

Original article

Release of prostaglandin D₂ and leukotriene C₄ in response to hyperosmolar stimulation of mast cells

Background: Mannitol-induced bronchoconstriction in subjects with exercise-induced asthma is associated with increased urinary excretion of 9 α , 11 β -PGF₂, a metabolite of prostaglandin D₂ (PGD₂) serving as a mast cell marker. It has however been questioned whether or not human mast cells release PGD₂ and leukotriene C₄ (LTC₄) after osmotic challenge with mannitol *in vitro*.

Methods: Cord blood-derived human mast cells were stimulated osmotically, immunologically or with a combination of both. Supernatants were analysed for PGD₂, LTC₄ and histamine contents with enzyme immunoassays.

Results: Significant release of *de novo* synthesized eicosanoids, predominantly PGD₂ [12 (8.8, 14) pmol/10⁶ cells; median (25th, 75th percentile) but also LTC₄ (0.1 (0.08, 0.15) pmol/10⁶ cells] were found in mast cells *in vitro* in response to 0.7 M mannitol stimulation. A massive release of histamine [70 (5.3)% of total; mean (SEM)] was also found. There were no correlations between the levels of released mediators after mannitol stimulation. In contrast, there was a correlation between release of PGD₂ and LTC₄, following immunological stimulation.

Conclusion: The findings support that hyperosmolar challenge activates mast cells, but different than antigen stimulation.

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Mast cells have a central role in the pathophysiology of asthma and in allergic diseases. In addition, the relative number of mast cells has been found to be increased in the airway smooth muscle layer of asthmatic subjects (1). Mast cells release a range of bioactive mediators following activation either by antigen cross-linking of the high-affinity immunoglobulin E (IgE) receptors (Fc ϵ RI) or by a number of other stimuli via IgE-independent pathways (2). It is not conclusively established however whether or not the pattern of mediator secretion in response to different activating stimuli is similar. For instance it has been reported that exposure of mast cells and basophils to hyperosmolar stimuli triggers degranulation and release of histamine without causing significant *de novo* synthesis of mast cell-derived lipid mediators such as prostaglandin D₂ (PGD₂) and cysteinyl-leukotrienes (CysLTs) (3). As IgE-dependent stimulation consistently leads to release of all three

classes of mediators (histamine, PGs and LTs) (2) the findings with hyperosmolar stimulation *in vitro* have been taken as evidence of distinct stimulation of histamine release. In fact, it was suggested that hyperosmolar stimulation partially inhibited IgE-dependent formation of PGD₂ and CysLTs (3). Prostaglandin D₂ and the CysLTs are potent bronchoconstrictors *in vivo* (4, 5). Furthermore, PGD₂ is almost exclusively formed in mast cells and is the predominant cyclooxygenase product (6, 7) whereas CysLTs can be formed by other inflammatory cells (5).

Local hyperosmolarity of the airway surface liquid is likely to be a key trigger factor in exercise-induced bronchoconstriction (EIB). Humidification of inspired air causes evaporation of water from the airway surface liquid and increased osmolarity in epithelial and submucosal cells as water is transported across the epithelium to the lumen, with subsequent activation of cells and release of mediators (8). Mast cells have been inferred to play a central role in EIB and evidence for mast cell mediator release in response to exercise has previously been reported with increased excretion of the urinary PGD₂ metabolite 9 α , 11 β -PGF₂ (9). Modification of airway response to exercise by inhibition of mast cell mediator effects *in vivo* utilizing different antagonists and stabilizers has been tested (10, 11).

Abbreviations: anti- λ , anti-lambda antibodies raised in rabbit; BSA, bovine serum albumin; CBMC, cord blood-derived mast cells; CysLTs, cysteinyl leukotrienes; EIB, exercise-induced bronchoconstriction; LDH, lactate dehydrogenase; LTC₄, leukotriene C₄; IgE, immunoglobulin E; IL-6, interleukin-6; PGD₂, prostaglandin D₂; PBS, phosphate-buffered saline; RP-HPLC, reversed phase-high performance liquid chromatography; SCF, stem cell factor.

