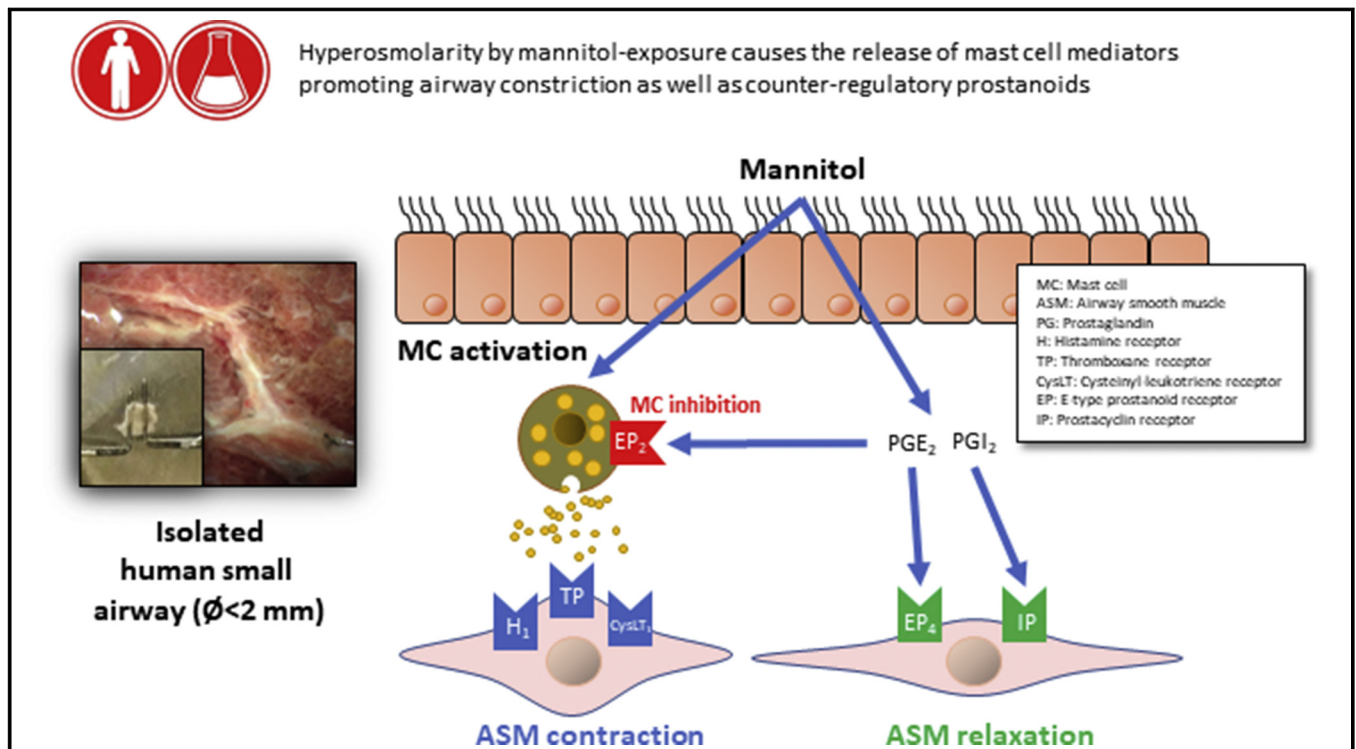


Mannitol triggers mast cell-dependent contractions of human small bronchi and prostacyclin bronchoprotection



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GRAPHICAL ABSTRACT



Background: Clinical research supports that exercise-induced bronchoconstriction (EIB) is caused by hyperosmolar triggering of mast cells. The reaction can be mimicked by inhalation of mannitol, but it has paradoxically previously not been possible to replicate this mode of action of mannitol in isolated airways. **Objective:** We sought to establish an *ex vivo* model of EIB in human small bronchi.

Methods: Small bronchi (inner diameter, 0.5-2 mm) from macroscopically healthy human lung tissue were obtained from 48 patients and mounted in organ baths. Contractions and mediator release were analyzed after challenge with hyperosmolar mannitol (850 mOsm).

Results: Ten minutes of exposure to mannitol caused a small initial contraction (12% \pm 1% of maximum) that was followed

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by a second and much larger contraction (maximum effect [E_{\max}], 47% \pm 5%) when mannitol was washed out. The mast cell stabilizer cromolyn reduced the second contraction (E_{\max} , 27% \pm 3%). Furthermore, this main contraction was abolished by the combination of antagonists of histamine and cysteinyl leukotrienes in the presence of indomethacin. Mannitol increased the release of the mast cell mediators histamine (9.0-fold), cysteinyl leukotrienes (4.5-fold), and prostaglandin (PG) D_2 (5.4-fold), as well as PGE_2 (6.3-fold) and the prostacyclin metabolite 6-keto $PGF_{1\alpha}$ (5.7-fold). In contrast, indomethacin alone enhanced the bronchoconstriction (E_{\max} , 68% \pm 6%). Likewise, receptor antagonists for PGE_2 (EP_2 and EP_4) and prostacyclin (IP) also enhanced the mannitol-induced bronchoconstriction (E_{\max} , 67% \pm 5%, 66% \pm 4%, and 68% \pm 3%, respectively). In bronchi precontracted by carbachol, the IP receptor agonist cicaprost induced profound relaxation.

Conclusion: This new protocol established an *in vitro* model for studies of EIB in isolated human bronchi. The IP receptor might be a new target for asthma treatment. (J Allergy Clin Immunol 2019;144:984-92.)

Key words: Hyperosmolar challenge, exercise-induced bronchoconstriction, prostaglandin D_2 , prostaglandin E_2 , prostaglandin I_2 , EP_2 receptor, EP_4 receptor, prostacyclin receptor, histamine, nonsteroidal anti-inflammatory drugs

Exercise-induced bronchoconstriction (EIB) can occur in elite athletes without asthma but is most commonly recognized as a form of airway hyperresponsiveness that is highly overrepresented in asthmatic patients.^{1,2} EIB is a consequence of the increased ventilation and water loss during exercise, which in turn leads to hyperosmolarity of the airway tissues and triggering of mast cells to release the mediators that cause contraction of the airway smooth muscle.³ Accordingly, leukotriene (LT) receptor antagonists and mast cell stabilizers can attenuate EIB, and functional antagonism by short-acting β_2 -adrenoceptor agonists has been the conventional strategy to prevent or reverse EIB.⁴ Thus far, however, no treatment can completely alleviate the symptoms or prevent the induction of EIB.

