMMON TRIGGER for an attack of asthma, and a majority of asthmatic subjects experience bronchoconstriction after exercise (4). The narrowing of the airways is thought to be caused by dehydration and a transient hyperosmolarity of the airway surface, which causes release of bronchoconstricting mediators from mast cells and other cells in the airways (2). In support of hyperosmolarity being the mechanism triggering exercise-induced bronchoconstriction (EIB), similar airway responses are seen following inhalation of mannitol (8, 22) and eucapnic voluntary hyperpnea (EVH) (5, 32). These challenges are, therefore, being used as surrogates for exercise to diagnose potential for EIB (17). The bronchoconstricting mediators released into the airways are rapidly removed by the circulation and are excreted into the urine (20, 27). Using enzyme immunoassay (EIA) methodology, our laboratory has previously demonstrated increased urinary excretion of cysteinyl leukotrienes (CysLTs) and the prostaglandin (PG) D2 metabolites following exercise (28), EVH (18, 19), and mannitol (22), respectively. Whereas CysLTs may be biosynthesized in many inflammatory cells, PGD2 is almost exclusively produced by the mast cells, and its release, therefore, provides objective evidence of mast cell activation. Previous knowledge about mediator excretion into the urine following EIB has, however, been restricted to those two lipid mediators.

Here we report on an extended spectrum of mediators using a newly developed platform for mass spectrometry, enabling us to simultaneously study the urinary excretion of metabolites of CysLTs, PGD2, PGE2, PGF2α, thromboxane (Tx) A2 (TxA2), as well as multiple isoprostane species (6). The methodology was applied to establish the profile of lipid mediators generated in response to repeated EVH challenges. When challenge by exercise, or with EVH/mannitol, is repeated within 4 h, a decrease in the bronchoconstrictor response is observed (11). This decreased responsiveness to repeated challenges is called refractoriness, and its duration, the refractory period. The occurrence and the degree of refractoriness decrease continuously with increasing time between the challenges (11). The mechanisms behind this protective response remain unclear. We hypothesize that identification of endogenous molecules that mediate refractoriness may help to define new targets for treatment of airway obstruction.

Our laboratory has previously observed that the subjects who were most refractory to repeated challenge with mannitol had the highest levels of CysLT and the PGD2 metabolite 11β-PGF2α during the refractory period (22). This finding suggested a decreased responsiveness to the released mediators at the level of the airway smooth muscle as one possible mechanism in refractoriness (21, 22). Because nonsteroidal anti-inflammatory drugs almost completely abolish refractoriness (26), it has been proposed that PGE2 may be of importance for the development of refractoriness (24, 26, 36). How-
ever, there is to date no direct evidence for release of PGE$_2$ in vivo during the refractory period.

Because the occurrence of refractoriness depends on the time between the challenges, and because the urinary excretion of mediators also has time-dependent kinetics, our study first defined the optimal conditions for this combined study of the bronchoconstrictor response and the urinary excretion of alleged mediators of bronchoconstriction and refractoriness. After the establishment of a suitable experimental design, it was possible to provide the first evidence of increased excretion of PGE$_2$ following EVH challenge. The study also discovered increased urinary excretion of metabolites of PGI$_2$ during the refractory period, possibly adding yet another potential endogenous protective factor to consider.

MATERIALS AND METHODS

Study Design

The urinary excretion of mediators is delayed compared with the airway response, necessitating us to determine the optimal interval between challenges to detect refractoriness while still being able to study the urinary mediator excretion. Therefore, we performed an initial study (study 1) to compare two different intervals between challenges. In this first study 16 asthmatic subjects were recruited to perform repeated 4-min EVH challenge either 1 or 3 h apart, in a randomized cross-over design (Fig. 1).

