

Release of chemical mediators and inflammatory cell influx during early allergic reaction in the nose: Effect of furosemide

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Background: We evaluated the effect of furosemide on allergen-induced rhinitis in a double-blind, crossover, placebo-controlled experiment.

Methods: Fourteen patients with rhinitis who were allergic to house dust were nebulized with an intranasal dose of 20 mg of furosemide or placebo before allergen challenge with an extract of *Dermatophagoides pteronyssinus* (100 BU). Clinical evaluation and nasal lavages with normal saline solution were performed at baseline; after placebo-furosemide nebulization; and at 10, 30, and 60 minutes after allergen challenge. Number of sneezes and a composite symptom score were recorded to evaluate clinical response. Prostaglandin E₂ (PGE₂), PGD₂, peptide leukotrienes and 15-hydroxy, 5,8,11,13-eicosatetraenoic acid (15-HETE) were measured by radioimmunoassay in nasal lavages. Cells were counted and classified as epithelial cells, neutrophils, eosinophils, and others.

Results: No differences in either clinical symptoms or cell influx after allergen challenge were found between furosemide and placebo groups. PGE₂ levels did not change after provocation, and furosemide had no effect on its production. Ten minutes after antigen challenge there was a marked increase of PGD₂ ($p < 0.01$), peptide leukotrienes ($p < 0.01$), and 15-HETE (not significant) on both study days. However, no significant differences in the release of eicosanoids were found between furosemide and placebo groups.

Conclusions: Our observations in the nasal mucosa suggest that furosemide has no effect on the release of proinflammatory and bronchoconstrictor metabolites (PGD₂, peptide leukotrienes, and 15-HETE). In contrast to bronchial asthma, allergen-induced rhinitis was not effectively prevented by furosemide. (*J ALLERGY CLIN IMMUNOL* 1993;92:248-54.)

Key words: Furosemide, *Dermatophagoides pteronyssinus*, nasal mucosa, perennial rhinitis, PGD₂, PGE₂, peptide leukotrienes, 15-HETE.

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Abbreviations used

15-HETE: 15-hydroxy, 5,8,11,13-eicosatetraenoic acid

PG: Prostaglandin

RIA: Radioimmunoassay

Furosemide, a loop diuretic, has been reported to inhibit the bronchoconstrictor response elicited by a variety of different stimuli such as allergen exposure, exercise, distilled water, adenosine, and sodium metabisulfite.¹⁻⁶ The mechanisms involved in this nonspecific, inhibitory effect of furosemide have not yet been clarified.¹⁻⁷ Different hypotheses have been suggested to explain the antiasth-

matic effects of this diuretic. Because furosemide increases the production of prostaglandin E_2 (PGE_2) in the kidneys⁸ and because PGE_2 is a potent immunomodulator and a moderate bronchodilator, it has been proposed that furosemide might prevent bronchoconstriction by enhancing PGE_2 synthesis.⁹ In addition, Anderson et al.¹⁰ have reported that furosemide may prevent the release of histamine and leukotrienes from human lung tissue that is passively sensitized to *Dermatophagoides pteronyssinus*. However, the relevance of these in vitro findings has not been evaluated in in vivo allergen-induced asthma. Inhibition of ion transport in the epithelial cells and the prevention of bronchospasms by action over airway sensory nerves have also been suggested as mechanisms to explain the antiasthmatic effect of furosemide.^{1, 3, 4, 6, 7} None of these hypotheses, however, have been proved.

Bianco et al.¹¹ have also described the preventive effect of furosemide in allergen-induced rhinitis. Because the mechanisms involved in allergic rhinitis and allergic asthma are very similar and because nasal lavage has proved to be a useful tool for the study of the role of inflammatory cells and chemical mediators in allergic and nonallergic reactions, we used the nose to investigate whether furosemide inhibits allergic responses by interfering with the release of arachidonic acid metabolites.