Inhaled mannitol is considered to induce airway narrowing with a mechanism similar to that observed during exercise by increasing the osmolarity in the airways (12). It was recently observed that inhalation of mannitol caused increased urinary excretion of 9α , 11β -PGF₂ and this response was more pronounced in subjects with asthma in association with bronchoconstriction (13). The mannitol response was also followed by increased excretion of CysLTs but to a similar extent in asthmatic and healthy subjects (13). Pharmacological intervention studies indicate histamine and leukotriene-dependent components in mannitol-induced bronchoconstriction (14). Recent data point to a role for PGD₂ in the airway response to mannitol (15). There is good evidence that PGD₂ in humans is almost exclusively generated by the mast cell (6, 7).

In order to shed light on the proposed activation of mast cells by hyperosmolar stimulation, the current study was undertaken to determine the effects of mannitol on basal and immunologically induced release of histamine, PGD₂ and CysLTs from cultured human cord blood-derived mast cells (CBMC). These human mast cells provide a good model for assessment of direct effects of stimuli on mast cells.

Methods

Materials

Mannitol and calcium ionophore A23187 were bought from Sigma-Aldrich (St Louis, MO, USA). Human recombinant stem cell factor (rhSCF) was purchased from PeproTech (London, UK) and rhIL-6 from Chemicon (London, UK). LDH assay cytotoxicity kit was from GBiosciences (St Louis, MO, USA). All reagents for enzyme immunoassay analyses of PGD₂-MOX and LTC₄ were purchased from Cayman Chemicals (Ann Arbor, MI). Histamine immunoassay kit was from Immunotech (Beckman Coulter, Marseille, France). Human myeloma IgE (ND) and rabbit anti-human lambda (λ) antibody were generous gifts from Dr T Karlsson (Pharmacia Diagnostics, Uppsala, Sweden).

Human cord blood-derived mast cells

All cord blood donors were anonymous and thus no individual data or information regarding atopy status or family history was available. Human CBMC were developed essentially as described (16), cells were cultured in RPMI 1640 medium supplemented with 50 μ M 2-mercaptoethanol, 10 mM HEPES, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 IU/ml penicillin, 100 μ g/ml streptomycin and 10% fetal calf serum (FCS). Cells were seeded at a density of 1×10^6 /ml medium with the addition of 50 ng/ml rhSCF and 10 ng/ml rhIL-6. After 8–10 weeks, >95% of the cells were found to be tryptase positive. The collection of human umbilical cord blood was approved by the ethical committee at the Karolinska Institutet; D-nr 01-374.

Stimulation of CBMC

Mannitol (0.3, 0.7 and 1.0 M) was dissolved in phosphate-buffered saline (PBS) and the calculated osmolality (calculating with the

osmotic coefficient of 1.01 for mannitol) were 577, 981 and 1284 mOsm respectively. Cells were passively sensitized with human myeloma IgE (ND) (1 μ g/ml) in culture medium for 12–16 h, followed by activation with an anti-lambda (λ) antibody (0.2–200 μ g/ml). IgE-sensitized cells were washed and stimulated with 2 μ g/ml anti-lambda in a 0.7 M mannitol solution for combined stimulation. Non-physiological, ionophore A23187 (5 μ M) was used to induce calcium-dependent activation. All cell incubations were prepared in isotonic sterile PBS with Ca²⁺ and Mg²⁺ (0.5×10^6 cells/ml and an osmolality of 274 mOsm/kg) supplemented with 0.1% bovine serum albumin. Stimulations were stopped by centrifugation at 4°C after 30 min of incubation, supernatants were stored at –70°C prior to analysis.

Measurements of eicosanoids

Cell supernatants were analysed with enzyme immunoassay for the content of LTC₄ using a CysLT rabbit polyclonal antiserum with cross-reactivity to LTC₄, LTD₄ (100%) and LTE₄ (67%). Acetyl cholinesterase (AChE)-linked LTC₄ was used as tracer (17). For analysis of PGD₂, cell supernatants were derivatized with methoxylamine-HCl and analysed for PGD₂-MOX using enzyme immunoassay. The assay was performed with a PGD₂-MOX AChE-linked tracer (17).