During screening, the subjects underwent a physical examination, skin prick test, and spirometry. A 4-min EVH challenge was performed using a slight modification of a protocol published by Smith et al. (35). Subjects who met the inclusion criteria of a maximum fall in forced expiratory volume in 1 s (FEV$_1$) ≥ 10% were included in the study. On the 2 study days, following baseline spirometry, repeated challenge with 4 min of EVH (challenges 1 and 2) was performed. Urine samples were collected 30 min before, immediately before the start of the first challenge, and then hourly until 240 min after the first challenge. Lung function was monitored repeatedly. To be able to calculate a percentage of protection, data were analyzed per protocol, excluding the subjects who did not achieve a 10% fall in FEV$_1$ on the first challenge on a particular study day. For the 1-h protocol, 5 of 16 subjects and, for the 3 h protocol, 1 of 16 subjects did not achieve a fall in FEV$_1$ ≥ 10% following challenge 1.

On the basis of this initial range-finding study, study 2 was designed. Now, a 6-min EVH challenge was performed, and only subjects who had a maximum fall in FEV$_1$ ≥ 15% were included. Nine subjects met the inclusion criteria, but one subject was excluded from analysis because of asthma deteriorating between the screening visit and the study day, indicating that she did not meet the inclusion criteria of having stable mild asthma. Thus eight subjects were eligible for further analysis. The screening day was performed in the same way as for study 1, with the exception of 6-min EVH instead of 4-min EVH. During the study day, the subjects performed repeated challenge with 6 min of EVH 3 h apart. Urine samples were collected 30 min before, immediately before the start of the first challenge, and then every hour until 300 min after the first challenge.

Subjects

Nonsmoking subjects with mild and stable asthma were eligible for participation. Asthma was defined by at least one of three criteria: response to asthma treatment, episodic wheezing, and variation in lung function over short periods of time. To be included, the subjects had to display baseline FEV$_1$ ≥ 70% of predicted value. Study subjects only used asthma medications as needed and were allowed to have used short-acting $\beta_2$-agonist only during the month before the study. Exclusion criteria included respiratory tract infection within the last 6 wk before inclusion. Subject characteristics are presented in Table 1.

All included subjects gave their written, informed consent, and the study was approved by the local ethics committee (Karolinska Institutet regional ethics committee Dnr 03-127, Ethics Board Stockholm 2012/1277-32).

EVH

Hyperpnea with dry, room temperature air containing 5% carbon dioxide was performed through a low-resistance, one-way valve in the sitting position (Ailos Asthma Test, Karlstad, Sweden) (5, 32). The target ventilation was 35 × FEV$_1$ × 0.75 (l/min) and was maintained for 4 or 6 min.

Lung Function

Lung function (forced vital capacity and FEV$_1$) was measured according to the American Thoracic Society criteria, using a wedge spirometer (Vitalograph, Buckingham, UK). FEV$_1$ was measured in duplicate before EVH, immediately after, at 2, 5, and 10 min, and then every 10 min during 1 h following each challenge. On each occasion, the highest value of two FEV$_1$ measurements was registered. The fall in FEV$_1$ was calculated in percentage of the prechallenge value.

Skin Prick Test

Skin prick test was performed during screening using the following allergens: birch, Timothy, mugwort, dog, cat, horse, dermaphagoides pteronyssinus, farinae, cladosporium, and alternaria (Soluprick SQ, ALK, Denmark). A positive response was defined as a measurable wheal of ≥3 mm in the absence of any equivalent reaction in the control test.

Urinary Mediators

After collection, urine samples were stored at −70°C until analysis. All urine samples were analyzed for creatinine using the modified Jaffe colorimetric method (22). Leukotriene (LT) E$_4$, 11β-PGF$_{2\alpha}$, 8-isoprostanep-PGF$_{2\alpha}$, 6-keto-PGF$_{1\alpha}$, PGE$_2$, TXB$_3$, and tetranor-PGD metabolite (PGDM) were analyzed using EIA kits commercially available (Cayman Chemical, Ann Arbor, MI), as previously described (22). In addition, the urine samples from study 2 were analyzed using ultra-performance liquid chromatography triple quadrupole mass spectrometry (UPLC-MS/MS), as described by Balgoma et al. (6), with the exception that prostacyclin metabolites were not detected due to a technical error. All levels of mediators were
corrected for dilution using creatinine and expressed as nanograms per millimole creatinine.