METHODS

Population

Fourteen volunteers (8 men and 6 women) aged 21 ± 3 years (mean $\pm$ SD) with a clinical history of house dust-induced allergic rhinitis and positive skin prick test results for *D. pteronyssinus* were included in the study. All subjects were medical students who gave informed consent to participate in the study. The protocol was approved by the research committee of our institution. Patients were asked not to use any treatment (antihistamines and glucocorticoids) for at least 3 weeks before the study. None of the volunteers had had a respiratory infection for at least 3 weeks.

Study design

A double-blind, crossover study design was used. Subjects were randomly assigned to 2 study days, 1 week apart. After a basal nasal lavage, 20 mg of furosemide or placebo (normal saline) was instilled in the nasal cavity by a hand-driven nebulizer. A second nasal lavage was carried out 10 minutes after furosemide and placebo instillation. Allergen challenge was carried out immediately afterward. Clinical evaluation and nasal washes were subsequently performed at 10, 30, and 60 minutes after allergen provocation. Because

the pH of furosemide solution used in our study was 9, the placebo solution (pH=7.3) was ranged to a pH of 9 by addition of 0.1 mol/L NaOH.

Nasal challenge procedure

Nasal provocations were carried out with the same batch of lyophilized *D. pteronyssinus* extract (Pharmalgen, Pharmacia, Uppsala, Sweden). The allergen extract was reconstituted and diluted to 10,000 BU/ml with phosphate-buffered saline that contained 0.3 mg albumin per milliliter. Mite extracts were instilled in the nose with a nebulizer valve (1 puff in each nostril).

The subjects were asked to rate the severity of rhinorrhea, nasal obstruction, and itching. Scores for each symptom ranged from 0 to 3 (none=0; mild=1; moderate=2; and intense=3). From these recordings, a composite nasal symptom score was calculated as the sum of the individual scores. The number of sneezes was also recorded and analyzed independently of the other symptoms.

Nasal lavage

Nasal lavages were carried out according to the method previously described by Naclerio et al.¹² Briefly, with the subject's head tilted backward, 8 ml of normal saline solution (0.9% NaCl) was instilled into the nose (4 ml in each nostril) while the subject refrained from either breathing or swallowing. After approximately 10 seconds, the subject expelled the mixture of saline and nasal secretions into polypropylene tubes. Nasal lavage recoveries in the present study were 5.3 ± 0.1 ml (66%).

Cell analysis

Nasal fluid obtained by means of nasal washes was centrifuged (500 g at 4° C for 10 minutes). The supernatant was immediately stored at -70° C for posterior mediator assays. The cell pellet was resuspended with 500 ml of phosphate-buffered saline and vigorously shaken with a pipette to dissolve the mucus plugs. Total cell count was obtained in a Neubauer's hemocytometer (Brand, Germany). A total of 3 to 6 cell smears were obtained by low-speed cytocentrifugation (400 rpm, 10 minutes) (Cytospin 2, Shandon, Scientific Ltd., Cheshire, England) with 25×10^3 cells in 75 to 150 μ l. One smear was stained with May-Grünwald Giemsa stain, and the differential cell count of 200 cells was then obtained. Cells were identified according to their structure as epithelial cells, neutrophils, eosinophils, and others.

Analysis of mediators in nasal lavage fluids

Cyclooxygenase metabolites. Extraction of PGE_2 and PGD_2 from nasal lavages was performed according to a method previously described.¹³ Briefly, nasal lavage supernatants were centrifuged at 20,000 g at 4° C for 15 minutes. Supernatant aliquots (0.5 ml) were acidified at a pH of 3 and processed through C_{18} cartridge columns

(J. T. Baker, Inc., Phillipsburg, N.J.). After washing with 10 ml acidified water and 20 ml petroleum ether, prostaglandins were eluted with 8 ml methyl-formate, which was vacuum-evaporated (Savant, Hicksville, N.Y.). Dried residues were redissolved in Tris buffer, and PGE₂ levels were determined by radioimmunoassay (RIA) as previously described with the use of an antisera provided by the Institute Pasteur (Marnes la Coquette, France). PGD₂ measurements were made by means of an RIA kit provided by Amersham International (Amersham, Buckinghamshire, England) as previously described. The recovery of radiolabeled (³H)-PGE₂ that was used as internal standard of the assay was 86%.

