Effects of inhaled furosemide on platelet-activating factor challenge in mild asthma

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ABSTRACT: Furosemide (Fur) may have an anti-inflammatory effect on airways in patients with asthma although its intrinsic mechanism remains elusive. Platelet-activating factor (PAF) is a potent proinflammatory mediator that induces systemic and respiratory effects in normal control subjects and asthmatics. The aim of this study was to assess whether pretreatment with nebulized Fur (40 mg) was able to modulate PAF-induced systemic and respiratory effects in asthma.

Eleven patients were studied (mean±sem 22±0.8 yrs) with mild asthma (forced expiratory volume in one second, 95±4%) in a randomized, double-blind, placebo-controlled, cross-over fashion, one week apart. PAF challenge (18 μg) was carried out 15 min after administration of Fur or placebo. Peripheral blood neutrophils, respiratory system resistance, and arterial blood gases were measured at baseline, and 5, 15 and 45 min after PAF; urinary cysteinyl leukotriene E_4 (uLTE$_4$) was also measured, at baseline and 120 min after PAF challenge.

Although Fur did not alter PAF-induced systemic and respiratory effects, it did partially inhibit (63%; $p<0.04$) the increments of uLTE$_4$ levels shown after PAF inhalation.

It is concluded that furosemide is not effective in protecting against platelet-activating factor challenge in patients with asthma despite its potential inhibition of leukotriene synthesis. These findings reinforce the view that the pulmonary effects of platelet-activating factor are mediated through different pathways.
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Platelet-activating factor (PAF) provokes, in both normal individuals and patients with asthma, neutropenia, bronchoconstriction, increased mucus secretion and microvascular leakage in the airways. In addition, PAF induces a ventilation-perfusion ($V'A/Q'$) imbalance which leads to gas exchange disturbances predominantly related to altered airway vascular permeability [1].

Several studies have demonstrated that inhaled furosemide (Fur) pretreatment reduces the severity of the asthmatic response to different types of constrictor stimuli, more specifically, those which act through indirect bron-choconstrictor mechanism. Likewise, it has been suggested that Fur may have an anti-inflammatory effect through inhibiting mediator release from various cell types [2]. However, its precise mechanism of action remains unclear. Recently, it has also been shown that Fur, when applied topically, dilates tracheal arterioles and venules by both cyclo-oxygenase- and nitric oxide-independent mechanisms [3].

Previous observations have suggested that Fur inhibits leukotriene (LT) synthesis from lung fragments [4] and after allergy-induced early reaction [5]. GÓMEZ et al. [6] have recently shown that 5-lipoxygenase selective inhibitor (e.g. zileuton) administration in patients with mild asthma attenuated PAF-induced systemic and pulmonary effects, thereby suggesting that these effects may be partially mediated by the LT pathway. Effective inhibition of PAF-induced effects by Fur might indicate that this drug, primarily, has an immediate anti-inflammatory effect, probably via LT inhibition. Alternatively, a failure by Fur to abolish PAF-induced abnormalities may indicate that the vasodilatory effects of Fur on airway vessels via enhanced abnormally increased microvascular leakage provoked by PAF, play a major role. The objective of this study was to investigate in patients with mild asthma whether Fur pretreatment could modulate the systemic, cellular, and pulmonary effects induced by PAF inhalation.

Methods

Study population

Eleven patients with mild asthma (table 1) were recruited from the Outpatient Dept, and the study was

Table 1. – Anthropometric and baseline function data on placebo and furosemide studies

	Baseline	Placebo	Furosemide
n	11		
Sex M/F	4/7		
Age yrs	21.5±0.80		
Height cm	165±1.7		
Weight kg	66.3±3.6		
FEV_1 L	3.5±0.2		
FEV_1 % pred	95±4.2		
FEV_1/FVC %	80±3.3		
PD_{20} μmol	0.98±0.4		
Neutrophils $\times 10^9$ cells·L^{-1}		3.1±0.4	3.3±0.3
$V'E$ L·min^{-1}		5.1±0.3	5.9±0.3
Rrs $cmH_2O·L^{-1}·s^{-1}$		3.7±0.3	3.8±0.5
Pa,O_2 mmHg		96.7±1.8	96.6±1.8
Pa,CO_2 mmHg		38.4±0.9	37.4±0.9
A-a PO_2 mmHg		8.2±1.1	9.1±1.5
$V'O_2$ mL·min^{-1}		195±11.1	214±9.8
HR min^{-1}		70±2.5	70±2.0
Ps mmHg		78.8±1.9	80.7±1.8
$uLTE_4$ pg·mg $creatinine^{-1}$		551±203	392±81