Many mechanisms that regulate airway tone after an exercise challenge remain unresolved. For example, a protective role for prostaglandin (PG) E_2 has been implicated in patients with EIB because inhalation of PGE_2 can attenuate the reaction,⁵ whereas treatment with nonsteroidal anti-inflammatory drugs blocks the reduced EIB that normally occurs when the exercise challenge is repeated within a few hours from the first challenge.^{6,7} This nonsteroidal anti-inflammatory drug-sensitive refractoriness indicates that some COX-derived prostanoids are protective, but it remains to be determined how such protective signaling pathways are triggered, which particular prostanoids are involved, and how bronchoconstrictive and bronchoprotective actions interact.

One strategy to investigate EIB in asthmatic patients is through inhalation of hyperosmolar concentrations of mannitol,⁸ which induces bronchoconstriction and release of mast cell mediators in a similar manner as exercise.^{9,10} It was recently established that bronchoconstriction induced by another EIB-mimetic, eucapnic voluntary hyperpnea with dry air, as expected, was associated with increased release of PGE_2 from the airways, but in addition, it was discovered that prostacyclin (PGI_2) also was released during the reactions.¹¹ In the *in vivo* setting in human

Abbreviations used

CysLT:	Cysteinyl leukotriene
EIB:	Exercise-induced bronchoconstriction
E_{\max} :	Maximal contractile response
E_{\min} :	Maximal relaxant response
IP:	Prostacyclin
LT:	Leukotriene
PG:	Prostaglandin

subjects, it is difficult to define the precise mechanisms involved because there are no available drugs to selectively intervene with biosynthesis or receptors of specific prostanoids. A general need for a better understanding of the underlying processes in patients with EIB was one major incentive for the current study aiming at development of a new experimental model for translational research on mechanisms in patients with EIB.

We have previously reported how isolated human small bronchi can be activated by anti-IgE to produce mast cell-dependent contractions that are inhibited by PGE_2 .¹² Therefore it was considered that mannitol challenge of this particular preparation might offer an experimental model suitable to investigate the role of prostanoids and other mediators from the point of view of mechanisms of EIB.

The strength of using explanted intact bronchi is that the smooth muscle appears in its natural context close to structural cells and mast cells. It has been established that isolated human mast cells indeed release their mediators in response to hyperosmolar mannitol.^{13,14} However, the approach involved challenges because previous investigations using prolonged exposure to hyperosmolar NaCl in bovine tracheal strips¹⁵ or mannitol in human bronchi¹⁶ found that the observed contractions were resistant to different pharmacologic agents, including mast cell inhibitors.

Knowing that mast cell-induced contractions of human airways develop within minutes after anti-IgE exposure¹² and that cumulative mannitol inhalation in human subjects uses a protocol in which doses are increased every minute,⁸ we hypothesized that a short-term exposure of mannitol might uncover the mast cell activation. Therefore a new “hit-and-run” protocol was established in which a brief 10-minute exposure of the bronchi to hyperosmolar mannitol was followed by a washout period. This procedure produced mannitol activation of mast cell mediator release and the appearance of an EIB-like contraction that was amenable to confirming pharmacologic antagonism resembling interventions with exercise challenge *in vivo*. In addition, we provide new pharmacologic implications that prostacyclin, as well as PGE_2 , are possible protective factors involved in the phenomenon of refractoriness to repeated exercise challenge.

METHODS

Isolated tissue preparation

With permission of the Regional Ethical Review Board in Stockholm (reference no. 2010/181-31/2), macroscopically healthy human lung tissue was collected after consent from patients undergoing lobectomy for neoplasms (92%) or other reasons (hamartoma, granuloma, or follicular bronchiolitis; n = 48, 22 female and 26 male subjects; median age, 70 years; age range, 32-82 years; Table I). One to 3 bronchi were dissected out and further cut into segments from each lung. In all investigations 1 segment from each bronchus was used as a control for paired comparisons.

TABLE I. Patients' characteristics and tissue responses

	Female subjects (n = 22)	Male subjects (n = 26)	P value
Age (y)	67 (57-79)	71 (32-82)	.43
Body mass index (kg/m ²)	25.6 (17.8-31.9)	26.2 (21.2-39.1)	.33
C-reactive protein (mg/L)	3 (1-75)	5 (1-109)	.58
Hemoglobin (g/L)	130 (110-145)	140 (104-163)	.01
Leukocyte particle concentration ($\times 10^9/L$)	7 (4.9-15.7)	6.7 (4.1-18.7)	.84
Current smoker (no.)	7	11	.56
Ex-smoker (no.)	10	13	.78
COPD (no.)	1	4	.36
Asthma (no.)	2	4	.67
Allergy (no.)	6	7	.99
E _{max} mannitol (10 min [% of maximum])	13.75 (0-27.4)	11.25 (0-24.3)	.78
E _{max} after mannitol washout (% of maximum)	45.3 (30.2-71.9)	37.3 (30.9-77.2)	.58

Statistical analysis was performed by using the Mann-Whitney *U* test. Numbers represent absolute values or medians and ranges. An ex-smoker is defined as a person who has not smoked for the last 12 months.

COPD, Chronic obstructive pulmonary disease.