Lipoxygenase metabolites. Peptide leukotrienes and 15-hydroxy, 5,8,11,13-eicosatetraenoic acid (15-HETE) were extracted from nasal lavages according to a method previously described.^{13, 14} Supernatant aliquots (2.5 ml) were processed without pH modification through C₁₈ Baker cartridge columns. After washing with 10 ml of water, peptide leukotrienes and 15-HETE were eluted with 5 ml methanol-water (9:1). The eluent was then vacuum-evaporated to dryness. Residues were redissolved in RIA buffer, and peptide leukotrienes and 15-HETE were determined by use of RIA kits provided by Amersham. Antibodies for 15-HETE were highly specific. Antibodies used for peptide leukotrienes antibody cross-reacted to 100% with leukotriene C₄, 46% with leukotriene D₄ and 64% with leukotriene E₄. Recovery of radiolabeled (³H)-peptide leukotrienes and (³H)-15-HETE used as internal standards of the assays was 59% and 97%, respectively.

Statistical analysis

Results are expressed as means $\pm$ SEM. The changes in the concentration of arachidonic acid metabolites and inflammatory cell numbers (expressed in percentages) after allergen challenge were assessed by nonparametric tests (Friedman two-way analysis of variance and Mann-Whitney U test). The Mann-Whitney U test was also used to compare mediator concentrations and influx of cells between placebo and furosemide days. A *p* value of less than 0.05 was considered significant.

RESULTS

Clinical symptoms

Allergen challenge induced symptoms in all patients. Sneezing appeared mostly within the first 10 minutes (10 $\pm$ 2 sneezes with placebo and 11 $\pm$ 3 with furosemide) (Fig. 1, A). The mean symptom score rose to 4.2 $\pm$ 2 after placebo administration and 4.7 $\pm$ 2 after furosemide administration at 10 minutes. The score progressively decreased with administration of furosemide and placebo (Fig. 1, B). No differences were found either in the number of sneezes or in the com-

posite score of symptoms between placebo and furosemide days.

Cell analysis

There were no significant changes in total cell count after allergen challenge either with placebo or furosemide (Fig. 2). Nasal eosinophilia (expressed as percentage of total cell count) increased progressively and was maximal at 60 minutes. This difference, however, was not statistically significant. No difference in the eosinophil response was seen between placebo and furosemide days (Fig. 3).

Neutrophil percentage, like eosinophil percentage, increased steadily and reached maximal values at 60 minutes. The change, however, did not reach statistical significance. There were no differences in neutrophil numbers between furosemide and placebo days (Fig. 3).

The percentage of epithelial cells significantly decreased after allergen challenge both with saline and furosemide at 60 minutes (*p* < 0.05) (Fig. 3). Only a few lymphocytes and unclassifiable cells were seen in cell smears before and after allergen challenge (data not shown).

Arachidonic acid metabolites analysis

Cyclooxygenase metabolites. Mean PGE₂ concentration in the nasal lavage was 58 $\pm$ 12.5 pg/ml on the furosemide day and 57 $\pm$ 12 pg/ml on the placebo day. Neither furosemide (50 $\pm$ 10 pg/ml) nor placebo (68 $\pm$ 14 pg/ml) nebulization caused changes in basal values of PGE₂. These PGE₂ levels did not change appreciably after allergen challenge either with furosemide (39 $\pm$ 10 pg/ml) or placebo (35 $\pm$ 10 pg/ml). With repeated lavages, PGE₂ concentrations showed a mild and nonsignificant drop (Fig. 4, A).