Data are presented as mean±SEM. M: male; F: female; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; PD_{20}: provocative dose of methacholine causing a 20% fall in FEV_1; $V'E$: minute ventilation; Rrs: respiratory system resistance; Pa,O_2: oxygen tension in arterial blood; Pa,CO_2: carbon dioxide tension in arterial blood; A-aPO_2: alveolar-arterial partial pressure of oxygen difference; $V'O_2$: oxygen uptake; HR: heart rate; Ps: mean systemic pressure; $uLTE_4$: urinary leukotriene E_4. Normal PD_{20} value=>4.0 mmol. 1 mmHg=0.133 kPa.

approved by the Ethical Research Committee of the Hospital clínic at the Universitat de Barcelona. All subjects gave informed written consent after the purpose, risks and potential benefits of the study were explained to them.

The inclusion criteria were: no respiratory infection or exacerbation of asthma within the preceding 6 weeks; forced expiratory volume in one second (FEV_1) >70% predicted and >1.5 L; positive methacholine (provocative dose causing a 20% fall in FEV_1 (PD_{20})<4.0 μmol) and PAF (20% increase of baseline respiratory system resistance (Rrs) after PAF (18 μg)) bronchial challenges; maintenance therapy with aerosol short-acting β_2-adrenergics on demand (8 patients), with or without regular inhaled glucocorticosteroids (800 μg daily) (2 patients) and/or long acting β_2-adrenergics (1 patient), but no previous treatment with oral steroids; and, absence of any systemic or cardiopulmonary disease other than asthma. All subjects were nonsmokers and atopic, as judged by the presence of a positive response to skin prick tests to one or more common aeroallergens.

Measurements

Blood samples were collected anaerobically through a catheter inserted into the radial artery. Arterial partial pressure of oxygen (PO_2), partial pressure of carbon dioxide (PCO_2) and pH were analysed in duplicate using standard electrodes, and haemoglobin concentration was measured by a Co-oximeter (Ciba Corning 860 System; Ciba Corning Diagnostics Corporation, Meadfield, MA, USA). Both O_2 uptake ($V'O_2$) and CO_2 production ($V'CO_2$) were calculated from mixed expired O_2 with a Zirconia analyser (MCG Medical Graphics Corporation, St. Paul,

MN, USA), and CO_2 concentrations by a nondispersive infrared analyser (NDIR) (Model CPX/D, MCG Medical Graphics Corporation). Both minute ventilation ($V'E$) and respiratory rate (RR) were measured using a calibrated Wright spirometer (Respirometer MK8; BOC-Medical, Essex, UK). The alveolar-arterial PO_2 gradient (A-aPO_2) was calculated according to the alveolar gas equation using the measured respiratory exchange ratio (R).

Total white cell counts in arterial blood were measured with a Technicon H.1^{TM} System (Technicon, Tarytown, New York, NY, USA). Patients received nebulized Fur (40 mg) (Hoechst AG, San Feliu de Llobregat, Spain) or placebo (P) (ClNa solution 0.9% adjusted to pH 9.0 by the addition of sodium hydroxide (NaOH) *via* an ultrasonic nebulizer (OMROM NE-U07; OMROM Corporation, Tokyo, Japan; volume: 4 mL; mass median aerodynamic diameter of the particles: 1–5 mm; output: 1 mL·min^{-1} frequency: 1–5 MHz)).

The measurement of Rrs was carried out *via* the forced oscillation technique and its analysis restricted to 8 Hz [6]. A three-lead electrocardiogram, heart rate (HR), systemic pressure (Ps) and arterial O_2 saturation were continuously recorded through a pulseoximeter (HP M1166A; Hewlett-Packard, Boblingen, Germany) throughout the whole study (HP 7830A Monitor and HP 7754B Recorder; Hewlett-Packard, Waltham, MA, USA). Measurements of urinary cysteinyl leukotriene E_4 ($uLTE_4$), were corrected for urinary creatinine and urine volume, were carried out with a validated enzyme immunoassay (EIA) [6, 7].

