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Different effects of inhaled amiloride and frusemide on airway responsiveness to dry air challenge in asthmatic subjects

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Different effects of inhaled amiloride and frusemide on airway responsiveness to dry air challenge in asthmatic subjects. L.T. Rodwell, S.D. Anderson, J. du Toit, J.P. Seale. ©ERS Journals Ltd 1993.

ABSTRACT: Amiloride, a Na^+ channel blocker, and frusemide, an inhibitor of the $Na^+/K^+/2Cl^-$ co-transporter on the basolateral surface of airway epithelial cells, have the potential to affect water transport across the airway epithelium. As isocapnic hyperventilation challenge (ISH) with dry air may provoke airway narrowing in asthmatic subjects by dehydrating the airways, inhaled amiloride and frusemide may reduce airway responsiveness by effecting airway hydration.

Fifteen asthmatic subjects (6 females, 9 males), who had a fall in forced expiratory volume in one second (FEV_1) of 20% after ISH, inhaled amiloride (11 mg), or its vehicle, from a Fisoneb™ ultrasonic nebulizer, within 10 min before ISH. On a separate day, eight of these subjects inhaled frusemide (38 mg), from the same Fisoneb™, 10 min before ISH. After breathing, 30 *l* at resting ventilation, subjects breathed at 30% of their maximum voluntary ventilation (MVV *i.e.* predicted $FEV_1 \times 35$), then at 60% MVV, and finally at MVV for 3 or 4 min. FEV_1 was measured 1, 3, 5, 7 and 9 min after each period, or until it was stable. Airway sensitivity was expressed as the ventilation ($l \cdot min^{-1}$) which provoked a 10, 15, 20 or 30% fall in FEV_1, (PVE_{10}, PVE_{15}, PVE_{20} and PVE_{30}, respectively).

There was no significant difference in the $PVE_{10,15,20,30}$ between the vehicle and amiloride treatment day; however, in the 8 subjects who inhaled frusemide, frusemide caused a significant increase in the PVE_{20} when compared to amiloride.

In conclusion, inhaled amiloride failed to protect against ISH, whereas frusemide was effective at reducing airway responsiveness. Further studies are needed to explain the mechanism of action of frusemide.

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Water loss from the airways as a result of conditioning inspired air (*i.e.* alveolar temperature and saturation) is the stimulus whereby hyperpnoea with dry air provokes airway narrowing [1–4]. The precise mechanism whereby the loss of water causes the airways to narrow is not known. There have been several proposals: 1) that dehydration and the consequent increase in airway osmolarity results in the release of substances that cause airway smooth muscle to contract [5, 6]; and 2) that airway cooling leads to a reactive hyperaemia of the bronchial circulation and oedema of the airway wall, both of which cause the airways to narrow [7, 8].

The amount of airway surface water available to humidify inspired air in the first ten generations of airways is thought to be less than 1 ml [5]. Water lost through evaporation from these airways must be replaced instantaneously, if isotonicity of the airway surface liquid is to be maintained. The most readily available source of water to replace this loss is from the epithelial cells and the submucosa below the basement membrane. Water may also move onto the airway surface following active transport of Cl^- ions across the apical membrane of the airway epithelium.

KILBURN [9] proposed that an important source of water is the fluid which moves continuously up the mucociliary escalator. Although this hypothesis has not been proved, it could occur, because the human airway epithelium is normally absorptive [10], whereby Na^+ moves from the lumen into the epithelial cell with water following. The inhibition of Na^+ absorption across the apical surface of epithelial cells by amiloride [11] stimulates Cl^- secretion [12]. These two events should act to increase the airway surface liquid (ASL) and, thus, reduce the dehyrating effects of evaporative water loss. We reasoned that amiloride could provide protection against a stimulus, such as dry air isocapnic hyperventilation, by maintaining airway surface liquid levels, and preventing its dehydration.