Measurement of histamine and lactate dehydrogenase

Measurements of histamine were performed using an enzyme immunoassay according to the manufacturer. For determination of intracellular amounts, the cell pellet was subjected to three freeze-thaw cycles. The amount of released histamine was expressed as percentage of total histamine. Release of lactate dehydrogenase (LDH) is considered proportional to necrotic cell death (18). The cytotoxic effect of mannitol (0.3–1.0 M) on CBMC was investigated by measurement of LDH from cell supernatants according to the manufacturer.

Statistical analyses

When data were not normally distributed, comparisons between more than two groups were made with nonparametric tests (Kruskal–Wallis one-way analysis of variance on ranks). If significant, further pairwise comparisons were performed with Mann–Whitney rank sum test. For normally distributed data, comparisons between more than two groups were made with parametric tests (one-way analysis of variance), further pairwise comparisons were performed with Student's *t*-test. Correlation was calculated with Spearman's rank order. *P* < 0.05 was considered as significant. All data are expressed as median (25th, 75th percentile) except for release of histamine after mannitol titration that are expressed as mean [standard error of the mean (SEM)].

Results

Mannitol-induced release

Hyperosmolar stimulation of CBMC with mannitol (0.3–1.0 M) elicited release of PGD₂, LTC₄ and histamine. The release of PGD₂ at baseline was 0.5 (0.3, 0.8) [median (25th–75th percentile)] pmol/10⁶ cells. The amounts were significantly increased at 0.7 and 1.0 M mannitol with 22

and 16 times elevated levels respectively (Fig. 1A). Furthermore, significantly higher levels of LTC₄ were found at 0.7 M and 1.0 M mannitol with 4 and 10 times increased release compared with the baseline value of 0.02 (0.02, 0.03) pmol/10⁶ cells (Fig. 1B). Hyperosmolar stimulation with mannitol also led to elevated levels of released histamine compared to unstimulated cells 8 (2) [mean (SEM)]% of total (Fig. 1C). Significantly increased release of histamine was documented at 0.7 and 1.0 M mannitol with nine- and sevenfold increases compared with unstimulated cells respectively. For the following experiments 0.7 M mannitol was chosen as hyperosmolar stimulus. Mannitol (0.3–1.0 M) was not cytotoxic as no LDH could be detected in supernatants from mannitol-stimulated cells.

IgE-dependent release

Cells, passively sensitized with human myeloma IgE (ND) (1 µg/ml) cross-linked with increasing doses of anti-λ (0.2–200 µg/ml), dose dependently released PGD₂, LTC₄ and histamine. As controls, release of PGD₂ and LTC₄ was also analysed from cells stimulated with anti-λ without prior sensitization as well as from sensitized cells without the addition of anti-λ. No increased release of any mediator could be detected in these control samples compared with unstimulated cells (data not shown). The release of PGD₂ at baseline was 0.7 (0.6, 0.8) pmol/10⁶ cells and the amounts were significantly increased at 0.2, 2.0, 20 and 200 µg/ml anti-λ to 29-, 65-, 88- and 55-times enhanced levels respectively. IgE-mediated activation further resulted in a dose-dependent generation of LTC₄ compared with unstimulated cells [0.02 (0.01, 0.02) pmol/10⁶ cells]. Stimulation with increasing doses of anti-λ (0.2–200 µg/ml) elicited significantly elevated amounts of LTC₄ with 55-, 220-, 570- and 2300-fold increase respectively (Fig. 2). For comparison, dose-dependent release of histamine after anti-λ stimulation

was analysed in two experiments. Under non-stimulated conditions 6.7 (4.9, 8.6)% of total histamine was released. Increasing doses of anti-λ (0.2–200 µg/ml) elicited more histamine, the increases were 1.1, 1.9, 2.5- and 2.8-fold basal level respectively. Data for 200 µg/ml anti-λ was obtained from duplicate experiments and are thus not shown in the figure. For the following experiments challenge with 2 µg/ml anti-λ was used for activation.