Study design

A randomized double-blind, placebo-controlled, crossover design was used. All patients were challenged on two occasions, one week apart, with inhaled PAF after the administration of either Fur or P, with patients breathing room air and seated in an armchair. All asthma medication was withheld for 48 h before arrival to the laboratory on the day of the study. After the establishment of adequate steady-state conditions, a set of duplicate measurements of arterial blood respiratory gases, O_2 and CO_2 fractions in mixed expired gases, white blood cell counts, urinary samples, ventilatory and haemodynamic parameters, and Rrs was carried out (baseline). Maintenance of steady-state conditions after PAF challenge was demonstrated by stability (±5%) of both ventilatory and haemodynamic variables and by close agreement between duplicate measurements of mixed expired and arterial O_2 and CO_2 (within ±5%). These conditions were met in all patients throughout the period of study. Immediately after approximately 15 min of Fur or P nebulization, another set of all of the measurements except that for $uLTE_4$, was performed and 15 min later, the patients were challenged with PAF (C_{16}) (1-O-Hexedecyl-2-acetyl-sn-glycero-3-phosphocholine) (18 μg) (Novabiochem AG, Laufelfingen, Switzerland). The preparation of the PAF solution and details of the PAF challenge have been previously reported in full [6, 8–10]. Duplicate measurements were taken at 5, 15, and 45 min following PAF inhalation, as described previously [6, 8–10]. All sets of measurements consisted of the following steps in sequence: haemodynamic and ventilatory recordings; respiratory gas and circulating white blood cells samplings and, Rrs measurements. No patient needed

rescue medication with short-acting β_2-adrenergics at the end of each study period.

Urine samples for $uLTE_4$ levels were collected at baseline (before Fur or P nebulization) and 120 min after PAF challenge. Because changes in urinary creatinine concentration (depend upon volume of urine) after Fur nebulization and PAF challenge may modulate the levels of $uLTE_4$, a third bronchoprovocation test with PAF (18 µg) was carried out, in 8 out of the 11 patients, at least 1 month later. All patients were pretreated with nebulized Fur (40 mg) following the same protocol (see above) before PAF challenge, and volume of urine in addition to urinary creatinine and LTE_4 levels were measured before and 120 min after PAF inhalation. There was no oral fluid intake during the period of study.

Statistical analysis

The results are expressed as mean±SEM. The effects of PAF challenge and those of pretreatment with Fur or P on the different end-point variables were assessed by a two-way repeated analysis of variance (ANOVA). This test was performed after checking for both homogeneity of variances and normal distribution of variables. When the F value of the ANOVA was significant, *post hoc* comparisons were performed using paired Student t-test. In the third PAF study, in which patients were pretreated with Fur only, a Student t-test was used to compare $uLTE_4$ before and after PAF inhalation. All analyses were performed with SPSS version 6.1.3 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at p<0.05.

Results

Baseline data before platelet-activating factor

In table 1, mean baseline anthropometric and functional measurements of all patients are shown. All were similar to those reported in previous investigations [6, 9, 10] without baseline differences between Fur or vehicle study days. Except for $uLTE_4$, which was not measured immediately after Fur or P (see above), no differences were shown between baseline and post-Fur or P measurements, before PAF inhalation, and accordingly, all comparisons after PAF challenge were performed taking into account baseline data measured before Fur or P nebulization.