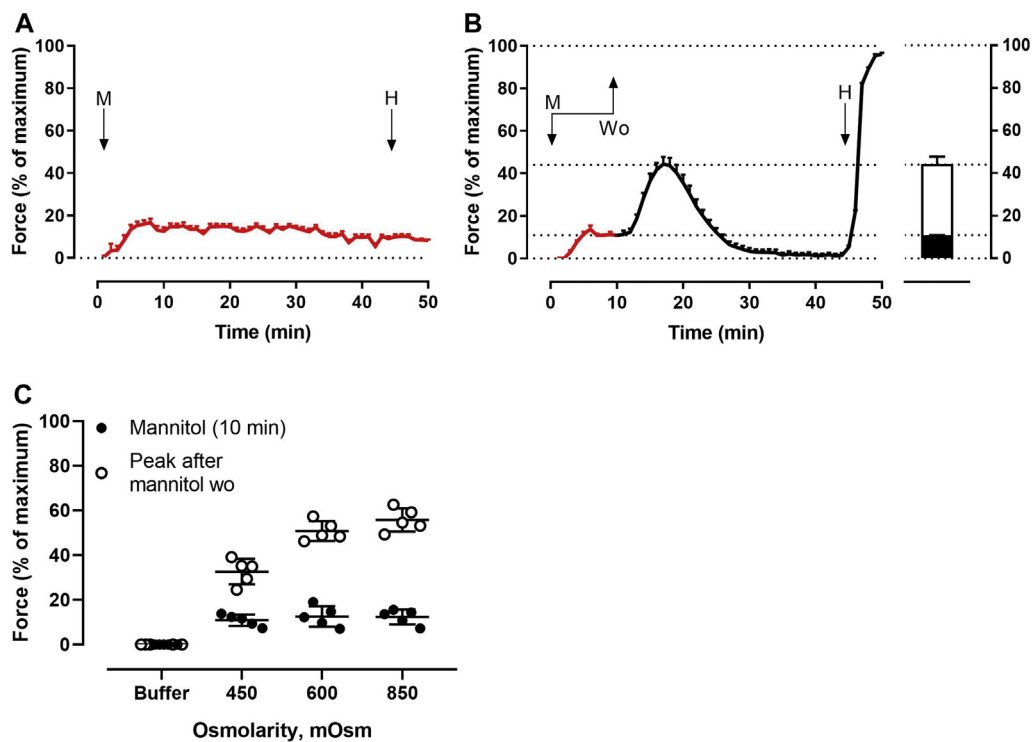


FIG 1. A, Force trace of human isolated bronchi with osmolarity increased to 850 mOsm by exposure to mannitol (*M*) during the 50-minute observation period. B, In this protocol mannitol (*M*) was only present for 10 minutes, followed by a washout (*Wo*) period that was followed by a second larger contraction. A supramaximal concentration of histamine (1 mmol/L) was administered after 45 minutes in both Fig 1, A and B (*H*), but only produced a response in Fig 1, B. C, Paired segments with osmolarity increased mannitol to either 450, 600, or 850 mOsm by using the protocol from Fig 1, B. Black dots/bars represent contraction after mannitol (10-minute exposure), and white dots/bars represent the maximal contraction after mannitol washout. Data represent means \pm SEMs (number of patients = 4-5, number of segments used = 5-15).

The specimens were immediately put in ice-cold Krebs-Henseleit buffer solution supplemented with 2.5 mmol/L calcium chloride and 2.1 g/L sodium bicarbonate. Within 1 hour of the resection, microscopy-aided dissection isolated bronchial rings with an inner diameter of between 0.5 and 2 mm. The bronchial ring segments were placed in separate culture plate wells containing Dulbecco modified Eagle medium (Gibco, Auckland, New Zealand) supplemented with 1% penicillin (100 IU/mL; Sigma-Aldrich, St Louis, Mo) and streptomycin (100 μ g/mL; Gibco) under sterile conditions in a humidified incubator (37°C at 95% O₂ and 5% CO₂), allowing the tissue to equilibrate overnight.¹²

Experimental set-up

On the experimental day, the rings were mounted horizontally between 2 metal prongs for recording of isometric tension (ADInstruments, Hastings, United Kingdom) by using myographs (Organ Bath Model 700MO; DMTA/S, Aarhus, Denmark) containing Krebs-Henseleit buffer solution and kept at 37°C and constantly bubbled with carbogen (5% CO₂ in O₂) to maintain pH at 7.4. Preparations were allowed to equilibrate for 30 minutes with buffer solution changed every 15 minutes, followed by a stepwise increase in tension over 60 minutes to a resting tension of 1.5 mN. KCl (60 mmol/L) was added twice with a washout in between to ascertain bronchial reactivity and viability. This

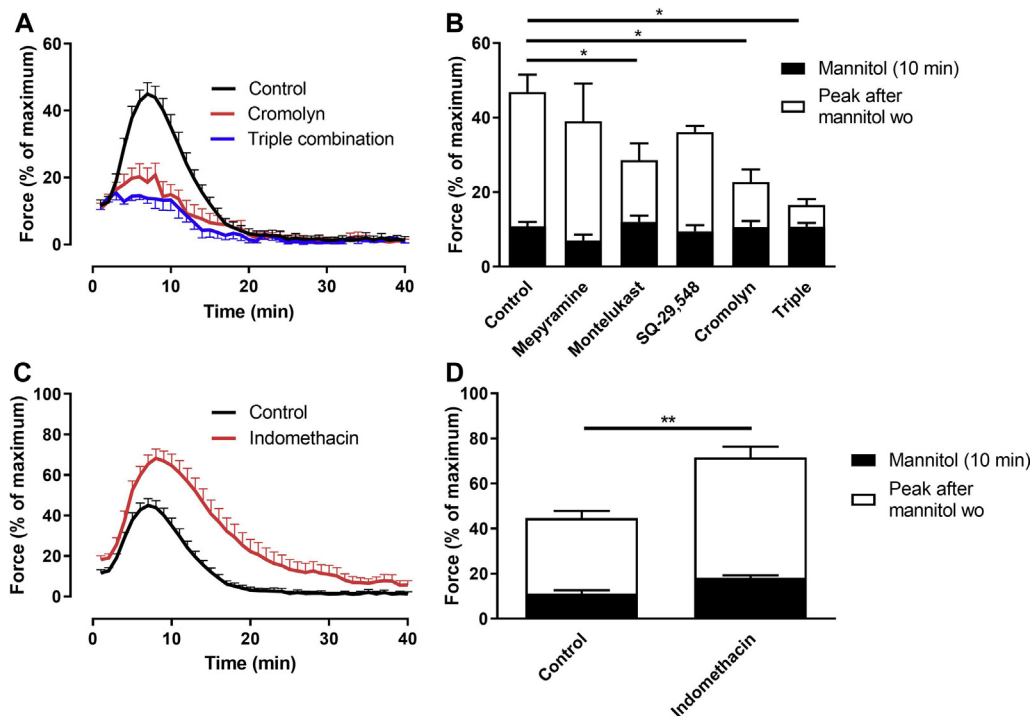


FIG 2. Contractions of human small bronchi in response to 10 minutes of increased osmolarity by mannitol. **A**, Time-response contraction over 40 minutes (number of patients = 5-15, number of segments used = 5-23). **B**, E_{max} displays contraction in relation to maximal contractility in the presence or absence of mepyramine (1 $\mu\text{mol/L}$), montelukast (1 $\mu\text{mol/L}$), SQ-29,548 (1 $\mu\text{mol/L}$), cromolyn (100 $\mu\text{mol/L}$), or a triple combination of mepyramine (1 $\mu\text{mol/L}$), montelukast (1 $\mu\text{mol/L}$), and SQ-29,548 (1 $\mu\text{mol/L}$). Data represent means \pm SEMs (number of patients = 5, number of segments used = 5). **C**, Time-response contraction over 40 minutes in the presence or absence of indomethacin (3 $\mu\text{mol/L}$; number of patients = 8, number of segments used = 16). **D**, E_{max} displays contraction in relation to maximal contractility in the presence or absence of indomethacin (3 $\mu\text{mol/L}$). Data represent means \pm SEMs. * $P < .05$ and ** $P < .01$.

was followed by 60 minutes of equilibration, including several washes to allow the segment to return to baseline tension.