Frusemide is another diuretic that can alter water transport by inhibiting the co-transport of Na^+ and Cl^- across the basolateral membrane of airway epithelial cells [13]. Frusemide is known to inhibit exercise-induced asthma [14, 15], responses to hyperosmolar saline [16, 17], and hyperventilation challenge [18, 19], and it may do so by reducing the water loss from the submucosa.

Theoretically this would protect the submucosa from partial dehydration [17], but result in a depletion of ASL. The different sites of action for these diuretics suggest that there would be differences in responses to dry air challenge.

The purpose of this study was to investigate the effect of inhaled amiloride and inhaled frusemide on airway narrowing provoked by isocapnic hyperventilation with dry air in asthmatic subjects.

Methods

Subjects

Fifteen asthmatic subjects (6 females, 9 males) participated in the amiloride study, and eight of these subjects took part in the study with frusemide. All subjects had clinically stable asthma, and had positive skin prick tests for at least one of eight common allergens. All asthma medications were withheld for 6 h before each visit. To be included in the study, all subjects had a 20% (or more) fall in forced expiratory volume in one second (FEV_1) from baseline, in response to isocapnic hyperventilation challenge on a control day. The study was approved by the Ethics Committee at Royal Prince Alfred Hospital (Protocol No. X984), and consent was obtained in writing from all subjects. Parental written consent was also obtained from the one subject under 18 yrs of age. Anthropometric details of subjects, regular asthma medications and the ventilation rate required to induce a 20% fall in FEV_1 (PVE_{20}) on the control day are given in table 1.

Study protocol

Isocapnic hyperventilation challenge (ISH). All subjects visited the respiratory laboratory on three separate days, with a minimum of three days and a maximum of 30 days between each visit. This was a double-blind, randomized, vehicle-controlled, cross-over design study, and was completed in 67 days.

Control visit. On arrival at the laboratory, FEV_1 was measured, and repeated 10 min later. If FEV_1 varied by less than 10% between measurements, the subject was allowed to continue in the study. Initially, subjects breathed 30 *l* of medical air at resting ventilation, and the FEV_1 was measured at 1, 3, 5, 7 and 9 min after, or until the FEV_1 was stable, where upon the subject performed isocapnic hyperventilation with a mixture of 4.9% CO_2, 21% O_2, and the balance N_2 [20, 21]. The predicted maximum voluntary ventilation (MVV) for each subject was calculated by multiplying the predicted FEV_1 by 35. The subject voluntarily hyperventilated at 30% of his predicted MVV, and FEV_1 was measured as before. If a 20% fall from baseline FEV_1 did not occur, then subjects continued the challenge and ventilated at 60% of their predicted MVV for 3–4 min. If a 20% fall was not recorded, then subjects ventilated at their MVV for 3–4 min until a 20% fall occurred. If a 20% fall was not recorded then subjects were excluded from further study, and those who recorded a 20% fall in FEV_1 returned for two further visits, when they inhaled either amiloride, or its vehicle, immediately before (Group 1, n=6), or 10 min before (Group 2, n=9), an ISH challenge.

Table 1. – Anthropometric details and baseline data

Pt no.	Age yrs	Sex	Height cm	FEV_1 Control day % pred	PVE_{20} FEV_1 Control day·min^{-1}	Rx	Dose of BDP µg	Group
1	22	F	166	92	48.0	S, BDP	1000	1
2	27	M	171	92	77.0	S, C	-	2
3	23	F	164	95	48.0	S	-	1
4	20	F	160	97	40.5	S, BDP	2000	2
5	17	M	175	70	69.0	S, BDP	1500	2
6	20	M	189	95	105.5	S, BDP	800	2
7	20	M	186	76	62.0	S	-	2
8	25	F	161	101	35.5	S, T, BDP	2000	2
9	23	M	178	70	61.0	S, C, T	-	1
10	21	F	164	71	28.5	S, BDP	1000	2
11	22	M	170	91	51.0	S	-	1
12	20	M	195	79	50.5	S	-	1
13	20	F	169	89	77.0	S, T, BDP	800	2
14	41	M	171	83	41.0	F	-	1
15	19	M	170	88	36.5	S	-	2
Mean	23			86	55.4			
±SD	6			11	20.3			