Mean PGD₂ basal concentrations were 33 $\pm$ 3.5 pg/ml on the placebo day and 34 $\pm$ 6 pg/ml on the furosemide day. Neither placebo (25 $\pm$ 4 pg/ml) nor furosemide (25 $\pm$ 5 pg/ml) nebulization modified basal values. A sevenfold significant increase in PGD₂ concentration was detected in nasal lavage fluid after antigen stimulation at 10 minutes on the 2 study days (223 $\pm$ 109 pg/ml for placebo, 243 $\pm$ 79 pg/ml for furosemide). PGD₂ concentrations decreased more quickly on the furosemide day than on the placebo day. As a result, PGD₂ levels, measured at 30 minutes after placebo administration, were higher and still significant with respect to baseline values. In contrast PGD₂ levels at 30 minutes after furosemide administration were close to basal values

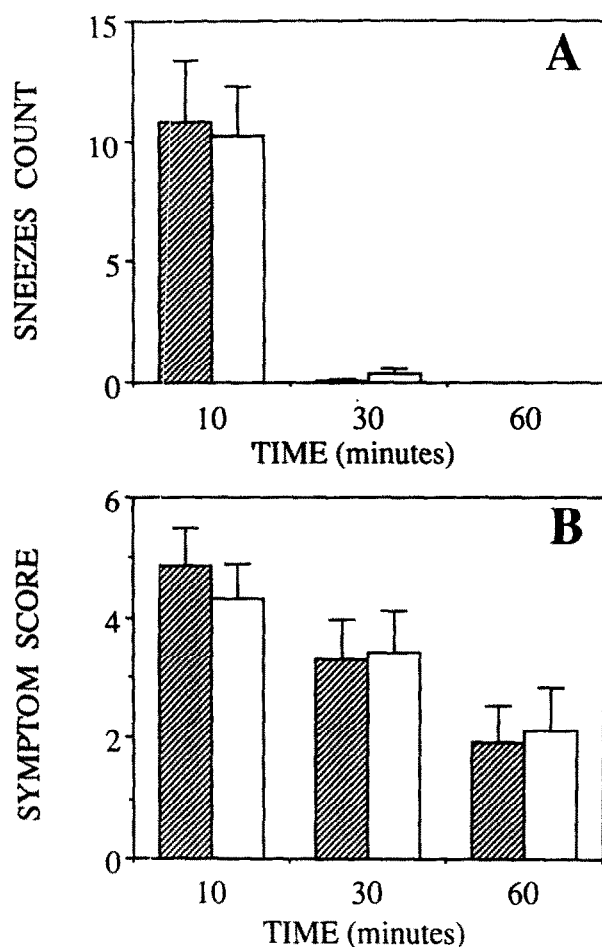


FIG. 1. A, Effect of furosemide on sneeze count after allergen time within three periods of time; **B,** effect of furosemide on rhinorrhea, nasal obstruction, and itching. Score for each symptom ranged from 0 to 3. Composite score was calculated as the sum of individual scores. No differences were observed between placebo (open bars) and furosemide (hatched bars). Values are expressed as means $\pm$ SEM.

(138 ± 88 pg/ml after placebo vs 57 ± 13 after furosemide, at 30 minutes). The difference between both days, however, was not significant. PGD_2 concentrations returned to prechallenge levels 60 minutes after antigen challenge on both the placebo day (49 ± 14 pg/ml) and the furosemide day (33 ± 6 pg/ml) (Fig. 4, B).