Assay development

Mannitol was dissolved in the buffer solution to the desired osmolarity (400, 650, and 850 mOsm) and exchanged with normosmolar (317 mOsm) buffer solution to study the response of increased osmolarity in the human airways. In the first experiments mannitol was administered during a 50-minute time period. For the main experiments, segments were exposed to 850 mOsm mannitol for 10 minutes, which thereafter was exchanged with buffer for observation of the smooth muscle response over an additional 40 minutes.

Receptor antagonists targeting TP (SQ-29,548), H_1 (mepyramine), and cysteinyl leukotriene (CysLT) type 1 (montelukast) or the mast cell-stabilizing agent cromolyn were added 45 minutes before changes in osmolarity to investigate the receptors involved in the mannitol-induced mast cell response.

Prostanoid contributions were investigated by means of administration of a nonselective COX enzyme inhibitor (indomethacin); receptor antagonists targeting EP_2 (PF-04418948), EP_3 (ONO-AE3-208), and prostacyclin receptor (IP; CAY 10441); and a receptor agonist targeting EP_2 (ONO-AE1-259) 45 minutes before changes in osmolarity. During the experiments, drug interventions were present during both the hyperosmolar and subsequent normosmolar period. All experiments were ended by addition of histamine (1 mmol/L) as a reference for the maximal contraction.

Bronchial relaxation was studied in segments exposed to receptor agonists targeting M_3 (carbachol; 1 $\mu\text{mol/L}$) to produce a stable precontraction before a cumulative addition of agonists targeting IP (cicaprost) or β_2 -adrenoceptor (salbutamol). These experiments were ended with a combination of the phosphodiesterase inhibitor (papaverin; 0.1 mmol/L) and the nitric oxide donor (sodium nitroprusside; 0.1 mmol/L) as a reference for maximal relaxation.

Separate segments were denuded from the epithelium by means of luminal brushing with a cotton swab to investigate epithelial involvement. The isolated bronchial rings were collected directly after the experiment and immersed in plain 4% buffered formaldehyde for at least 24 hours, embedded in paraffin, and stained with hematoxylin and eosin solution (catalog nos. GHS316 and E4382) to investigate involvement of the epithelium. This was followed by sectioning and examination with a light microscope.

Media collection and measurement of released mediators

The buffer solution surrounding the preparations was collected 20 minutes after exposure to 850 mOsm mannitol, snap-frozen in liquid nitrogen, and stored in -80°C until analysis. Histamine levels were measured with glass fiber-coated microtiter plates (RefLab ApS, Copenhagen, Denmark) and prepared according to the manufacturer's instructions. CysLTs, PGD_2 , PGE_2 , and 6-keto $PGF_{1\alpha}$ levels were measured by using an ELISA express kit (Cayman Chemical, Ann Arbor, Mich), according to the manufacturer's instructions. Detection limits were 20 pg/mL (CysLT), 3.1 pg/mL (PGD_2 MOX), 15 pg/mL (PGE_2) and 6 pg/mL (6-keto $PGF_{1\alpha}$), and data were normalized to tissue wet weight.

Drugs and suppliers

NaHCO_3 , CaCl_2 and KCl were obtained from VWR (West Chester, Pa). Histamine dihydrochloride, mepyramine maleate, mannitol (D-mannitol), salbutamol, papaverine, sodium nitroprusside, Krebs-Henseleit buffer, hematoxylin and eosin solution, and indomethacin were purchased from Sigma-Aldrich. ONO-AE3-208 and ONO-AE1-259 were kind gifts from ONO Pharmaceuticals (Osaka, Japan). Montelukast and cicaprost were obtained from