Anthropometric details, provocative ventilation rate (l·min^{-1}) required to induce a 20% fall in FEV_1 (PVE_{20}) on the control day, and regular medication including doses of beclomethasone dipropionate (BDP), are given for the 15 subjects who participated in this study. Group 1 inhaled amiloride immediately before isocapnic hyperventilation challenge (ISH), and Group 2 inhaled amiloride 10 min before ISH. Rx: prescription; S: salbutamol; F: fenoterol; C: cromolyn; T: theophylline. Subjects 1–8 only participated in both the amiloride and frusemide sections of the study. FEV_1: forced expiratory volume in one second.

Amiloride pretreatment. FEV_1 was measured before amiloride or vehicle inhalation, and measured again immediately before ISH challenge. It was originally intended that all subjects inhale the amiloride or its vehicle, and then immediately commence the ISH challenge. As some subjects complained of "wheeze" after inhaling the amiloride or vehicle, a 10 min period was allowed for recovery before the ISH challenge.

Frusemide pretreatment. Eight of the 15 subjects returned to the laboratory and performed an identical ISH challenge to that performed in the amiloride study. These eight subjects inhaled frusemide 10 min before ISH challenge.

Preparation of amiloride and its vehicle

A 10 $mmol \cdot l^{-1}$ solution of amiloride (pH=6.4, 116 MOsm) was prepared each week. The vehicle was 0.3% NaCl (pH = 6.69, 98 MOsm). It was difficult to increase the osmolality of the amiloride solution to iso-osmolar conditions, as amiloride has limited solubility in electrolyte solutions [22] and, thus, has the potential to cause airway narrowing [23]. The 10 $mmol \cdot l^{-1}$ solution of amiloride and its vehicle were stored in dark glass vials and at room temperature because flocculation occurred if the solution was cooled. If the solution did flocculate, the glass vial containing the amiloride was left to stand in hot water for 5 min, and then manually shaken until the flocculated particles had dissolved. On some occasions this procedure had to be repeated several times.

Six ml of the amiloride solution (10 $mmol \cdot l^{-1}$) or its vehicle were drawn up into a 10 ml syringe, through a 0.2 μm filter (Minisart, Sartorius, Gottingen, Germany), and injected into a Fisoneb™ ultrasonic nebulizer (UN). Subjects wore noseclips and breathed the nebulized aerosol at tidal volume *via* a mouthpiece for 7 min. The Fisoneb™ UN, with a cork placed in the output hole during weighing, was weighed before and after nebulization. Approximately 10.6±2.5 mg of amiloride was delivered.

Preparation of frusemide

The Hoescht preparation of injectable frusemide was used (pH 9.07, osmolarity 303 MOsm). Six ml was placed in the Fisoneb™ UN, which was the same one used to deliver the amiloride. The dose of frusemide delivered from the nebulizer was 37.8±5.0 mg.

Statistical analysis

Airway sensitivity at $PVE_{10,15,20,30}$ FEV_1

Amiloride. To test the overall effect of amiloride and its vehicle on airway responsiveness, the mean difference (±95% confidence interval (CI)) between the vehicle and amiloride treatment days at the rates of ventilation ($l \cdot min^{-1}$) causing a 10, 15, 20 or 30% fall in FEV_1 ($PVE_{10,15,20,30}$) was calculated. An analysis of variance for repeated measures (SAS statistical package, SAS Institute Inc, Cary, NC, USA) was performed on the rates of ventilation ($l \cdot min^{-1}$) causing a 10, 15, 20 or 30% fall from baseline FEV_1 values (*i.e.* $PVE_{10,15,20,30}$) after pretreatment with amiloride and vehicle. This analysis was also performed to test for any interaction between the time the amiloride or vehicle was delivered before ISH challenge (*i.e.* immediately, or 10 min before), and values for $PVE_{10,15,20,30}$.