Lipoxygenase metabolites. Peptide leukotriene basal levels before and after nebulization of placebo (basal = 41 ± 7 pg/ml; after nebulization = 45 ± 12 pg/ml) and furosemide (basal = 70 ± 21 pg/ml; after nebulization = 32 ± 13 pg/ml) did not differ significantly. Nasal lavage peptide leukotrienes levels were found to be significantly elevated 10 minutes after allergen provocation both with placebo (530 ± 196 pg/ml) and

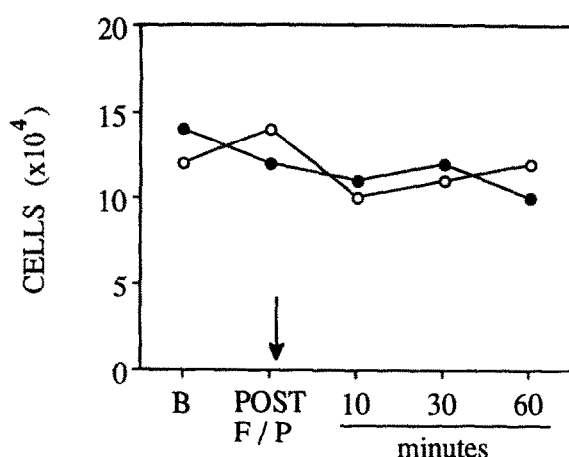


FIG. 2. Total cell count in nasal lavage after allergen challenge. No differences were found between furosemide ($\bullet$) and placebo ($\circ$). Challenge test (arrow). P, Placebo; F, furosemide.

furosemide (586 ± 209 pg/ml). The decrease of peptide leukotrienes at 30 minutes was less marked after placebo administration (104 ± 39 pg/ml) than after furosemide administration (63 ± 13 pg/ml); the difference with respect to basal values was still significant with placebo but not with furosemide. No statistically significant differences were found, however, in the release of peptide leukotrienes between placebo and furosemide days (Fig. 5, A).

Basal concentrations of 15-HETE before and after nebulization of placebo (basal = 676 ± 239 pg/ml; after nebulization = 612 ± 204 pg/ml) and furosemide (basal = 403 ± 65 pg/ml; after nebulization = 345 ± 50 pg/ml) did not change. Nasal lavage 15-HETE levels were found to be elevated, although not significantly, 10 minutes after allergen provocation with placebo (1112 ± 596 pg/ml) and furosemide (1205 ± 571 pg/ml). The levels of 15-HETE decreased more quickly on the furosemide day than on the placebo day. On the furosemide day 15-HETE concentrations returned to baseline values (540 ± 168 pg/ml at 30 minutes and 353 ± 78 pg/ml at 60 minutes), whereas the placebo day 15-HETE levels remained elevated (1002 ± 596 pg/ml at 30 minutes and 1239 ± 624 pg/ml at 60 minutes). The difference in 15-HETE levels at 60 minutes between the placebo and furosemide days was close to statistical significance ($p = 0.056$) (Fig. 5, B).

DISCUSSION

Our results are in keeping with those of previous studies, which demonstrated that the imme-