Stimulus-dependent release

The mediator release pattern was compared after stimulation with anti-λ (2.0 µg/ml) in sensitized cells, mannitol (0.7 M) or a combination of both. The amounts of PGD₂ produced at baseline was 0.3 (0.2, 1.0) pmol/10⁶ cells

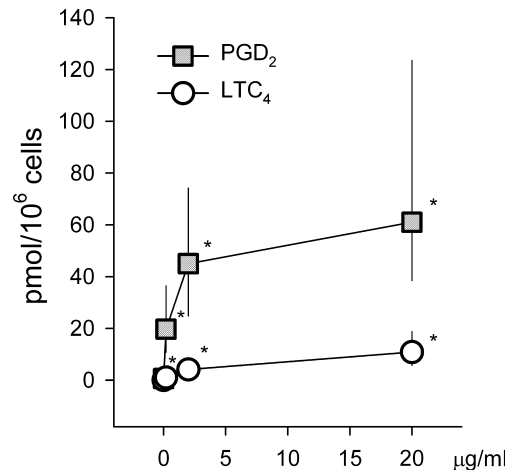


Figure 2. Release of PGD₂ (square) and LTC₄ (circle) after increasing doses of anti-λ. Values are presented as median (25th, 75th percentile) from four different experiments ($n = 8-10$). * $P < 0.05$ when compared with control level.

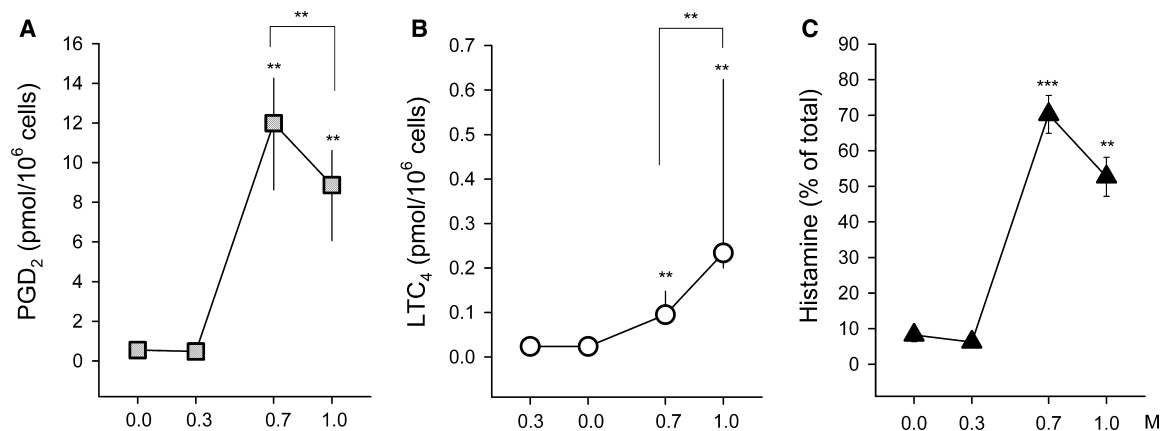


Figure 1. Release of PGD₂ (A), LTC₄ (B) and histamine (C) after increasing doses of mannitol. Values in (A) and (B) are presented as median (25th, 75th percentile) and in (C) as mean (SEM) from five and three individual experiments ($n = 9-12$ and $n = 5-6$ respectively). ** $P < 0.01$, *** $P < 0.001$ when compared with control level or as indicated.