Frusemide. The mean difference (±95% CI) in PVE_{20} was calculated between the frusemide and amiloride days, the frusemide and vehicle days, and the amiloride and vehicle days, for the eight subjects who inhaled frusemide, amiloride and vehicle. A paired t-test was performed on the mean difference between PVE_{20} measurements on the frusemide and vehicle day, and the mean difference between the PVE_{20} on the amiloride and vehicle ISH challenge days.

Baseline FEV_1 (expressed as a % of the predicted value)

The mean changes in FEV_1 (% pred) (±95% CI) after inhalation of the vehicle and of amiloride were calculated, and the significance of any differences were determined by an analysis of variance for repeated measures. The effect of commencing ISH challenge immediately, or 10 min after, inhaling amiloride was tested using the analysis of variance for repeated measures.

A paired t-test was performed on the post-drug, pre-challenge FEV_1 (% predicted) on the frusemide day and amiloride day to test for differences in lung function between challenge days.

Differences were regarded as significant at $p<0.05$.

High performance liquid chromotography (HPLC)

HPLC was performed on a solution of amiloride that had flocculated, and then been dissolved back into solution, and a sample of amiloride solution taken from the reservoir of the Fisoneb™ UN after nebulization. This was to investigate the possibility that flocculation or ultrasonic nebulization might render the amiloride inactive.

Results

Data are summarized as mean±SD or SEM and, where indicated the mean change ±95% CI.

Amiloride, when given as an aerosol, had no significant effect on airway sensitivity to ISH challenge in this group of 15 asthmatic subjects. The values for sensitivity, measured as the rate of ventilation ($l \cdot min^{-1}$) causing a 10, 15, 20 or 30% fall from baseline FEV_1 were not significantly different between the days of pretreatment with amiloride or vehicle (p=0.99) (fig. 1).

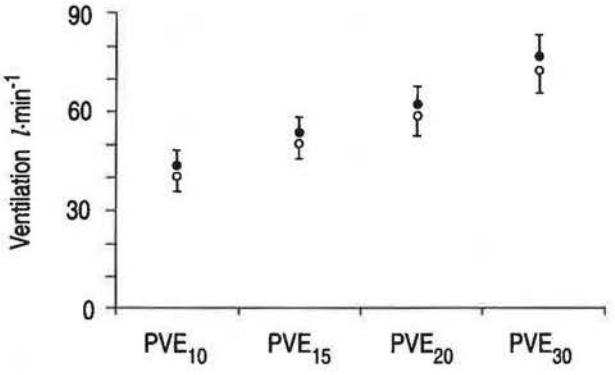

Fig. 1. – Isocapnic hyperventilation challenge. The mean provoking ventilation ($l \cdot min^{-1}$) (±SEM) causing a 10, 15, 20 and 30% fall in FEV_1 (PVE_{10}, PVE_{15}, PVE_{20}, and PVE_{30}, respectively) from the prechallenge FEV_1 after inhaling amiloride (●) or vehicle (○), in the 15 asthmatic subjects who participated in this study. FEV_1: forced expiratory volume in one second.

The mean difference in the rate of ventilation between the vehicle and amiloride days was not significant at PVE_{10} (3.1 $l \cdot min^{-1}$, 95% CI -2.8 to 8.9); PVE_{15} (2.6 $l \cdot min^{-1}$, 95% CI -3.4 to 8.6); PVE_{20} (3.1 $l \cdot min^{-1}$, 95% CI -4.2 to 10.4); or PVE_{30} (4.6 $l \cdot min^{-1}$, 95% CI -3.7 to 12.9).

