The occurrence of refractoriness and mast cell mediator release following mannitol-induced bronchoconstriction

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Larsson J, Perry CP, Anderson SD, Brannan JD, Dahlén SE, Dahlén B. The occurrence of refractoriness and mast cell mediator release following mannitol-induced bronchoconstriction. J Appl Physiol 110: 1029–1035, 2011. First published January 20, 2011; doi:10.1152/japplphysiol.00978.2010.—For several hours after exercise-induced bronchoconstriction, there is diminished responsiveness to repeated challenge. The mechanism causing this refractoriness is unclear. Inhalation of dry powder mannitol is a new bronchial provocation test that has been suggested as a surrogate for an exercise challenge. Refractoriness to repeated mannitol challenge has however not been established. Our objective was to investigate if repeated challenge with mannitol is associated with refractoriness and diminished release of mast cell mediators of bronchoconstriction. Sixteen subjects with asthma underwent repeated inhalation of mannitol 90 min apart. Lung function was assessed by forced expiratory volume in 1 s (FEV1). The urinary excretion (ng/mmol creatinine) of the mediators was measured. The group mean fall in FEV1 after the second challenge was 48.5 ± 5.8% of the first (P < 0.001). The protection afforded by the initial challenge, however, varied considerably between subjects (range 88–0%). Furthermore, the urinary excretion of the two mediators was increased after both challenges. The average excretion of mediators after the challenges was significantly higher for the six most refractory subjects. This was observed both for LTE4 (95.6 ± 5.2 vs. 58.0 ± 2.4 for the 6 least refractory) (P < 0.001) and for 9α,11β-PGF2α (137.6 ± 6.7 vs. 50.1 ± 1.1 for the 6 least refractory) (P = 0.002). As occurs with exercise-induced bronchoconstriction, repeated inhalation of mannitol induced refractoriness. We propose that refractoriness is due to tachyphylaxis at the level of the airway smooth muscle responsiveness to mediators of bronchoconstriction rather than due to fatigue of their release from mast cells.

prostaglandins; leukotrienes; exercise; tachyphylaxis; mast cell activation

EXERCISE-INDUCED bronchoconstriction (EIB) occurs in the majority of subjects with asthma (2, 7, 24). Evidence has accumulated to support a role for mast cell mediators in EIB (18, 19, 31). The stimulus for mast cell release of mediators is thought to be hyperosmolarity of the airway surface liquid following evaporative water loss during the increased ventilation of exercise (1). In support of this theory is the finding of mast cell activation after inhalation of the osmotically active substance mannitol (5), and that human mast cells, in vitro, release histamine, prostaglandin (PG) D2, and cysteinyl-leukotrienes (CysLT) when exposed to an hyperosmolar environment (13). Moreover, the mast cell stabilizing agent sodium cromoglycate is an effective inhibitor of EIB (35) as well as the airway response to mannitol (6).

Following repeated exercise challenge within 2 h, ~50% of asthmatics will have <50% of the initial response (33). The refractory period is also observed after other provocation tests that cause bronchoconstriction by the same mechanism as exercise, including eucapnic voluntary hyperpnea (EVH), and hypertonic saline (12, 28, 32, 33). The mechanism for the refractoriness is still unclear. Decreased release of mediators from the mast cells during the second challenge is one possible explanation (12, 33). Release of a protective PG is another (22). Receptor desensitization to the mediators released by the mast cell at the site of the smooth muscle is yet another possibility (17).

The aim of this study was to investigate if inhalation of mannitol was followed by refractoriness to a repeated challenge and if this was associated with differences in the urinary excretion of mediators. The PGD2 metabolite 9α,11β-PGF2α was measured as it is a sensitive marker of mast cell activation (11). Urinary excretion of leukotriene (LT) E4 was followed as it is established as an important mediator of bronchoconstriction in EIB (9, 21).

In this study, the second mannitol challenge was performed 90 min after the first. The choice of this interval was based on studies of refractoriness to other surrogates of exercise (12, 28, 33, 33) since no previous studies have investigated refractoriness to mannitol.