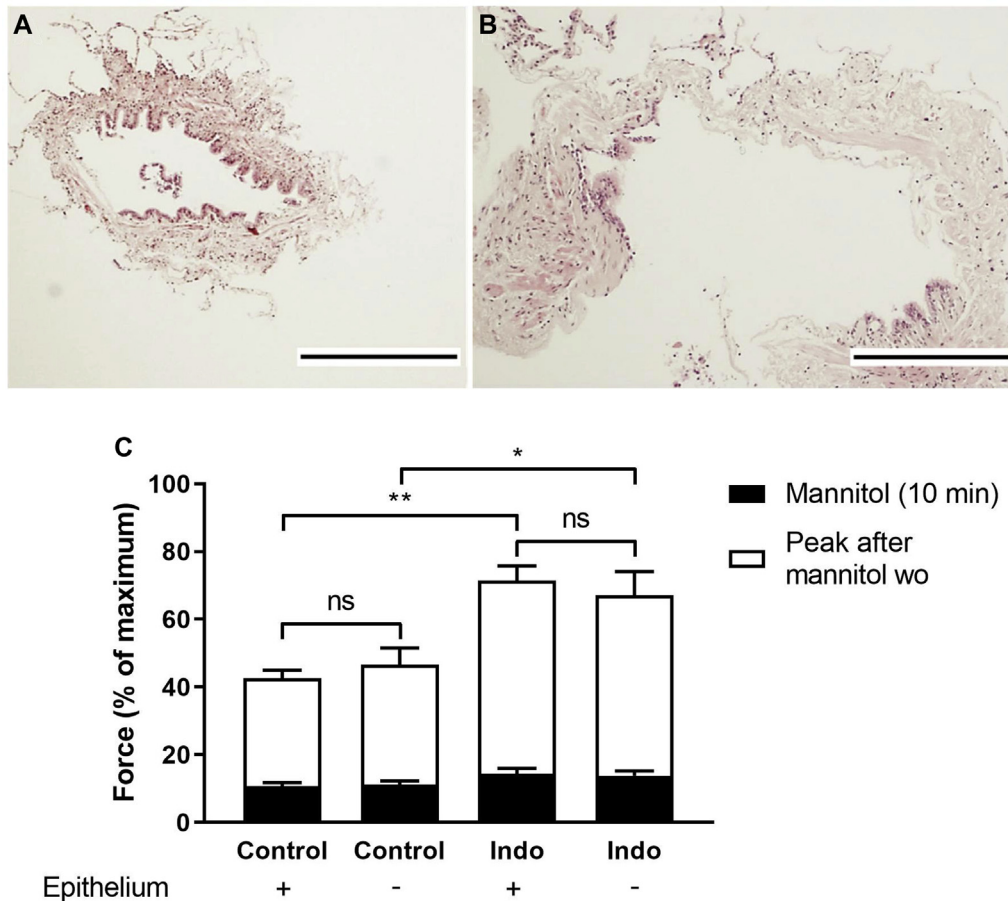


FIG 3. **A** and **B**, Photomicrographs of isolated human bronchial preparations with hematoxylin and eosin staining depicting intact (Fig 3, **A**) and denuded (Fig 3, **B**) bronchial epithelium. Scale bars = 1 mm. **C**, Contractions of human small bronchi in response to 10 minutes of increased osmolarity by mannitol. E_{max} displays contraction in relation to maximal contractility in the presence or absence of indomethacin (*Indo*) in bronchial segments with (+) or without (-) intact epithelium. *wo*, Washout. Data represent means \pm SEMs (number of patients = 5, number of segments used = 5). * $P < .05$ and ** $P < .01$. *ns*, Not significant.

Cayman Chemical. PF-04418948 was a kind gift from Pfizer Central Research Division (Groton, Conn). Dulbecco modified Eagle medium were obtained from Gibco.

Calculations and statistics

Statistical analysis was performed by using paired 1-way ANOVA, followed by the Bonferroni multiple comparison test, with P values of less than .05 considered significant. For nonparametric data of patient groups, the Mann-Whitney U test was used. For analysis, GraphPad Prism 6.01 (GraphPad Software, San Diego, Calif) was used.

RESULTS

Sustained exposure to hyperosmolar mannitol causes smooth muscle paralysis

Segments were exposed to 850 mOsm mannitol, which induced a small sustained contraction (E_{max} , $12.1\% \pm 1.6\%$; Fig 1, **A**). This contraction, which ensued after 1 hour of maintained exposure to hyperosmolarity, was not inhibited by antagonists and inhibitors that blocked the effect of histamine, CysLTs, and COX products (1 $\mu\text{mol/L}$ mepyramine, 1 $\mu\text{mol/L}$ MK-886, and 3 $\mu\text{mol/L}$ indomethacin; $n = 5$; data not shown). Moreover, the preparations were seemingly paralyzed and did not respond to addition of histamine (Fig 1, **A**).

Brief exposure to hyperosmolar mannitol causes mast cell-mediated contraction

In contrast, when bronchi were exposed only briefly to mannitol for 10 minutes, a strong contraction developed as soon as mannitol was washed away (Fig 1, **B**). This response reached its maximum 7 ± 1 minutes after the washout period (ie, 17 ± 1 minutes from the initial mannitol addition; Fig 1, **B**). With this protocol, the preparation displayed normal responsiveness to a supramaximal concentration of histamine (Fig 1, **B**). Responses to mannitol were also observed for lower concentrations (450 and 600 mOsm), but 850 mOsm consistently produced the greatest response and was therefore used for the remainder of the study (Fig 1, **C**).

This substantial contraction to the highest dose of mannitol (E_{max} , $46.9\% \pm 4.6\%$) was attenuated by antagonizing the CysLT₁ receptor with montelukast (E_{max} , $28.6\% \pm 4.5\%$; $P < .05$; Fig 2, **B**). The response to mannitol was numerically smaller in the presence of either the H₁ receptor antagonist mepyramine (E_{max} , $39.0\% \pm 10.2\%$) or the thromboxane TP receptor SQ-29,548 (E_{max} , $36.1\% \pm 1.7\%$), but neither of these 2 interventions reached significance ($P = .64$ and $.75$, respectively; Fig 2, **B**). However, the response was almost abolished by the triple-receptor antagonism provided by the combination