There was a small reduction in FEV_1 (% predicted) in response to inhaling both the amiloride (3.4%, 95% CI 1.3 to 5.5, p=0.0038) and its vehicle (2.3%, 95% CI -0.6 to 5.3, p=0.11). This reduction in baseline FEV_1 (% pred) was not significantly different between the amiloride or vehicle inhalation days (p=0.42).

When comparing the $PVE_{10,15,20,30}$ between Group 1 (those who commenced ISH challenge immediately after inhaling the aerosol) and Group 2 (those who commenced ISH challenge 10 min after inhaling the aerosol), there was no significant difference between the two groups after amiloride or vehicle inhalation (p=0.74). Therefore, the time between aerosol inhalation and commencement of the ISH challenge had no significant effect on airway sensitivity to ISH.

Although amiloride did not have a statistically significant effect on airway sensitivity to ISH challenge for the group, some individuals experienced reduced responses to ISH challenge after amiloride inhalation. At the ventilation rate which provoked a 20% fall in FEV_1 from prechallenge values, three subjects (nos 7, 9, 15) had a greater than, or equal to, 20 $l \cdot min^{-1}$ improvement in this ventilation rate after inhaling amiloride, and a further two subjects (nos 6 and 14) had a 9.0 and 11.0 $l \cdot min^{-1}$ improvement, respectively. In two subjects (nos 2 and 12) the PVE_{20} was worse after inhaling amiloride by 17.5 $l \cdot min^{-1}$ and 22 $l \cdot min^{-1}$, respectively. In the other eight subjects (nos 1, 3, 4, 5, 8, 10, 11 and 13) there was a mean difference in PVE_{20} (±1SD) of -0.13±3.27 $l \cdot min^{-1}$ on the vehicle and amiloride days.

All subjects complained of the bitter taste of amiloride aerosol, which made it difficult to maintain the double-blind nature of this study.

Frusemide inhibited airway responses in the majority of the 8 subjects, when compared with the responses observed on the day the amiloride was given. Six subjects had a difference in PVE_{20} greater than 21 $l \cdot min^{-1}$ (fig. 2), and two of these subjects (nos 3 and 6) had less than 15% fall in FEV_1 even after ventilating at their MVV for 3–4 min. The mean difference in PVE_{20} between frusemide and amiloride days was 20.9 $l \cdot min^{-1}$ (95% CI 13.6 to 28.1; p<0.001, n=8). The mean difference in PVE_{20} between frusemide and the amiloride vehicle was 21.5 $l \cdot min^{-1}$ (95% CI 7.0 to 36.0; p<0.01, n=8). The corresponding mean difference between amiloride and its vehicle was 1.31 $l \cdot min^{-1}$ (95% CI -7.7 to 10.3, p=NS, n=8).