METHODS

Subjects. Sixteen subjects with asthma, a positive response to inhaled mannitol, and a spontaneous recovery within 90 min after the challenge were included in the study (Table 1). The protocol was approved by the institutional Ethics Committee (protocol no. X05-0068), and the study was carried out under the Therapeutic Goods Administration of Australia Clinical Trial Notification scheme (CTN No. 2005/362). Informed consent was obtained from all subjects in writing. Exclusion criteria were forced expiratory volume in the first second (FEV1) <65% of predicted, age <14 yr, exacerbation of asthma or respiratory infection within the last 4 wk, subjects who had varied their inhaled corticosteroid therapy within the last 4 wk, pregnant females or females at risk of becoming pregnant or breast feeding, current smokers, or known hypersensitivity to mannitol. The subjects were permitted to continue their inhaled corticosteroid treatment, but they were asked not to take their inhaled steroid on the day of the challenge. Inhaled

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β2-adrenoceptor agonists were withdrawn before the study day (short acting at least 8 h, long acting 48 h).

Study design. At visit 1 (screening) a skin prick test was performed to establish presence of atopy. Mannitol challenge was performed to identify subjects with a fall in FEV1 from baseline value >15% following the administration of 635 mg of mannitol, or less. In case of a positive response to mannitol, FEV1 was measured at 5 min, then at 10-min intervals for 90 min. If spontaneous recovery of FEV1 to at least 95% of baseline occurred within 90 min, the subject was asked to return to the lab for the study day. The interval between the screening and study day was at least 5 days, and while most subjects returned within 14 days, one came back 17 days later, one 26 days later, and the interval for one was 83 days.

Visit 2 (study day) included two challenges with mannitol. The second challenge was repeated 90 min after the end of the first challenge and ceased at the same cumulative dose of mannitol that provoked the 15% fall on the first challenge. Urine was collected 90 min after the first challenge and was used as the baseline before the second challenge. Urine collected 90 min after the first challenge was used as the urine collected 90 min after the second challenge was repeated 90 min after the end of the first challenge and ceased at the same cumulative dose of mannitol that provoked the 15% fall on the first challenge. Urine was collected 90 min after the first challenge and was used as the urine collected 90 min after the second challenge. The interval between the screening and study day was at least 5 days, and while most subjects returned within 14 days, one came back 17 days later, one 26 days later, and the interval for one was 83 days.

Table 1. Subject characteristics

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<th>Subject</th>
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<th>Sex</th>
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<th>Weight, kg</th>
<th>Medications</th>
<th>%Predicted FEV1</th>
<th>No. of Positive Skin Prick Tests</th>
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M, male; F, female; FEV1, forced expiratory volume in 1 s; AH, antihistamine; SABA, short-acting β-receptor agonist; LABA, long-acting β-receptor agonist; prn, as required; Y, yes.

Atopic status. Skin prick testing was performed with standardized extracts of the following allergens: house dust, house dust mite, dog, cat, Alternaria, Aspergillus niger, Aspergillus fumigatus, timothy grass, perennial rye grass, and cockroach. Test was considered positive if a wheal > 3 mm in diameter was seen.

Statistical analysis. All data are presented as mean values ± standard error of the mean (SE), if not otherwise stated. As the study results documented that the degree of refractoriness varied and appeared to be a continuous variable, urinary excretion of the mediators of bronchoconstriction was compared for the six most refractory and the six least refractory subjects. This extreme group analysis (26) was performed using an unpaired t-test. To establish within-group variation between time points we used paired t-test to find differences between baseline and postchallenge values. When the data were not normally distributed, analysis with Wilcoxon signed rank test was performed. Correlations were calculated using Pearson product-moment correlation. As a limit for considering differences significant, we used a P value of <0.05. All statistical analyses were made using SigmaStat 3.5 software (Systat Software).

RESULTS

Airway response. For all 16 subjects, the fall in FEV1 (% of baseline) was 26.9 ± 1.7% (mean ± SE) after the first challenge and 13.3 ± 1.3% after the second challenge (P < 0.001). Accordingly, the average percent protection at the second challenges was 48.5 ± 5.8 (Fig. 1A). The range of protection, however, varied from almost complete to no protection at all (87.6 to −0.5%).