Statistical Analysis

All data are presented as mean value ± SE, unless otherwise stated. Statistical significance was determined using paired t-test to identify differences in the maximum fall in lung function and to compare baseline with peak mediator excretion. The Wilcoxon signed-rank test was performed for data that were not normally distributed. Correlations were calculated using Pearson product-moment correlation. Significance was defined as the commonly accepted level of P value of <0.05. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS

Study 1: Defining Optimal Time Between Challenges

Subject characteristics of the 16 subjects included in this first part of the study are presented in Table 1. Skin prick test was positive in 13 of the subjects. There were no significant differences in prechallenge lung function (forced vital capacity and FEV1) or exhaled nitric oxide levels on the different study days (data not shown).

Airway response. For the 1-h protocol \( (n = 11) \), the mean maximal decrease in FEV1 was 19.6 ± 2.4% after EVH challenge 1 and 15.4 ± 1.2% following challenge 2, 1 h later \( (P = 0.06) \). For the 3-h protocol \( (n = 15) \), the mean maximal decrease in FEV1 was 19.5 ± 2.1% after challenge 1 and 16.3 ± 2.5% after challenge 2, 3 h later \( (P = 0.02) \) (Table 1). These results correspond to an attenuation of 15% when challenge 2 was performed after 1 h, and 19% attenuation when challenge 2 was done 3 h after challenge 1. No correlation was found between percent fall following challenge 1 and the protection afforded for neither the 1-h nor the 3-h study day. Comparing the degree of protection between the 1-h and 3-h protocols for the 11 subjects who had data from both study days, we found no differences: the mean protection was 15 ± 9 and 15 ± 8%, respectively.

Urineary CysLT and PGD2 excretion. With the 1-h protocol \( (n = 11) \), there were no significant differences in the excretion [ng/mmol creatinine (±SE)] of LTE4 or 11β-PGF2α after either of the challenges (Fig. 2, A and C).

In contrast, for the 3-h protocol \( (n = 15) \), 11β-PGF2α increased significantly both after challenge 1 \( (29 ± 4 \text{ vs. } 24 ± 4 \text{ ng/mmol creatinine, } P = 0.0054) \) and after the challenge 2 \( (28 ± 3 \text{ vs. } 24 ± 3 \text{ ng/mmol creatinine, } P = 0.0209) \) (Fig. 2B). Also, there was an increase of LTE4 after challenge 1 \( (40 ± 3 \text{ vs. } 32.5 ± 3 \text{ ng/mmol creatinine, } P = 0.0011) \) and a strong tendency for an increase after challenge 2 \( (39 ± 4 \text{ vs. } 33 ± 3 \text{ ng/mmol creatinine, } P = 0.0582) \) (Fig. 2D).
Repeatability of the EVH challenge. The agreement between challenges was, in general, good; however, in a few patients, the standard deviation was sometimes large, with differences >20% when comparing the maximum percent fall in FEV₁ following challenge 1. Bland-Altman plots for demonstration of the variability revealed that the larger the fall in FEV₁, the greater the variability becomes (7). There were, however, no significant differences between the screening day and the study days (Table 1).

Study 2: Extended Analysis of Urinary Mediator Excretion

From the results of study 1, it was obvious that the 1-h interval between the challenges was too short and unsuitable because the mediator levels do not return to baseline within this time frame. Also, since the timing of the peak excretion of mediators after a challenge differ between subjects, it is not satisfactory with only one sample following challenge. Therefore, another eight subjects were recruited to perform repeated EVH 3 h apart. To increase the probability to catch the peak excretion following the second challenge, a sampling of urine was added at 300 min after the first challenge. Also, to try to enhance the response, we chose to use 6 min of EVH instead of 4 min and included only subjects with a fall in FEV₁ ≥ 15% at screening. Subject characteristics are presented in Table 1. Skin prick test was positive in seven of the subjects.