Effects of platelet-activating factor after placebo

All but 3 patients noticed facial flushing, 4 coughed and 8 felt shortness of breath immediately after PAF challenge. There were no differences in the response to PAF between patients treated with or without inhaled glucocorticosteroids. Peripheral blood neutrophils fell at 5 min after PAF inhalation (from $3.09\pm0.4 \times 10^9$ cells·L^{-1} to $2.07\pm0.5 \times 10^9$ cells·L^{-1}; p<0.01), followed by a rebound neutrophilia at 15 and 45 min (to 4.29 ± 0.6 and $4.52\pm0.7 \times 10^9$ cells·L^{-1}; p<0.03 and 0.04, respectively; fig. 1). Total Rrs increased at 5 min after PAF challenge (from 3.68 ± 0.3 cmH$_2$O·L^{-1}·s^{-1} to 6.24 ± 1.2 cmH$_2$O·L^{-1}·s^{-1}; p<0.04). Moreover, arterial PO$_2$ decreased and A-aPO$_2$
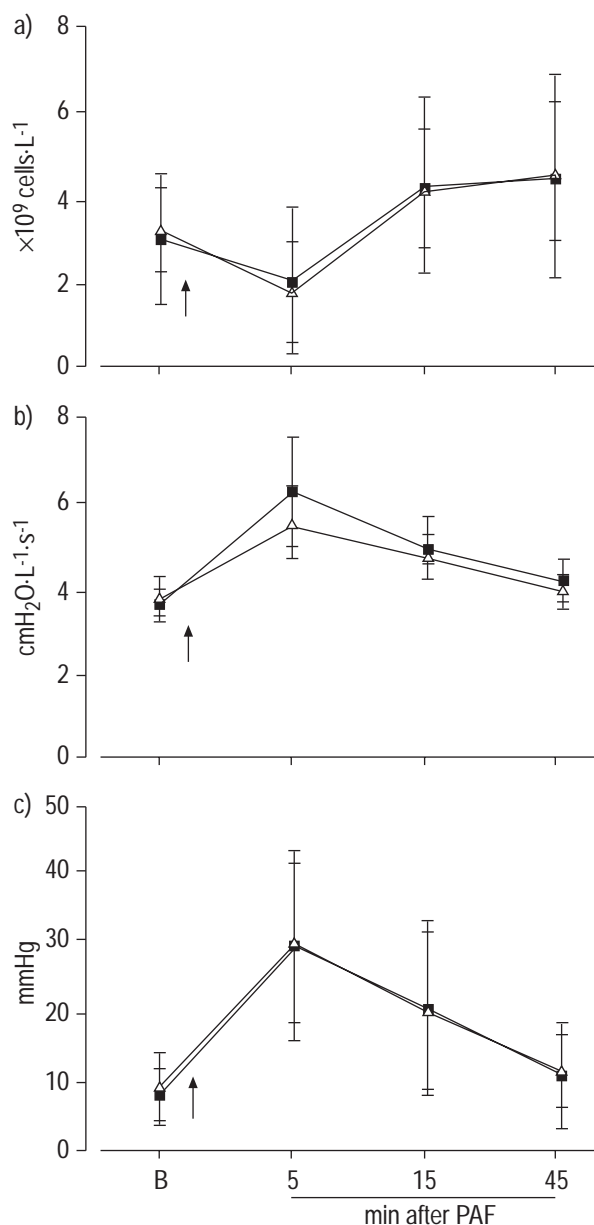

Fig. 1. – Mean(±SEM) values of: a) neutrophils, b) resistance of the respiratory system, and c) alveolar-arterial partial pressure of oxygen difference, after inhaled platelet-activating factor (PAF) (arrow) in placebo (■) and furosemide (△) studies at baseline (B) and at 5, 15 and 45 min. No differences were shown in any of the variables (see text). 1 mmHg= 0.133 kPa.

increased at 5 min (from 12.9 ± 0.2 kPa (96.7 ± 1.8 mmHg) and 1.1 ± 0.2 kPa (8.2 ± 1.1 mmHg) to 10.3 ± 0.5 kPa (77.7 ± 3.7 mmHg) and 4.0 ± 0.5 kPa (29.7 ± 4.0 mmHg), respectively; p<0.001 each), probably reflecting the development of low V'A/Q' ratios [6, 8–10]. Additionally, ventilatory and haemodynamic variables and the other gas exchange indices, including arterial pH, did not change. Compared with Fur, all of these changes were not significantly different.