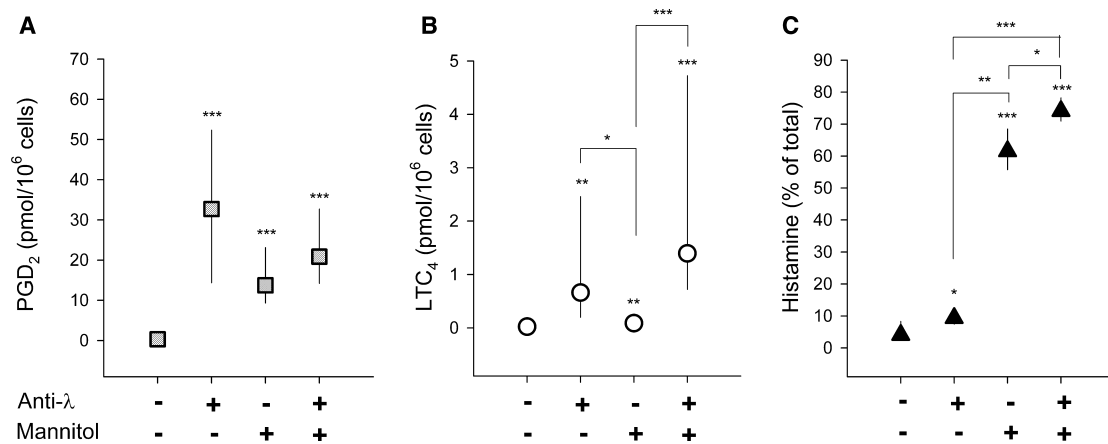


Figure 3. Release of PGD₂ (A), LTC₄ (B) and histamine (C) after stimulation. Values in (A–C) are presented as median (25th, 75th percentile) and represent eight and seven individual experiments ($n = 10$ –15 and $n = 8$ –12 respectively). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared with control level or as indicated.

(Fig. 3A). The increased productions after stimulation with anti- λ , mannitol or a combination of both were 104-, 44- and 66-times the basal level respectively. There was no statistically significant difference between the groups. Release of LTC₄ from unstimulated cells was 0.02 (0.02, 0.03) pmol/10⁶ cells and after anti- λ , mannitol or combined stimulation the amounts were increased 29-, 4- and 61-times compared with control levels respectively (Fig. 3B). Stimulation with anti- λ alone or in combination with mannitol elicited significantly more LTC₄ than mannitol alone, with no significant difference between anti- λ and the combined stimulation (Fig. 3B). Histamine release in response to anti- λ , mannitol or combined stimulation is shown in Fig. 3C. The release at control level was 4.1 (4.0, 7.5)% of total histamine. After anti- λ , mannitol or combined stimulation the increases were 2.3-, 15- and 18-times respectively. The histamine release was significantly different between groups. Both hyperosmolar mannitol stimulation itself and the combination with anti- λ had a more pronounced effect on histamine release than anti- λ itself. There was also a significantly increased degranulation with the combination compared with mannitol alone. Thus a synergistic effect was found for histamine release with the combination of immunological and mannitol stimulation compared with each stimulus alone.

Release in percentage of ionophore A23187-induced production

Measurements of released PGD₂, LTC₄ and histamine expressed as percentage of the release after ionophore (5 μ M) stimulation are shown in Table 1. Stimulation with anti- λ (2 μ g/ml) had the strongest effect on PGD₂ release with almost 50% of the A23187 induced release. For LTC₄, the amount of released mediator was only up to 3% compared with A23187-induced release. For histamine release, mannitol stimulation alone or in

Table 1. Mediator release expressed as percentage of ionophore (A23187) – induced secretion

	Anti- λ	Mannitol	Combination	Control
PGD ₂	44 (25, 62)	14 (13, 14)	23 (17, 30)	0.4 (0.3, 0.8)
LTC ₄	2.3 (1.0, 9.0)	0.2 (0.1, 0.5)	3.2 (1.5, 5.7)	0.04 (0.02, 0.07)
Histamine	22 (16, 31)	133 (104, 136)	157 (115, 175)	12 (9.3, 16.5)

Values are presented as median (25th, 75th percentile) from four individual experiments ($n = 5$ –8).

combination with anti- λ elicited over 130% of the one induced by A23187.

Differentiated eicosanoid release

Correlation between the release of LTC₄ and PGD₂ with anti- λ , mannitol or a combination of both was investigated (Table 2). A significant correlation was only found after anti- λ stimulation ($r_s = 0.91$, $P < 0.001$).