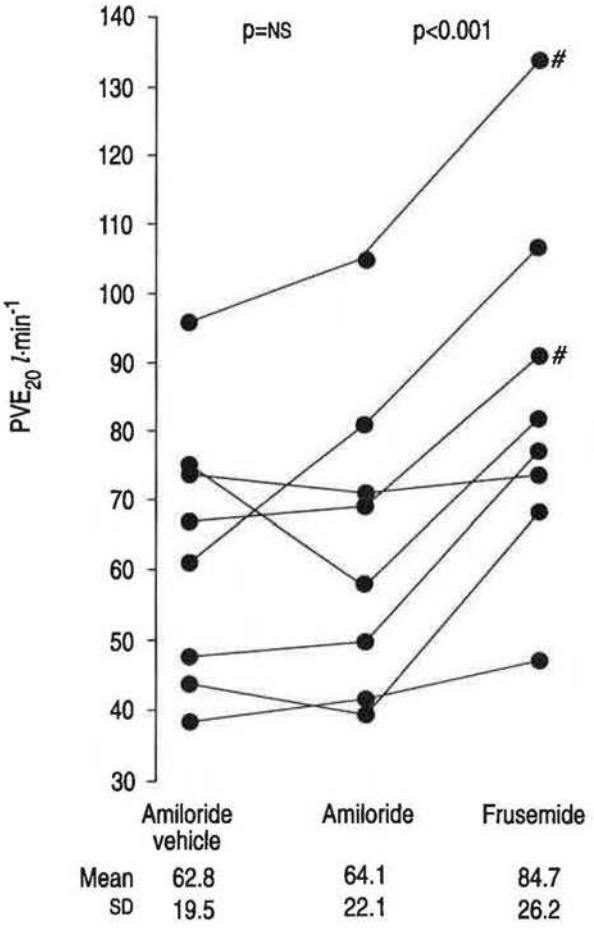

Fig. 2. – Individual PVE_{20} values for the eight subjects (nos 1–8) who performed the isocapnic hyperventilation (ISH) challenge on three separate occasions, *i.e.* after inhaling the amiloride vehicle, amiloride, and frusemide. #: PVE_{20} not achieved, therefore the highest ventilation rate achieved on the study day is given as the PVE_{20}. For abbreviation see legend to figure 1.

The mean difference between the PVE_{20} measurements on the frusemide and vehicle day, and the mean difference between the PVE_{20} on the amiloride and vehicle was significantly different (t=5.59, p<0.001, n=8).

These HPLC analyses revealed no evidence of chemical degradation, structural changes or oxidation of the amiloride solution due to flocculation or nebulization.

Discussion

The results of this study, clearly demonstrated that inhaled amiloride does not alter airway responsiveness to isocapnic hyperventilation with dry air in the majority of asthmatic subjects studied. By contrast, inhaled frusemide significantly reduced or inhibited the airway responsiveness to dry air challenge in all but two of the subjects.

These findings with frusemide confirm the observations of BIANCO *et al.* [14], who used exercise as a means to develop hyperpnoea. We found frusemide to be more effective against ISH with air at ambient temperature than GRUBBE *et al.* [18], and SEIDENBERG *et al.* [19], who investigated the effects of frusemide on hyperventilation with air at subfreezing temperatures.

Other investigators, using metabisulphite challenge (MBS), have also recorded similar findings with frusemide and amiloride [24, 25]. As MBS is likely to work through nerve endings [26], the effects of these two drugs may be independent of water transport.

There are many technical reasons why amiloride may not have had a significant effect. Firstly, the drug may not have reached the airways, or may not have been given in sufficient concentration to inhibit Na^+ absorption. This is unlikely, as inhaled frusemide inhibited airway responses in the same subjects in whom inhaled amiloride did not. Both drugs were delivered from the same Fisoneb™ UN, suggesting that generation and deposition of the amiloride aerosol in the airways would have been adequate, using this nebulizer. The Fisoneb™ UN also delivers amiloride more effectively to the conducting airways than a jet nebulizer [27].

Flocculation and ultrasonic nebulization of the amiloride may have rendered it inactive. For this reason, HPLC analysis was performed on the dissolved flocculate and nebulized amiloride taken from the reservoir of the Fisoneb™ UN. The HPLC analysis of both solutions revealed unchanged amiloride. Therefore, it is unlikely that the generated aerosol or its deposition in the conducting airways could explain the lack of efficacy of the amiloride.

Amiloride is known to be rapidly cleared from the airways of sheep [22], having a half-time in the airways of only 10.5 min. In the present study, we took 7 min to deliver the amiloride, and in six subjects the ISH challenge was performed immediately. In this group, we would have expected a reduction in the response at least at low levels of ventilation, as the first challenge period would have been completed within 5–8 min of delivering the aerosol. There was no difference in responses between the group who were challenged immediately after amiloride and those who had a 10 min wait before the challenge commenced.