Urinary mediators. Excretion of LTE4 (ng/mmol creatinine) was increased both after the first and the second challenge (Fig. 1B). The levels at 30, 60, and 90 min after the first challenge were all significantly higher than at baseline (69.7 ± 7.9, 85.2 ± 12.5, and 81.4 ± 14.1, respectively, vs. baseline 43.1 ± 4.0; P = 0.003 for all time points). The value 30 min after the second challenge (i.e., 120 min) was significantly higher than second baseline (i.e., time point 90 min after first challenge) (95.3 ± 15.3 vs. baseline 81.4 ± 14.1) (P = 0.011), whereas the values at 150 (90.6 ± 12.2) and 180 min (77.1 ± 11.9) did not differ significantly from the second baseline.
Increased values from baseline were also observed for urinary excretion (ng/mmol creatinine) of the mast cell marker 9α,11β-PGF₂ (Fig. 1C). The levels of 9α,11β-PGF₂ at all time points 30, 60, and 90 min after the first challenge were also significantly higher than the baseline value (82.9 ± 17.1, 89.8 ± 26.5, and 84.2 ± 28.3 respectively, vs. baseline 39.9 ± 4.0, \textit{P} < 0.001). The value at 30 min after the second challenge (i.e., at 120 min) was significantly higher than the second baseline (99.7 ± 32.8 vs. baseline 84.2 ± 28.3, \textit{P} = 0.021), whereas the value at 150 (97.6 ± 32.2) and 180 min (86.8 ± 30.5) did not differ significantly from second baseline.

**Comparison of the most and the least refractory subjects.** To test the hypothesis that the differences between subjects in the degree of refractoriness (Table 2) reflected different patterns of excretion of urinary mediators, comparisons were made between the six most and the six least refractory subjects. Neither the average percent fall on the first challenge, nor the total dose of mannitol used was significantly different between the groups (\textit{P} > 0.05). There were also no differences in baseline FEV₁ between the groups before the first and the second challenge (\textit{P} > 0.05). For the six most refractory subjects, the mean percent protection was 69.2 ± 3.9. The maximum percent fall in FEV₁ from baseline after the first and second challenge was thus 30.8 ± 3.4 and 9.4 ± 1.7, respectively (\textit{P} < 0.001). In contrast, for the six least refractory, the mean percent protection was 24.8 ± 7.0, with the percent fall in FEV₁ after the first and second challenge being 23.2 ± 0.7 and 17.5 ± 1.6, respectively (\textit{P} = 0.014) (Fig. 2A). The difference in the percent protection between the groups was highly significant (\textit{P} < 0.001).

The average postchallenge concentration of urinary LTE₄ (ng/mmol creatinine) (i.e., mean of all time points 30–180 min) was significantly higher for the six most refractory subjects than for those six least refractory subjects (95.6 ± 5.2 vs. 58.0 ± 2.4, \textit{P} < 0.001) (Fig. 2B). There was a difference in the pattern of mediator excretion, with sustained high concentrations in the most refractory group, whereas the least refractory group was approaching baseline concentration before the second challenge was performed (at 90 min) and generally displayed only numerically greater concentrations postchallenge. For the most refractory group, the concentrations at 30 and 150 min were significantly higher than at first baseline (Table 3), and the values at 60, 90, 120, and 180 min showed the same tendency (\textit{P} = 0.055, 0.073, 0.071, and 0.063, respectively).

Likewise, the average postchallenge values over 30–180 min for the urinary concentration of 9α,11β-PGF₂ (ng/mmol creatinine) were higher for the six most refractory subjects compared with the six least refractory subjects (137.6 ± 6.7 vs. 50.1 ± 1.1, \textit{P} = 0.002) (Fig. 2C). With respect to the pattern of excretion, as for LTE₄, 9α,11β-PGF₂ was also found to be excreted in a greater concentration and in a more sustained fashion for the most refractory subjects compared with the least refractory subjects. Within the most refractory group, the concentrations at 30, 120, 150, and 180 min were significantly higher than at first baseline concentration (Table 3). There was a leveling off at 90 min, just before the second challenge, and the values at 120 and 150 min just failed to reach significance compared with second baseline value (\textit{P} = 0.067 and 0.066, respectively). At 180 min the concentrations for 9α,11β-PGF₂ had started to fall again. For
the least refractory group, the concentrations of 9α,11β-PGF₂α were lower and the variability was smaller compared with the most refractory group, but the values at all time points 30–180 min were nevertheless significantly higher than first baseline (Table 3).

For all sixteen subjects, neither the fall in FEV₁ nor the rise in urinary mediators during the first challenge showed any correlation with the percent protection. However, the peak change in the levels of LTE₄ and 9α,11β-PGF₂α showed a significant correlation after the first challenge \((r = 0.818, P < 0.001)\). Removing the outlier subject, there was still a correlation between LTE₄ and 9α,11β-PGF₂α \((r = 0.686, P = 0.005)\) (Fig. 3).