The ratio between released PGD₂ vs LTC₄ is presented at baseline and following stimulation with anti- λ , mannitol, a combination of both or A23187 (Table 3). At baseline and after combined stimulation the ratio is almost in the same range, with 15-fold higher levels of PGD₂ than LTC₄. Anti- λ stimulation shifts the release to almost 50 times more PGD₂ than LTC₄. Stimulation with mannitol has an even stronger effect on PGD₂ release

Table 2. Spearman's Rank Order Correlation (r_s) investigated between LTC₄ and PGD₂ release after stimulation with anti- λ , mannitol or a combination of both

	r_s	P -value
Anti- λ	0.9	<0.001
Mannitol	–0.2	0.662
Combination	0.7	0.059

Values are presented r_s from eight individual experiments ($n = 13$ –15).

Table 3. Ratio of released PGD₂ and LTC₄

	PGD ₂ :LTC ₄
Control	14–1
Anti- λ	49–1
Mannitol	156–1
Combination	15–1
A23187	1.2–1

Values are presented as ratio of released PGD₂ vs LTC₄ in cells from four to eight individual experiments ($n = 5–15$).

with almost 160 times more PGD₂ when compared with LTC₄.

Discussion

This is the first study demonstrating significantly increased secretion of both PGD₂ and LTC₄ in response to hyperosmolar activation of human mast cells *in vitro*. The physiological osmolality during quiet respiration in the tracheal fluid is around 360 mOsm/kg H₂O (19) compared with 280 mOsm/kg H₂O in plasma. After exercise, the airway surface liquid may reach an osmolality of 900 mOsm/l H₂O (20), which is in the same range as 0.7 M mannitol (981 mOsm). The difference between osmolality and osmolarity can be considered as insignificant in diluted solutions. Osmotic activation of mast cells or basophils might therefore be important in the pathophysiology of exercise-induced asthmatic responses.

Mannitol has previously been shown to cause significant release of histamine from dispersed human lung mast cells (3) reaching a peak at 770 mOsm. This indicates that significant release of histamine can be achieved at osmolarities twofold the physiological osmolality, and at similar levels as reached in the airways after exercise (20). In our study both histamine and PGD₂ release was markedly elevated and peaked at a concentration of 0.7 M (981 mOsm) mannitol. For LTC₄, the release dose dependently increased up to the highest concentration tested (1.0 M mannitol, 1284 mOsm). The dose-dependent release of LTC₄ compared with the peak of released PGD₂ confirms our finding that there is a differentiated release after mannitol stimulation as no correlation between released mediators (LTC₄, PGD₂ or histamine) was found. Moreover, mannitol seemed to be a rather weak stimulus for LTC₄ formation and indeed higher concentrations were released after ionophore (A23187) stimulation. All three mediators were released at a similar level of osmolarity as reported for EIB (20).

Eggleston et al. did not find any significant release of either PGD₂ or LTC₄ after mannitol stimulation at either 460 or 770 mOsm/kg in dispersed human lung mast cells (3). However, they reported significantly increased release of histamine at 770 mOsm/kg with 15% release of total histamine. In contrast, we found that histamine release

from CBMC reached 70% of total at 0.7 M (981 mOsm). Cells were still viable despite the profound degranulation as no LDH was found in mannitol-stimulated cell supernatants and thus no cytotoxic effect was determined. The discrepancy between the findings reported by others and the present results may be due to the different type of mast cells used. The present study utilized almost 99% tryptase-positive mast cells when compared with the cells prepared from human lung parenchyma with a purity of 1–10% mast cells (3).

In normal human subjects, the bronchoconstrictive effect of LTC₄ is 1000 times greater than that of inhaled histamine (21) and inhaled PGD₂ is 10 times more potent than inhaled histamine (4). Thus, even though small amounts of LTC₄ were released when compared with PGD₂ after mannitol stimulation of CBMC, the amounts may have effects *in vivo*. Furthermore, inhaled LTC₄ in combination with PGD₂ or histamine can synergistically provoke bronchoconstriction in asthmatic subjects (22). In our experiments, the amounts of released PGD₂ were 156 times the amounts of LTC₄ after mannitol stimulation, thus indicating that PGD₂ produced by mast cells may contribute to bronchoconstriction elicited by hyperosmolarity. This is also suggested by data using the cyclooxygenase inhibitor flurbiprofen in hypertonic saline challenges (23). Furthermore, mannitol challenge of atopic asthmatic patients with EIB has been shown to increase the urinary excretion of the PGD₂ metabolite 9 α , 11 β -PGF₂ in connection with bronchoconstriction (13). Pretreatment with the proposed mast cell stabilizer cromoglycate or a long-acting β 2-agonist 15 min before mannitol inhalation significantly inhibited increased urinary 9 α , 11 β -PGF₂ as well as bronchoconstriction (15). Taken together, these findings support that PGD₂ and its metabolite might be involved in the bronchoconstriction in subjects suffering from EIB.