Airway response. The maximum fall in FEV₁ following challenge 1 was 30.0 ± 5.4% and 21.2 ± 4.5% following challenge 2, *P* = 0.0076 (Table 1). The mean degree of protection was 30%, and all subjects showed some degree of protection, with the range being 2–70%. Thus, overall, the bronchoconstriction induced in study 2 was greater than in study 1 (30.0 ± 5.4 vs. 19.5 ± 2.1%, *P* = 0.0029), indicating the 6-min EVH to be more powerful than the 4-min one.

Extended urinary mediator analysis using both UPLC-MS/MS and EIA. The results of the extended analysis are presented in Table 2 (UPLC-MS/MS), Table 3 (EIA), and Fig. 3 (UPLC-MS/MS and EIA). Of the 30 metabolites in the platform, 13 were found at measureable levels. The most abundant mediator was tetranor-PGE metabolite (PGEM) (~3,500 ng/mmol creatinine), followed by the main isoprostane metabolite, 8,12-IPGF₂α-VI (~500 ng/mmol creatinine). The tetranor metabolite of PGD₂ displayed levels around 200–250 ng/mmol creatinine, whereas the levels of PGF₂α were around 100 ng/mmol creatinine. Metabolites of thromboxane were slightly less abundant, and CysLTs were found in the lowest concentrations of all measured compounds. In general, the levels detected in EIA were higher compared with the levels found using UPLC-MS/MS. There was, however, a good correlation between the EIA and UPLC-MS/MS results.

PGD₂ metabolites. Measuring the peak excretion of 11β-PGF₂α using EIA, the increase from baseline did not reach significance following challenge 1. Following challenge 2, there was, however, a significant increase from baseline (Table 3, Fig. 3A). The levels of 11β-PGF₂α were back to baseline before challenge 2. With the use of UPLC-MS/MS, while the early metabolite 11β-PGF₂α was undetectable, its metabolite 2,3-dinor-PGF₂α was found in similar concentrations as the 11β-PGF₂α values indicated by the EIA. The 2,3-dinor-PGF₂α increased to an equal magnitude after both challenges, indicating a similar level of mast cell activation (Table 2, Fig. 3B). The levels of these two early PGD₂ metabolites returned to baseline before challenge 2 was initiated.

The EIA for tetranor-PGDM, a later and more abundant metabolite of PGD₂, showed increased levels following challenge 1, but failed to reach significance following challenge 2 (Table 3, Fig. 3C). Conversely, with UPLC-MS/MS, the increase of tetranor-PGDM failed to reach significance following challenge 1, but increased significantly following challenge 2 (Fig. 3D).

CysLTs. Using EIA, for LTE₄ there was an increase from baseline following challenge 1, but not following challenge 2 (Table 3, Fig. 3E). The levels of LTE₄ were still somewhat
Table 2. UPLC-MS/MS (ng/mmol) creatinine