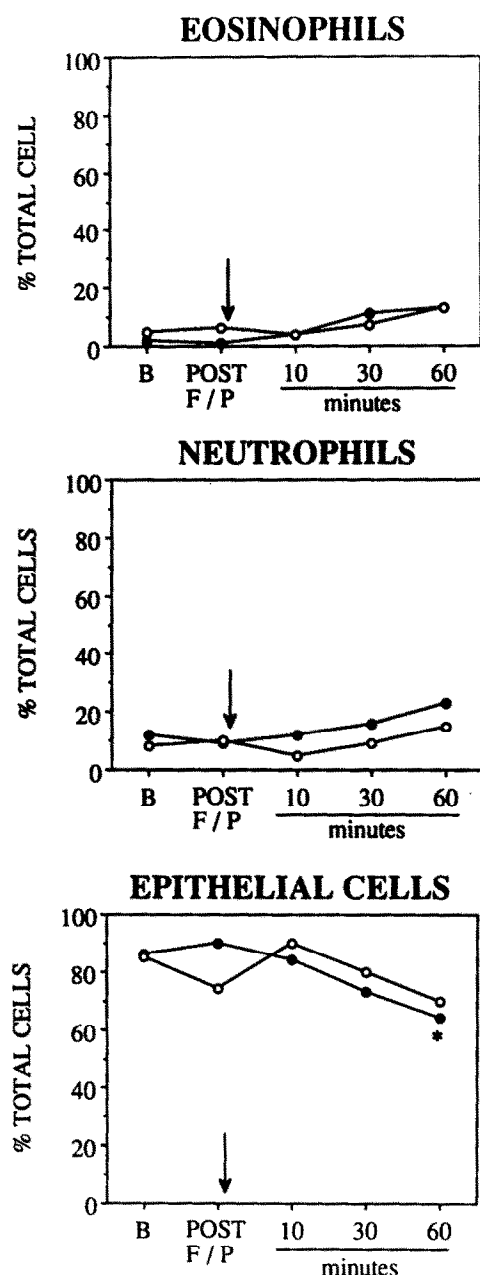


FIG. 3. Effect of placebo (○) and furosemide (●) on cell influx in nasal lavage. Each point represents the mean count expressed as percentage of the differential cell count. Allergen challenge (arrow) caused a progressive but not significant increase in eosinophils and neutrophils (upper and middle panels). A statistically significant decrease in epithelial cell count was observed at 60 minutes (lower panel). No differences in cell counts were found between placebo and furosemide. Challenge test (arrow). P, Placebo; F, furosemide. * $p < 0.05$.

diate allergic response is usually accompanied by an increased production of PGD_2 , peptide leukotrienes, and 15-HETE in asthma^{15, 16} and rhinitis.^{12, 17} Influx of eosinophils and neutrophils after allergen challenge has also been described in allergic asthma¹⁸ and rhinitis.¹⁹

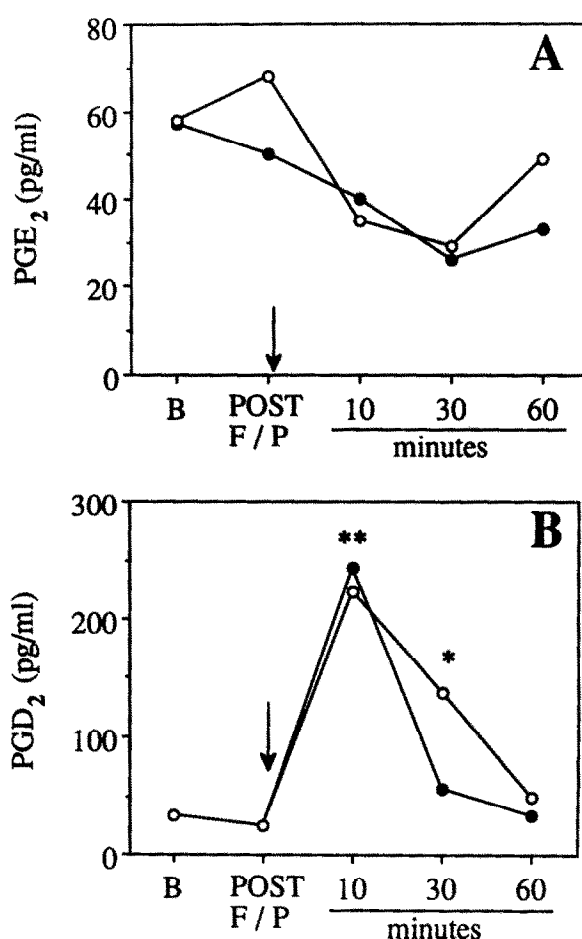


FIG. 4. Time course of cyclooxygenase metabolites after allergen challenge with placebo (○) and furosemide (●). **A**, No significant differences were found between mean PGE_2 levels before and after challenge or between furosemide and placebo days. **B**, PGD_2 mean levels increased significantly after allergen challenge at 10 minutes and remained significant after placebo administration but not after furosemide administration at 30 minutes. No significant differences were noted between furosemide and placebo. Challenge test (arrow). P, placebo; F, furosemide. ** $p < 0.01$, * $p < 0.05$.