In our experimental study, we also found that IgE-dependent activation was an effective stimulus for release of both PGD₂ and LTC₄ in CBMC, confirming other reports (24, 25). There was a tendency of higher levels of released PGD₂ after anti- λ stimulation compared with mannitol, and for LTC₄ this increase after anti- λ stimulation compared with mannitol was significant. The profile of released eicosanoids after IgE-dependent stimulation is skewed. Already at baseline there was a 14 : 1 predominance for PGD₂ release vs LTC₄, and this was further increased to 49 : 1 when cells were stimulated with 2 μ g/ml anti- λ . An increased PGD₂ release compared with LTC₄ has previously been reported in mast cells isolated from different human tissues as lung, intestine and uterus and found to be 3 : 1, 5 : 1 and 2 : 1, respectively, after anti-IgE stimulation (24). Hsieh et al. have shown a PGD₂/LTC₄ ratio of 287 : 1 in CBMC following anti-IgE stimulation, and after priming of the cells with IL-4 the ratio was decreased to 25 : 1 due to increased LTC₄ release. This was suggested to be due to induced expression of the LTC₄ synthase (24).

For secretory granule-associated release of histamine, anti- λ was a weak stimulus. A volume of 2 and 200 $\mu\text{g/ml}$ anti- λ stimulation resulted in 10% and 20% of total histamine release respectively. Similar results have been reported previously for CBMC (24, 26). Further, stimulation with anti-IgE in dispersed human lung mast cells released approximately 20% of total histamine (3, 6, 19).

In response to IgE-dependent activation, the increase in $[\text{Ca}^{2+}]_i$ in the cell is accomplished from both intracellular and extracellular stores. In contrast, at non-IgE activation (compound 48/80 or substance P) Ca^{2+} is mainly released from intracellular stores, thus reaching lower levels in the cytosol (27). The 5-lipoxygenase enzyme requires more Ca^{2+} than the cyclooxygenase to reach enzymatic activities and this might explain the preferential production of PGD_2 when compared with LTC_4 (28). This could also be the explanation for the higher PGD_2 release when compared with LTC_4 after anti- λ stimulation. The release of LTC_4 was much higher when stimulated with A23187 when compared with stimulation with anti- λ , mannitol or the combination of both where only 2–3% of the A23187-induced release was reached. For PGD_2 , anti- λ stimulation almost reached 50% of A23187-induced release. Interestingly, mannitol was a more effective stimulus for granulae release of histamine than A23187.

We found no difference in the release of PGD_2 in CBMC when stimulated with anti- λ in a hyperosmolar solution when compared with anti- λ stimulation alone. On the contrary, the combined stimulation significantly increased the LTC_4 release in CBMC compared with mannitol alone. This is in contrast to previous results in human lung mast cells where significantly decreased levels of both PGD_2 and LTC_4 were found after combined stimulation compared with anti-IgE alone (3). We confirmed a synergistic effect of stimulation with anti-IgE in a hyperosmolar solution for histamine release as previously found in human lung mast cells (3). No correlation between released LTC_4 , PGD_2 or histamine was found after mannitol or combined stimulation, thus indicating a differentiated release.

In summary, this is the first study documenting significantly elevated levels of released PGD_2 and LTC_4 after mannitol stimulation in mast cells. These results suggests a differentiated release of PGD_2 and LTC_4 in CBMC and indicate that different eicosanoid mediators released from mast cells may be of importance in asthmatic and inflammatory reactions. The results, with a predominant release of PGD_2 in response to mannitol, further confirm the *in vivo* data suggesting mast cell activation after hyperosmolar challenge of the airways.

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