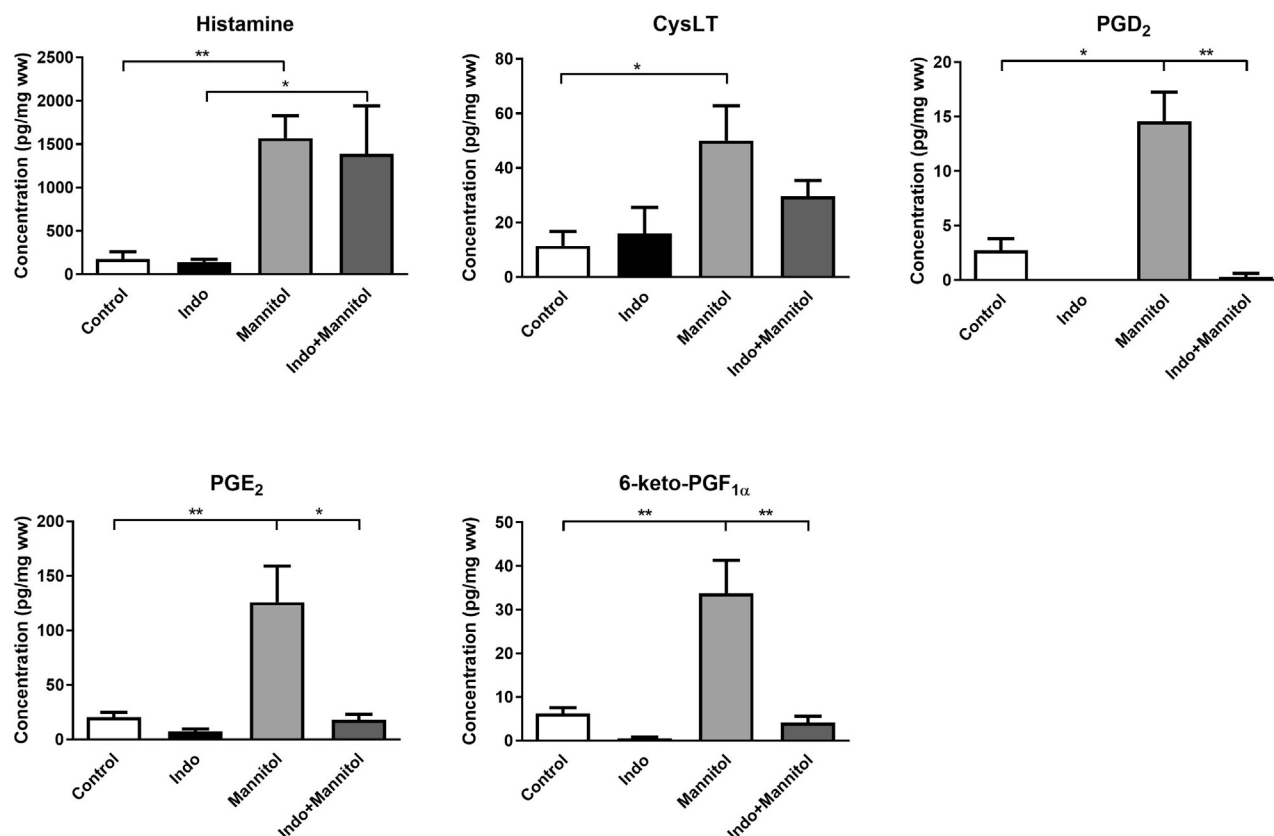


FIG 4. Measurement of mannitol-induced release of histamine, CysLT, PGD₂, PGE₂, and 6-keto PGF_{1α}. Media were collected in the absence (number of patients = 7, number of segments used = 7) or presence (number of patients = 4; number of segments used = 4) of indomethacin (*Indo*; 3 μmol/L) 20 minutes after addition of mannitol. Data represent means ± SEMs, **P* < .05 and ***P* < .01.

of montelukast, mepyramine, and SQ-29,548 (E_{max} , 16.6% ± 1.6%; P < .05; Fig 2, A and B). Likewise, the mast cell-stabilizing agent cromolyn inhibited the mannitol-induced contraction (E_{max} , 22.7% ± 3.4%; P < .05; Fig 2, A and B). In line with the first results, the initial small contraction in the presence of mannitol was not inhibited by the triple antagonism or cromolyn (Fig 2, B). On the other hand, the unselective COX inhibitor indomethacin augmented the contraction induced by mannitol exposure (E_{max} , 71.6% ± 4.8%; P < .05; Fig 2, C and D).

The histologically verified removal of the airway epithelium in segments (Fig 3, A and B) did not affect the control response to mannitol or the enhanced response by indomethacin (E_{max} , 67.1% ± 7.0%; Fig 3, C).

Mannitol induces release of mast cell mediators

Tissue bath buffer was collected for analysis 20 minutes after continuous exposure to mannitol to document the release of mast cell mediators. In medium from mannitol-exposed segments, marked increases in values of histamine (1570 ± 258 vs 175.2 ± 85.3 pg/mg, 9.0-fold; P < .05), CysLTs (50.0 ± 12.8 vs 11.4 ± 5.3 pg/mg, 4.4-fold; P < .05), PGD₂ (14.6 ± 2.7 vs 2.7 ± 1.1 pg/mg, 5.4-fold; P < .05), PGE₂ (125.9 ± 32.9 vs 20.2 ± 4.7 pg/mg, 6.2-fold; P < .05), and the hydrolysis product of prostacyclin 6-keto PGF_{1α} (33.8 ± 7.5 vs 6.2 ± 1.3 pg/mg, 5.5-fold; P < .05) were found compared with values in control segments.

Mannitol exposure was performed in the presence of the unselective COX inhibitor indomethacin (3 μmol/L) to further investigate the involvement of prostanoids. Release of PGD₂ (0 ± 0 pg/mg), PGE₂ (18.0 ± 5.2 pg/mg), and 6-keto PGF_{1α} (4.1 ± 1.5 pg/mg) was inhibited by indomethacin (P < .05), whereas neither CysLTs nor histamine were affected (Fig 4).

Evidence that endogenous PGE₂ and PGI₂ repress mannitol-induced contractions

The possible involvement of COX products responsible for increased contractility after treatment with indomethacin were first pharmacologically characterized by using specific receptor antagonists and agonists for PGE₂. Pretreatment with the EP₂ receptor antagonist PF-04418948 (1 μmol/L; E_{max} , 67.8% ± 3.8%) or the EP₄ receptor antagonist ONO-AE3-208 (1 μmol/L; E_{max} , 65.6% ± 6.4%) caused an increase of the maximal contraction to mannitol (P < .05). Consistent with the effects of the EP₂ antagonist, treatment with the selective EP₂ receptor agonist ONO-AE1-259 abolished contraction after the mannitol washout period (Fig 5, A).

Next, evidence was obtained implicating that endogenous PGI₂ also modulated the response to mannitol. Thus pretreatment with the IP receptor antagonist CAY 10441 (1 μmol/L; E_{max} , 68.6% ± 4.6%) induced a similar increase in maximal contraction to mannitol (P < .05) as seen with the EP₂ and EP₄ antagonists (Fig 5, B and C). The relaxant property of the IP receptor was confirmed in bronchial segments precontracted with carbachol