In the study of APP *et al.* [28], amiloride was given in a concentration of 1 $mmol \cdot l^{-1}$ to patients with cystic fibrosis. Whilst the greatest improvement in mucociliary clearance (reflecting improved airway hydration) occurred in the first 10 min, improvement continued for a further 30 min. We used a 10 $mmol \cdot l^{-1}$ solution and probably delivered a much greater dose than APP *et al.* [28], because we used an ultrasonic nebulizer rather than a jet nebulizer. Furthermore, only low concentrations of amiloride are needed to inhibit Na^+ movement. KNOWLES *et al.* [12] demonstrated that amiloride of 10^{-5} M was effective in blocking Na^+ when applied to the mucosal surface. APP *et al.* [28] estimated that 70 μg of amiloride was deposited in the airways, whereas in our study, assuming that only 10% of the dose nebulized was deposited in the airways, then approximately 1,000 μg amiloride was delivered.

Amiloride may not have been effective against challenge with dry air because of its ability to release histamine [29, 30]. Furthermore, the amiloride aerosol that we delivered was hypoosmolar, which may have caused some airway narrowing [23], counteracting any beneficial effects due to inhibition of Na^+ transport.

Our finding, that amiloride afforded no protection against dry air challenge in patients with asthma, contrasts with the observation that amiloride improves airway hydration in patients with cystic fibrosis (CF) [28]. In addition to amiloride inhibiting Na^+ absorption, one may have expected an increased rate of Cl^- secretion in subjects with asthma, thus improving airway hydration by two mechanisms. Patients with CF would not have benefited by any Cl^- secretion, as they have a defect in the secretion of this ion [31]. If the dose of amiloride inhibited Na^+ absorption, then we would have expected more airway surface water to be available for humidification, and a delay in the osmotic effects of dehydration. It is possible that amiloride is effective only at low rates of ventilation, when only a fraction of the water required for humidification comes from below the pharynx. For the high rates of ventilation which we used (30% MVV and above), the water loss may have been sufficient to overwhelm any benefit amiloride may have had in delaying water absorption (*via* inhibition of Na^+ absorption) and stimulating its secretion (*via* increased Cl^- secretion). Alternatively, water loss from the ASL may not be important to the airway response to dry air. Furthermore, it is probable that any evaporative water loss could have rendered the ASL hyperosmolar more quickly, as the concentration of Na^+ and Cl^- was increased as a result of inhibiting Na^+ absorption and enhancing Cl^- secretion.

The mechanism by which frusemide acted to prevent the responses to airway drying is not clear, but there are many possibilities. A direct effect on $Na^+/K^+/2Cl^-$ co-transport seems unlikely to explain its efficacy, as bumetanide, a more potent co-transport inhibitor, does not protect against dry air challenge [32]. Frusemide, however, is known to cause vasodilation [33] and may, therefore, enhance the delivery of water to the airway submucosa *via* the bronchial circulation. Whilst frusemide, by its inhibitory action on co-transport may have limited the transport of water across the epithelial cell, its vasodilating effect may have improved water availability. This improved availability of water in the submucosa may have enhanced paracellular movement of water in response to an osmotic stimulus [34]. When frusemide is added to sheep fetal lung, there is a decrease in fetal lung secretion [35], due to frusemide blocking Cl^- channel on the apical surface of the airway epithelial cell. By blocking the Cl^- channel, this would decrease the movement of water into the airway lumen, thus

protecting both the epithelial cell and the submucosa from dehydrating and increasing in osmolarity [17]. This may explain why frusemide inhibits the airway response to hyperventilation challenge.

There are several other mechanisms whereby frusemide could have inhibited or prevented the airway responses to dry air. Firstly, frusemide, in low concentrations, has been shown to inhibit the release of histamine and leukotrienes from human lung tissue in response to allergen challenge [36], implying that it may prevent mast cell release of mediators. Secondly, it is possible that frusemide has an indirect mode of action in protecting against dry air, and by releasing prostaglandin E_2 [37] a known bronchodilator. This study has clearly demonstrated the failure of inhaled amiloride to protect against isocapnic hyperventilation with dry air challenge in most subjects with asthma. The beneficial response to frusemide suggests that further studies are warranted to explain its mechanism of action.

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