Because the mechanisms involved in allergic reactions in the nose appear to be similar to those responsible for allergic asthma, the lack of effect of furosemide on allergen-induced rhinitis in contrast to its proven efficacy on bronchial asthma was an unexpected result of our study. At least two explanations may account for our findings: (1) differences in the methods currently used to investigate the effect of furosemide in bronchial asthma and those used in this study and (2) differences between the organs used to evaluate the effects of furosemide, namely the nose and the bronchi.

With respect to the methods, it should be noted that the dose of furosemide used in this study (20 mg) was apparently similar to the dose previously

tested in bronchial asthma. In our study, however, furosemide was directly nebulized in the nose. With this method it can be expected that almost all of the drug will reach the nasal mucosa, whereas in the best of cases, only 15% of a similar dose administered by nebulization will be deposited on the bronchial epithelium. Because the area of the bronchial mucosa is larger than that of the nasal mucosa, it can be assumed that the dose of furosemide administered in the nose was much greater than the dose nebulized in the bronchi in the studies carried out in patients with asthma. It seems therefore very improbable that the lack of effect of furosemide on allergen-induced rhinitis may be due to the administration of a low dose of drug. In addition, nasal mucosa is characterized by its ability to readily absorb substances deposited on the epithelial surface. It is unknown whether a very short-lasting presence of furosemide near the inflammatory cells, mainly responsible for production of arachidonic acid metabolites, such as eosinophils and mast cells, may account for the lack of drug effect in the nose.

Differences in the severity of allergy attacks, which were usually provoked in allergen-induced rhinitis with respect to asthma attacks precipitated by bronchial challenge, might account for the lack of furosemide effect in the nose. For ethical reasons, experimentally induced asthma must be limited to the precipitation of mild or moderate episodes of bronchoconstriction. In contrast, the higher tolerance of rhinitis induced by allergen challenge allows for the precipitation of more severe allergic responses. Since furosemide has proved to be a mild to moderate antiasthmatic drug, its lack of effect in preventing clinical symptoms in experimentally induced allergic rhinitis in comparison with results of similar studies in the bronchi might be because the severity of allergen response in the nose may hide the mild antiallergic effect of furosemide.

To our knowledge, the effect of furosemide on nasal arachidonic acid metabolite production has not yet been evaluated. Release of PGD_2 , peptide leukotrienes, and 15-HETE after allergen challenge has been demonstrated in rhinitis and asthma.^{12, 15-17} In our study furosemide showed no significant effects on the release of these metabolites. These findings contrast with the results obtained *in vitro* by Anderson et al.¹⁰ in human lung tissue passively sensitized to house dust mite. The striking differences in the methods used in both studies may account for the different outcome. In the *in vitro* model of Anderson et al.¹⁰ furosemide was in contact with the effector cells for a longer period than in our *in vivo* model in