**DISCUSSION**

This is the first study to document that mannitol challenge induces refractoriness to a second challenge with mannitol. The bronchoconstriction following the second challenge was, on average, about half of that obtained after the first. This finding is similar to that observed following EIB (2, 33). We also report for the first time the effect of repeated challenge with mannitol on the urinary excretion of mast cell mediators. The finding of increased urinary 9α,11β-PGF₂α after challenge with mannitol, similar to findings with EIB (31). The data for the first challenge confirm our previous observations that mannitol inhalation causes mast cell activation (5, 6). The study, however, provides the new finding that the urinary excretion of the stable metabolite (9α,11β-PGF₂α) of the mast cell product PGD₂ was increased following both challenges. This occurred even though refractoriness of the airway response followed the repeated challenge with mannitol. Moreover, the urinary excretion of LTE₄ was also increased after the two challenges. There was a strong correlation between the release of 9α,11β-PGF₂α and LTE₄, suggesting that the mast cell activation is a central event in the evoked release of cysteinyl-leukotrienes. Our findings suggest a new interpretation of the mechanisms of refractoriness to repeated challenge with stimuli that provoke release of mast cell mediators.

Whereas the lung function returned to baseline before the second challenge, the urinary excretion of the mediators of bronchoconstriction did not. The effects of the first and second challenge on urinary excretion of mediators are therefore superimposed and more difficult to analyze. We interpret the data to support the contention that refractoriness to mannitol challenge is unlikely to be caused by depletion of mast cell mediator secretion for reasons discussed below. Rather, we propose that the release of the mast cell mediators induced a protective response that rendered the airway smooth muscle less responsive or “tolerant” to a second challenge. This new hypothesis will need to be evaluated in future studies that include direct assessment of the influence of mannitol challenge on bronchial responsiveness to subsequent challenge with the mast cell mediators histamine, PGD₂, and the cysteinyl-leukotrienes.

When assessed at the group level, there was a significant increase in both urinary mediators at 30 min after the second challenge. This is in keeping with the interpretation that depletion of mast cell mediators does not explain refractoriness. However, the findings for all subjects should be interpreted with caution: first, because the curves for excretion of urinary mediators were superimposed, and second, because all subjects did not display refractoriness to repeated challenge with mannitol. In fact, the percent protection during the second challenge appeared to be a continuous variable ranging from no protection to almost complete inhibition of the second inhalation of mannitol. This did not appear to be related to the dose of mannitol administered, nor the degree of bronchoconstriction following the first challenge. Data supporting that refractoriness to EIB and hypertonic saline is a variable phenomenon have been provided by others (3, 14).

Taking these differences in degree of refractoriness of the bronchoconstriction into account, we compared the patterns
of urinary excretion of mast cell mediators in the six most refractory subjects with the six least refractory subjects. From this extreme group analysis (26) it was clear that the most refractory subjects not only had higher levels of urinary mediators in response to the first challenge, but they also displayed more sustained elevations of mediator excretion than the least refractory subjects. This relationship was most pronounced for LTE\(_4\) and provides the basis for the hypothesis we put forward to explain the mechanism of refractoriness.

Cysteinyl-leukotrienes, with the end-product LTE\(_4\) measured in urine, is recognized as an important group of mediators in EIB. For example, leukotriene receptor antagonists attenuate the airway response to exercise (9, 21) and to mannitol (4, 8). Tachyphylaxis to repeated inhalations of LTD\(_4\) has been demonstrated both in normal subjects (17) and in subjects with asthma (22). Manning et al. found that refractoriness to exercise was closely correlated with the refractoriness to repeated LTD\(_4\) inhalations, and also demonstrated cross refractoriness between LTD\(_4\) and exercise (22). The refractoriness to LTD\(_4\) was abolished by the cyclooxygenase inhibitor flurbiprofen. These findings led the authors to suggest that refractoriness to repeated exercise challenge involves leukotriene and prostaglandin interdependent pathways. It is established that activation of cysteinyl-leukotriene receptors causes dose-dependent secondary release of prostaglandins and other cyclooxygenase products in the lung (10, 29). In our study, the most refractory group had the highest levels of urinary mediators. One possibility is that the higher levels of LTE\(_4\) observed in the most refractory subjects caused a greater stimulation of leukotriene receptors and hence greater local release of bronchoprotective PGE\(_2\) in the airways of these particular subjects.