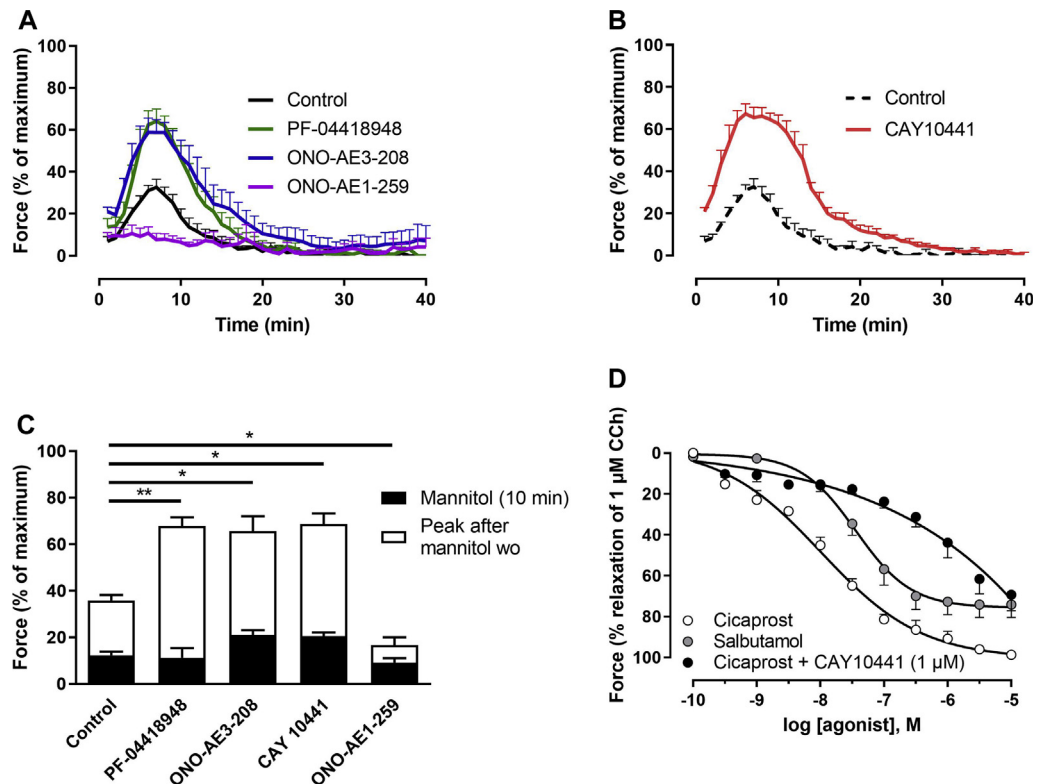


FIG 5. Responses of human small bronchi. **A** and **B**, Time-response contractions after 10 minutes of increased osmolarity by mannitol in the presence or absence of PF-04418948 (1 $\mu\text{mol/L}$), ONO-AE3-208 (1 $\mu\text{mol/L}$), or ONO-AE1-259 (1 $\mu\text{mol/L}$; Fig 5, **A**) or CAY 10441 (1 $\mu\text{mol/L}$; Fig 5, **B**). **C**, E_{max} displays contraction in relation to maximal contractility during (black bar) or after (white bar) normalized osmolarity (number of patients = 5, number of segments used = 5). * $P < .05$ and ** $P < .01$. **D**, Cumulative addition of salbutamol or cicaprost in the presence or absence of the selective IP receptor antagonist CAY 10441 (1 $\mu\text{mol/L}$) on segments precontracted with carbachol (1 $\mu\text{mol/L}$) in the presence of the EP₂ receptor antagonist PF-04418948 (1 $\mu\text{mol/L}$) and EP₄ receptor antagonist ONO-AE3-208 (1 $\mu\text{mol/L}$; number of patients = 5, number of segments used = 5-8). Data represent means \pm SEMs, and in Fig 5, **B**, the control (dotted line) is reproduced from Fig 5, **A**.

(1 $\mu\text{mol/L}$) in the presence of the EP₂ receptor antagonist PF-04418948 (1 $\mu\text{mol/L}$) and the EP₄ receptor antagonist ONO-AE3-208 (1 $\mu\text{mol/L}$). In this setting, in bronchi unexposed to mannitol, the selective IP agonist cicaprost caused a concentration-dependent relaxation (pEC₅₀, 7.8 ± 0.1 ; minimum effect [E_{min}], $94.3\% \pm 1.6\%$). Pretreatment with the selective IP receptor antagonist CAY10441 (1 $\mu\text{mol/L}$) produced both a rightward shift of the concentration-response relationship, as well as reduced relaxation to cicaprost (pEC₅₀, 6.1 ± 0.1 ; E_{min} , $73.3\% \pm 6.1\%$; $P < .05$). In comparison, the clinically used β_2 receptor agonist salbutamol was both less potent and efficacious (pEC₅₀, 7.3 ± 0.1 ; E_{min} , $72.0\% \pm 6.6\%$; $P < .05$) in the same tissue (Fig 5, **D**).

Bronchial responses in relation to donor characteristics

Table I summarizes characteristics of the subjects from whom small bronchi were obtained. There were no sex differences between clinical baseline data nor did the response of segments to mannitol relate to sex, age, body mass index, allergy, or the indication for surgery (data not shown). None of the preparations from subjects with doctor-diagnosed asthma (6/48) or spirometry indices suggesting chronic obstructive pulmonary disease (5/48)

responded qualitatively or quantitatively differently from other preparations.

DISCUSSION

This study introduces a new experimental protocol in explanted human small airways in which brief exposure to hyperosmolar mannitol triggers mast cell-dependent contractions. The supporting evidence included both characteristic pharmacology and actual measurement of mast cell mediators in the tissue bath. Furthermore, applying both global inhibition of COX biosynthesis with indomethacin and specific pharmacologic antagonism of EP₂, EP₄, and IP receptors, the investigation indicates that endogenous PGE₂ and PGI₂ exert negative feedback functions that dampen the consequences of mast cell activation.

First, the conundrum that mannitol is an effective stimulus for bronchoconstriction when inhaled in asthmatic patients but not in the previously published work on isolated airways was resolved. It was confirmed that persistent hyperosmolar stimulation causes a contracture that cannot be altered by mast cell inhibitors or relaxant agents.¹³ The preparation appeared paralyzed and did not react to supermaximal concentrations of histamine. Previous work with bovine tracheal strips suggests that this unresponsiveness can be explained by a block of the excitation-contraction

coupling caused by membrane hyperpolarization.¹² Presumably, the solution is that brief exposure of the isolated bronchi is enough to cause a strong release of mediators without arresting the smooth muscle in a contractile state. Furthermore, the concentration found to have the strongest effect, 850 mOsm, can simulate the conditions for EIB because it has been estimated that the osmolarity of the airway liquid surface is around 900 mOsm after exercise.¹⁷

Second, it was demonstrated that the main contraction after the transient mannitol exposure was due to release of mast cell mediators based on 3 lines of evidence. First, the contraction was associated with release of histamine, CysLTs (LTC₄, LTD₄ and LTE₄), and PGD₂ into the tissue bath. Second, combined pharmacologic antagonism of the H₁ receptors for histamine, the TP receptor for PGD₂, and the CysLT₁ receptor for the CysLTs completely abolished the response. Finally, the mast cell stabilizer cromoglycate attenuated the reaction. The same set of support has been shown for IgE-dependent contractions of this human small airway preparation.¹² Thus the main contraction induced by mannitol was partly inhibited by montelukast or cromolyn, findings that are similar to the effects of interventions with these mechanisms in patients with EIB.¹⁸⁻²¹

Although the level of histamine was the greatest of the measured mediators after mannitol stimulation (31-fold greater than CysLTs), the contraction was not significantly reduced by the H₁ receptor antagonist alone. This agrees with data for single antagonism after anti-IgE challenge in human bronchi²² or EIB in adult asthmatic patients²³ showing that antagonism of CysLTs alone was more efficacious than antihistamines, which is in line with the consistent findings that the CysLTs are about 1000-fold more potent than histamine in human bronchi and, in addition, cause more long-lived contractions.^{24,25} Likewise, TP antagonism of PGD₂ alone did not significantly inhibit the peak response, although the value was numerically smaller.