<table>
<thead>
<tr>
<th>Compound</th>
<th>Baseline 1</th>
<th>Peak 1</th>
<th>Baseline 2</th>
<th>Peak 2</th>
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<tr>
<td>PGD2</td>
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<tr>
<td>11β-PGF2α</td>
<td></td>
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<tr>
<td>2,3-donor-PGF2α</td>
<td>60 ± 14</td>
<td>88 ± 18*</td>
<td>56 ± 13</td>
<td>89 ± 20†</td>
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<tr>
<td>Tetranor-PGDM</td>
<td>202 ± 30</td>
<td>234 ± 23</td>
<td>190 ± 21</td>
<td>240 ± 20†</td>
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<td>PGE2</td>
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<tr>
<td>13,14-dihydro-15-keto-PGE1</td>
<td></td>
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<tr>
<td>17 ± 3</td>
<td></td>
<td></td>
<td>19 ± 4</td>
<td>25 ± 4†</td>
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<tr>
<td>13,14-dihydro-15-keto-PGE2</td>
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<tr>
<td>Tetranor-PGEM</td>
<td>3,193 ± 1,015</td>
<td>3,435 ± 966*</td>
<td>3,087 ± 932</td>
<td>3,957 ± 1,016</td>
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<tr>
<td>PGE2α</td>
<td>107 ± 18</td>
<td>161 ± 41</td>
<td>154 ± 43</td>
<td>164 ± 34</td>
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<tr>
<td>13,14-dihydro-15-keto-PGF2α</td>
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<tr>
<td>Tetranor-PGFDM</td>
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<tr>
<td>TXB2</td>
<td>14 ± 6</td>
<td>15 ± 5</td>
<td>13 ± 5</td>
<td>13 ± 3</td>
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<td>11-dihydro-TXB2</td>
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<td>9 ± 2</td>
<td>7 ± 2</td>
<td>9 ± 1</td>
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<td>2,3-donor-TXB2</td>
<td>68 ± 18</td>
<td>81 ± 17</td>
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<td>8-iso-PGF2α</td>
<td>26 ± 2</td>
<td>33 ± 4</td>
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<td>33 ± 4</td>
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<tr>
<td>2,3-donor-8-iso-PGF2α</td>
<td>147 ± 7</td>
<td>184 ± 15</td>
<td>163 ± 18</td>
<td>187 ± 10†</td>
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<tr>
<td>8,12-IPF2α-IV</td>
<td>430 ± 48</td>
<td>615 ± 116</td>
<td>547 ± 96</td>
<td>577 ± 61</td>
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<td>LTxB, LTxB4 metabolites§</td>
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<tr>
<td>LTCa, LTD4</td>
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<tr>
<td>LTE4</td>
<td>7 ± 1</td>
<td>10 ± 1</td>
<td>6 ± 2</td>
<td>12 ± 3†</td>
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<tr>
<td>EXCa, EXD4, EXE4</td>
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</table>

Values are means ± SE in ng/mmol. UPLC-MS/MS, ultra-performance liquid chromatography triple quadrupole mass spectrometry; ND, not detected; PG, prostaglandin; PGDM, prostaglandin D metabolite; PGEM, prostaglandin E metabolite; Tx, thromboxane; LT, leukotriene; EX, eoxin. §6-Trans-LTB4, 20-OH-LTB4, and 20-CO2H-LTB4. *Significantly different from baseline 1 (P < 0.05). †Significantly different from baseline 2 (P < 0.05).

elevated from baseline before challenge 2, making interpretations of excretion following challenge 2 more difficult. In UPLC-MS/MS, LTE4 was the only CysLT that could be detected (Table 2). Most of the subjects displayed increased levels following challenge 1, with a strong tendency for the whole group, although not statistically different. The levels returned to baseline before challenge 2, and significantly increased levels were seen following challenge 2 (Fig. 3F).

Thromboxanes and isoprostanes. TXB2 and its metabolites, the isoprostanes and PGF2α, failed to display consistent increases, irrespective of whether analyzed by EIA or UPLC-MS/MS (Tables 2 and 3).

PGE2. The EIA for PGE2 showed increased concentrations following challenge 1, but not following challenge 2 (Table 3, Fig. 3G). In UPLC-MS/MS, the levels of PGE2 were significantly increased following both of the challenges, with no differences between the two peaks (Table 2, Fig. 3H). Tetranor-PGEM increased significantly following challenge 1, whereas the increase following challenge 2 failed to reach significance (Table 2, Fig. 3I). There were no differences in the levels of urinary PGE2 between male and female subjects, whereas the levels of PGEM were significantly higher in male subjects. (The women are nos. 22, 23, and 24 in Fig. 3J). The peak levels of PGE2 or PGEM after the first or second challenge did not correlate with the degree of refractoriness.

Prostacyclin. With the use of the EIA, clear increases were seen of the prostacyclin metabolite 6-keto-PGF1α following both challenges (Table 3, Fig. 3J). This metabolite was not included in the UPLC-MS/MS. The peak levels of PG2 after the first or second challenge did not correlate with the degree of refractoriness.