Further support for a protective role of prostaglandins in EIB has been provided by the finding that premedication with the cyclooxygenase inhibitors indomethacin or flurbiprofen attenuated the refractoriness after exercise challenge (22, 23, 27). Inhalation of PGE\(_2\) has been shown to inhibit EIB (25), and such an effect could be due to relaxation of the smooth muscle and/or inhibition of mast cell mediator release (16, 22).

Another explanation of the refractoriness might be development of tolerance to the bronchoconstricting effects of leukotrienes. A possible mechanism is desensitization or downregulation of the leukotriene receptor. It has previously been observed that desensitization to aspirin challenge in aspirin-intolerant asthma is associated with decreased expression of leukotriene receptors (34).

As mentioned, one limitation in this study is that the levels of urinary levels of mediators had not returned to baseline before the second challenge after an interval of 90 min. This makes it difficult to use 90 min as baseline before second challenge, but the levels of both mediators were falling at 90 min and therefore the size of the second peak is underestimated when comparing it to the second baseline. The 90-min interval had been sufficient for return of urinary mediator concentration of 9α,11β-PGF\(_2\) but not LTE\(_4\) to baseline in the previous studies with mannitol (5, 6). However extending the interval between the challenges to allow the urinary mediator concentration to return to baseline would have risked missing the refractory period which is
usually less than 3 h (12). Further, as implicated by our hypothesis to explain the results, it may be that the sustained high concentration of LTE₄ actually contributes to the relatively greater refractoriness.

The levels of the PGD₂ metabolite 9α,11β-PGF₂ were also high and prolonged after the initial challenge in the most refractory group. It is therefore possible that PGD₂ also played a role in the development of refractoriness and tolerance of the smooth muscle to repeated stimulation as PGD₂ also is a potent bronchoconstrictor. In humans, the biosynthetic capacity for production of PGD₂ is much higher in mast cells than in other cells that ex vivo may be stimulated to release PGD₂ (11). Furthermore, sodium cromoglycate, known to inhibit mast cell mediator release selectively, also blocked the mannitol-induced release of the PGD₂ metabolite 9α,11β-PGF₂ (6). Interestingly, it has also been observed that sodium cromoglycate prevented refractoriness to exercise-induced bronchoconstriction (2). Together, our present findings and previous data (2, 5, 6) lend strong support to the interpretation that mast cell activation with release of PGD₂ is caused by mannitol challenge.

In conclusion, refractoriness to repeated challenge with mannitol was observed, and this supports mannitol as an appropriate surrogate for exercise-induced bronchoconstriction. Furthermore, refractoriness to mannitol was associated with the maintained release of bronchoconstrictive mediators, as monitored by 9α,11β-PGF₂ for PGD₂ and LTE₄ for the cysteinyl-leukotrienes. The data support the hypothesis that the mechanism for the refractoriness is tachyphylaxis at the site of the airway smooth muscle rather than at the level of mast cell mediator release. Our finding supporting preserved mast cell mediator release is in keeping with findings by Belcher and colleagues who studied the refractory period to exercise and did not find decreased histamine levels (3). One mechanism that would be compatible with the proposal that refractoriness occurs at the level of the airway smooth muscle is leukotriene-induced release of a bronchoprotective prostaglandin, presumably PGF₂. Another is that the muscle becomes tolerant to the mediators of bronchoconstriction, for example by the development of desensitization to the effects of the leukotrienes or PGD₂ at their receptors (10) in a similar way as for β₂-agonists, where exposure causes downregulation and internalization of the receptors.

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GRANTS

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DISCLOSURES

S. D. Anderson is the inventor of the mannitol test; however, the intellectual property is owned by her employer, the Sydney South West Area Health Service (SSWAHS), and licensed to Pharmaxis Ltd. She receives a 10% share of the royalties distributed to SSWAHS and holds shares in Pharmaxis that she purchased herself, but she does not hold any options. She has acted as a consultant to Pharmaxis Ltd. and has received fees for this service since April 2009.

J. D. Brannan is a visiting research fellow and not an employee of the SSWAHS. He holds shares in Pharmaxis Ltd. that he purchased himself, but he does not hold any options. He receives 10% of the royalties distributed to SSWAHS. He has acted as a consultant to Pharmaxis Ltd. and has received fees for this service.

C. P. Perry is employed by SSWAHS and holds shares in Pharmaxis Ltd. that she purchased herself, but she does not hold any options. She has acted as a consultant to Pharmaxis Ltd. and has received fees for this service.