The phenomenon that more powerful contractions of main agonists (CysLTs in this case) can mask responses to comediators that are less potent has been described for mast cell-dependent contractions in other models.²⁶ Thus the complex activation of several contractile receptors during hyperosmolar exposure in bronchial segments might be similar in patients with EIB and support the need of treatment with a combination of receptor antagonists. The approach to inhibit all 3 main classes of mast cell mediators (CysLTs, histamine, and PGD₂) was demonstrated to be effective here but has not yet been tested in asthmatic patients either for EIB or allergen-induced bronchoconstriction.

Third, there was also release of PGE₂ and the metabolite of PGI₂, 6-keto PGF_{1α}, after mannitol challenge. This is another similarity with the *in vivo* situation. Metabolites of these 2 prostanoids have been recovered in urine from asthmatic patients after eucapnic voluntary hyperpnea with dry air to mimic EIB.¹¹ Although not proved in this first study, it is presumed that these 2 bronchoprotective prostanoids are released from other sources than mast cells, such as epithelial cells, fibroblasts, macrophages, and endothelial cells. We base this assumption on data we recently published on the kinetics of lipid mediator release after challenge with anti-IgE in human bronchi.²⁷ Whereas release PGD₂ and LTC₄ peaked at 15 minutes after challenge, PGE₂ and 6-keto PGF_{1α} showed slower increments that increased with time.

Furthermore, in line with *in vivo* studies²⁸ and work using anti-IgE stimulation of the isolated small bronchi,¹² PGE₂ was clearly

implicated as one component of the indomethacin-enhanced response to mannitol. The study showed that both the EP₂ and EP₄ receptors were involved and had the same overall effects on the reaction. Based on the previous study of mast cell-dependent contractions in this model,¹² it is suggested that the EP₂ effect involves inhibition of mast cell mediator release,^{29,30} whereas the EP₄ response is due to smooth muscle relaxation.^{12,31} Again, it remains for future work to define the mode of action also in this model. However, the contraction was almost completely abolished when the segments were pretreated with the EP₂ receptor agonist. This inhibitory effect replicates the effect of inhaled PGE₂ before exposure to EIB.⁵

Concerning PGI₂, it was somewhat surprising that antagonism of the IP receptor had such a profound enhancing effect on the mannitol reaction. For the first time, these data demonstrate that activation of the IP receptor by an endogenous agonist has a protective role on the hyperosmolar-induced contraction. Therefore we further characterized the influence of IP receptor activation on the small airways. The previously described relaxant properties of PGI₂³² and its analogues³³ were confirmed by relaxation induced by the IP receptor agonist cicaprost, which was blocked by the antagonist CAY10441. Compared with previous studies,³³ cicaprost had a 10-fold greater potency and a markedly stronger relaxant effect in the present study. This might be due to the small airways used in the current study and more central airways in previous studies. This study has not addressed whether PGI₂ in addition to bronchorelaxation also can modulate release of mast cell mediators.

Absence of the epithelium did not modify the contraction response to mannitol challenge in this study either at baseline or after treatment with indomethacin. This was a surprise because data generally suggest that the epithelium is the major source of PGE₂.³⁴ Because the study found that antagonism of 3 different receptors (EP₂, EP₄, and IP) provided similar effects as the COX inhibitor indomethacin, it appears that there are parallel pathways for endogenous protection against hyperosmolar activation of mast cells. However, the comparison with indomethacin is not straightforward because the intervention also removes contractile prostanoids, such as PGD₂, thromboxane A₂, and PGF_{2α}. Therefore the relative role of the inferred prostanoids in the phenomenon of refractoriness to repeated exercise will require new studies taking into account the amounts released, their kinetics, their interactions, and responses to specific interventions with distal enzymes catalyzing the formation of individual primary prostanoids.

Induction of contractions to mannitol in bronchi that were mostly from nonasthmatic donors might seem contradictory to the clinical use of mannitol for diagnosis of asthma and airway hyperresponsiveness. However, we have previously documented that nonasthmatic subjects *in vivo* release mast cell mediators when inhaling mannitol, although they do not react with bronchoconstriction.⁹ Responsiveness to hyperosmolar challenge also occurs in nonprimed isolated mast cells,^{13,14} but it remains to be explained why the nonasthmatic isolated bronchi contracted in response to released mediators. One possibility is that cutting the bronchi into segments and placing them in tissue baths disrupted the structural barriers and created improved opportunities for the released mediators to reach smooth muscle cells. Thus experimental procedures might mimic typical factors associated with the asthmatic phenotype, such as diminished integrity of the airway epithelium,³⁵ infiltration of mast cells into the smooth muscle compartment,³⁶ and increased airway responsiveness.³⁷

Taken together, using the introduced new *ex vivo* protocol for mannitol challenge of isolated human bronchi, the results mimicked airway constriction, mediator release, and pharmacologic responses when EIB is triggered in asthmatic patients *in vivo*. We found that hyperosmolar exposure caused contractions explained by the release of contractile mediators from mast cells in the tissue but also the release of prostanoids repressing the contraction. In addition to the expected inhibitory effect of PGE₂, for the first time, this study found evidence that endogenous prostacyclin should be considered as a potent bronchoprotective mediator. Because potent IP agonists are used to treat pulmonary hypertension, it would seem possible in the near future to translate our findings into proof-of-concept studies of whether the IP receptor is a suitable new target for treatment of asthma in general and EIB in particular.

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Key messages

- A new protocol for *ex vivo* challenge of explanted human small airways with mannitol has been developed.
- The challenge triggers a mast cell–dependent contraction mimicking EIB *in vivo*.
- PGE₂ and prostacyclin are released as well, and they act as bronchoprotective modulators.

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