**DISCUSSION**

In this study, we report on extended analysis of mediator excretion during the refractory period following repeated EVH challenge. For the first time in this setting for EIB, we could demonstrate increased urinary excretion of metabolites of PGE2 and PGD2 following EVH. In contrast, the levels of metabolites of thromboxane and isoprostanes remained unchanged, indicating specificity in the excretion of eicosanoids. Using the mass spectrometry platform, we also documented increased levels of CysLTs, and metabolites of PGD2 following the EVH challenge, thus replicating and validating by mass spectrometry our laboratory’s previous findings using EIA only (18, 19). The increased urinary excretion of those two bronchoconstrictive mediators following both of the two repeated EVH challenges is similar to our previous findings of mediator excretion following repeated mannitol inhalation challenge (22). Taken together, the main findings support that excretion of mast cell mediators is maintained also during the second challenge, and that there is significant excretion of two lipid mediators with bronchoprotective properties during the refractory period, namely prostacyclin and PGE2. It is noteworthy that the urinary levels of the main metabolite of PGE2 were higher than for any of the other lipid mediators detected in the urine.
The primary eicosanoids are potent biologically active mediators; however, they are troublesome to measure since they are very rapidly metabolized and cleared from the circulation (34). Also, following withdrawal of blood, it has been shown that, e.g., TxB2 can be generated ex vivo (29). This often makes the measurement of these primary compounds very difficult, and the interpretation of such data ambiguous. Urine has emerged as a noninvasive alternative, and metabolism and urinary excretion of these compounds has been extensively studied (23, 38). The use of urinary excretion of eicosanoid metabolites is now well established (10). As we used a mass spectrometry platform, which has previously been applied to study the urinary mediator excretion following allergen challenge (9), the present results allow for comparison of differences and similarities with respect to the patterns of excretion in response to these two different indirect triggers of bronchoconstriction. Following allergen challenge, the levels of CysLT, and metabolites of PGD2 and TxB2, were all increased.

Fig. 3. Extended analysis of the mediator excretion during study 2. Enzyme immunoassay (EIA; A, C, E, G, J) and ultra-performance liquid chromatography triple quadrupole mass spectrometry (UPLC-MS/MS) data (B, D, F, H, I) are shown. A: 11β-PGF2α; B: 2,3-dinor (DN)-PGF2α; C and D: tetranor-PGD metabolite (PGDM); E and F: LTE4; G and H: PGE2; I: tetranor-PGE metabolite (PGEM); J: 6-keto-PGF1α. All values are presented as ng/mmol creatinine. Lines represent individual subjects; nos. next to lines are subject nos. Thick horizontal lines, means.
but there was no increase in levels of PGE2 or its metabolites. For the bronchoconstrictive mediators PGD2 and CysLT, the results in this EVH study are thus concordant with the findings in the allergen inhalation challenge study, whereas we could not find significant increases of TxB2 or its metabolites following EVH. The discrepancies in mediator excretion following EVH and allergen challenge suggest differences in the cells activated by the different challenges.

For PGE2, we observed increases of both the primary mediator and its most abundant metabolite, tetranor-PGEM. This is distinctly different to what was found following allergen challenge (9). The finding of PGE2 excretion following EVH, but not allergen challenge, might be explained by the mechanisms of the challenges. The reactions to both of the challenges are initiated by mast cell activation, as evident by the uniform excretion of PGD2, but, whereas the allergen challenge is an IgE-dependent specific mast cell activation, EVH activates mast cells through changes in osmolarity (2). The change in local tissue osmolarity is likely to induce excretion of PGE2 from other cells in the airways and in particular from airway epithelial cells (14, 16). In contrast, there was no increase in the excretion of PGF2α, which is consistent with previous findings in plasma following exercise challenge (3) and again underscores the specificity of the pattern of excretion of lipid mediators.