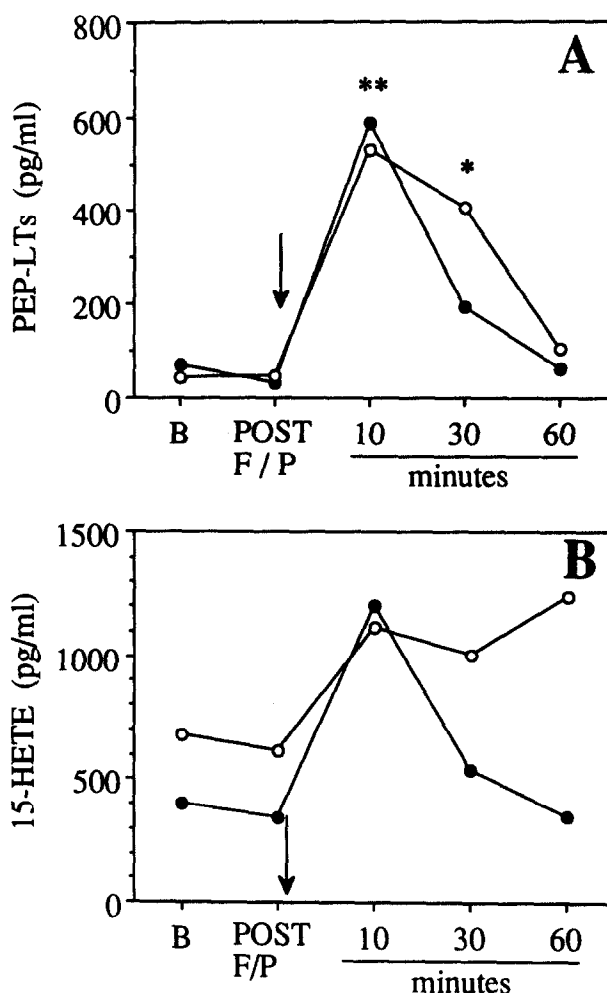


FIG. 5. Time course of lipoxigenase metabolites after allergen challenge with placebo (○) and furosemide (●). **A.** Peptide leukotrienes mean levels increased significantly after allergen challenge at 10 minutes and remained significant after placebo administration but not after furosemide administration at 30 minutes. No significant differences were noted between furosemide and placebo. **B.** 15-HETE mean levels increased without reaching statistical significance ($p = 0.1$) after allergen challenge at 10 minutes and remained elevated after placebo administration at 30 and 60 minutes. In contrast, 15-HETE levels returned to prechallenge levels after furosemide administration at 30 minutes. Difference in the levels between furosemide and placebo at 60 minutes was close to statistical significance ($p = 0.056$). Challenge test (arrow). P, Placebo; F, furosemide.

which the drug was probably readily absorbed, allowing for a brief interaction between furosemide and nasal inflammatory cells.

We could not detect any effect of furosemide on PGE_2 release. Our results therefore do not support the hypothesis suggesting that furosemide may prevent allergic responses by increasing the production of the bronchodilator PGE_2 .

The mechanisms involved in nasal and bronchial allergic reactions are similar, but the struc-

ture of the nose differs in several aspects with respect to the bronchial tree. Whereas the regulation of the bronchial patency is dependent, at least in part, on the activity of airway smooth muscle, air flow through the nose is mainly regulated by vessels. With these premises and according to the results of this study it can be hypothesized that furosemide might exert its preventive asthmatic effect by abrogating the contraction of smooth muscle during asthma attacks. In vitro studies, however, could not demonstrate any effect of furosemide on bronchial smooth muscle, suggesting that the antiasthmatic effect of the drug is not related to any direct action on smooth muscle contraction.²⁰ Furthermore, furosemide attenuates asthmatic responses to a variety of different bronchoconstrictor stimuli but does not inhibit bronchoconstriction induced by inhaled methacholine or histamine.⁴ These findings suggest that the antiasthmatic effect of furosemide might be mediated through inhibition of neurally mediated airway muscle contraction.^{1, 4, 6} Whether the different effects of furosemide in the nose and bronchi are due to the effect of furosemide on the airway smooth muscle, which is present in the bronchi but not in the nose, is speculative.

In conclusion, we have shown that furosemide is ineffective in inhibiting clinical symptoms in allergen-induced rhinitis in contrast to its proven efficacy in bronchial asthma. Because the allergen responses induced by nasal challenges may be stronger than those induced during bronchial challenges, it may well be that during experimental rhinitis a weak to moderate preventive effect of furosemide could not be detected. Our findings also suggest that furosemide may exert its antiasthmatic function by acting on structures present in the bronchi but not in the nose, such as airway smooth muscle, perhaps by inhibiting neurally induced bronchoconstriction.

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