The observation that pretreatment with inhalation of PGE2 has been shown to inhibit the response to exercise challenge (25), as well as the inhibiting effect of nonsteroidal anti-inflammatory drug pretreatment on the development of refractoriness (24, 26, 36), has led to the speculation that PGE2 is the key mediator of refractoriness. However, there are no previous reports on increased excretion of PGE2 following exercise challenge in asthmatic subjects, rather a decrease in the levels of PGE2 has been seen in induced sputum (13). Increased levels have been seen in male subjects following exercise in exhaled breath condensate, but the same was not seen in female subjects (31). As lipid mediators in exhaled breath condensate to a significant extent may reflect salivary admixture, the data are inconclusive (12).

In our study, the excretion of the abundant metabolite of PGE2, tetranor-PGEM, increased following challenge 1 but not following challenge 2. This supports increased excretion of PGE2 from the lung following EVH challenge. Interestingly, we also found that primary PGE2 increased in the urine following both of the challenges. The present concept is that the kidney itself is the exclusive source of urinary PGE2. This view is based on previous metabolic studies showing rapid metabolism of systemic PGE2. Thus primary PGE2 was only seen in the urine following renal artery infusion, but not after brachial vein infusion (38). However, the metabolic studies were done with relatively low doses of PGE2, and it is likely that the systemic load of PGE2 following massive excretion from the airways during the EVH challenge is much greater, explaining that a small proportion is excreted in the urine unmetabolized.

Comparing the levels of urinary mediators between EIA and UPLC-MS/MS for LTE4, there were good correlations, but absolute values were generally higher in EIA. For 11β-PGF2α, however, this metabolite was not at all detected in UPLC-MS/MS, but rather the levels in EIA seemed to correspond to the levels of 2,3-dinor-PGF2α, the metabolite to which the antibody is cross-reactive. What is actually measured with the EIA for 11β-PGF2α, therefore, appears to be 2,3-dinor-PGF2α. This has previously been noted in work from our group (27) and confirmed by others (15), but the commonly used term has still been 11β-PGF2α because this is the name of the antibody in the commercially available kit. For the other mediators analyzed both using EIA and UPLC-MS/MS, there was in general a good agreement between the methods, as can be seen by similar patterns of excretion (Fig. 3) and good correlations. In the Bland-Altman analysis (7), it was evident that, with increasing concentration, the discrepancies between the EIA and UPLC-MS/MS results became larger.

We performed an initial study to optimize the conditions for mediator analysis during the refractory period. From previous studies, we know that refractoriness is greater the sooner after the first challenge the second challenge is performed (11). However, since there is a lag between the excretion of mediators in the lung and the excretion in urine, we needed to extend the interval. Considering most mediators were back to baseline, and refractoriness was still found, the 3-h interval was sufficient. The subjects in study 2 displayed from no to almost complete protection (2–70%) at the second challenge, which is in line with previous findings that the degree of refractoriness is indeed a continuous response (22).

Concerning the repeatability of the EVH challenge, Bland-Altman analysis (7) revealed a high variability between challenges, which is in line with findings by Price and colleagues (30). Considering that the between-study day difference for several subjects was >10%, the question arises about the usability of the EVH challenge as a predictive tool in the diagnosis of EIB. The number of subjects with large differences was, however, smaller for the 6-min protocol, which also caused a greater response. This makes the 6-min challenge more suitable for diagnosis and drug intervention trials.

In conclusion, the consistent finding of increased levels of the bronchoconstrictive mediators following two repeated eu-capnic hyperventilation challenges makes decreased mediator excretion following the second challenge an unlikely mechanism of refractoriness. Our findings lend further support to the importance of PGE2, and possibly also PG12, in the development of refractoriness and provide circumstantial support for our laboratory’s previous suggestion of decreased responsiveness at the level of the airway muscle (21, 22). The next step will need to be specific interventions with, e.g., subtype-specific PGE2 receptor (EP) agonists to define the mechanisms in greater detail. Our laboratory has recently found that low doses of PGE2 via EP2 receptor activation has a long-lived inhibitory effect on mast cell-dependent constriction of human small airways (33). A better understanding of the mechanism of this unique natural protective mechanism may aid in the search for new treatment targets in asthma.

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