By contrast, the administration of P induced a marked increase of $uLTE_4$ corrected by urinary creatinine (by 63%) 120 min after PAF inhalation, compared with Fur pretreatment (from 551 ± 203 pg·mg^{-1} to $2,254\pm679$ pg·mg^{-1}
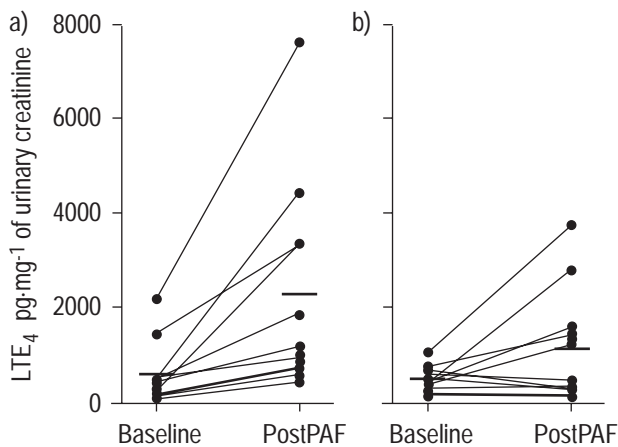
Fig. 2. – Individual and mean (solid bars) values of urinary cysteinyl leukotriene E_4 (uLTE$_4$) at baseline (B) and 120 min after platelet-activating factor (PAF) inhalation pretreated with a) placebo of b) furosemide. The uLTE$_4$ levels were markedly reduced after PAF challenge when patients were pretreated with furosemide (p<0.04).

(an increase of 474±129%) and from 392±81 pg·mg^{-1} to 1,017±366 pg·mg^{-1} (an increase of 148±85%), respectively; p<0.04) (fig. 2).

Effects of platelet-activating factor after furosemide

As compared with vehicle, pretreatment with Fur did not abolish PAF-induced systemic effects: six patients noticed facial flushing, three coughed, and seven felt shortness of breath. Similarly, PAF-induced responses on cellular and lung function abnormalities were not attenuated (fig. 1), and no significant changes in uLTE$_4$ levels were observed (see above) (fig. 2). Interestingly enough, uLTE$_4$ levels were not significantly increased after PAF when corrected either by urinary creatinine concentration (from 949±135 pg·mg^{-1} to 1,547±393 pg·mg^{-1} an increase of 63±31%; not significant) or urinary volume (from 97,004±13,972 pg·mL^{-1} to 160,503±59,676 pg·mL^{-1} an increase of 61± 44%; p<0.3) in the third PAF challenge carried out 1 month later.

Discussion

The novel finding of the present study is that previous nebulization of 40 mg of Fur (a dose similar to that used in previous studies [11]) does not modify either the systemic or the pulmonary responses provoked by PAF inhalation in patients with mild asthma [6, 9, 10]. When PAF was given after Fur, patients exhibited systemic effects (feeling of warmth, cough, and/or dyspnoea), a fall in peripheral blood neutrophils followed by an intense rebound kinetic effect, bronchoconstriction (increased R_{rs}), and hypoxaemia and/or increased A-aP_{O_2}, provoked by $V'A/Q'$ imbalance most likely characterized by the development of areas with low $V'A/Q'$ ratios [1, 6, 9, 10]. By contrast, Fur pretreatment was successful in inhibiting the increased urinary excretion of leukotriene (LT)E$_4$, the stable whole body metabolite of LT, after PAF challenge.

Fur has been found to be protective against bronchoconstriction induced by several types of bronchial chal-

lenges in patients with asthma, especially those which act through indirect mechanisms when the release of paracrine bronchoconstrictor mediators is implicated [2]. In this sense, it has been suggested that Fur may have a similar profile of action to disodium cromoglycate [12]. However, no protection was observed against direct bronchoconstrictive agents, such as methacholine or histamine, suggesting therefore that Fur may not have a direct effect on airway smooth muscle [2, 13]. There are studies that demonstrate that PAF induces the release of certain cytokines from cells. such as interleukin (IL)-4, IL-6, IL-8, and tumour necrosis factor-alpha (TNF-α) [14]. While the authors agree that it is plausible that these cytokines may mediate some of the effects that have been observed with PAF inhalation in asthmatics *i.e.* the neutropenia through the secondary release of IL-8 from bronchial epithelial cells [14], they suggest that it is unlikely that Fur may be acting by inhibiting such release. Indeed, it has been found that Fur did not alter PAF-induced neutrophil kinetic abnormalities. Similarly. the authors are not aware of any data implicating a direct effect of Fur in inhibiting cytokine release from inflammatory cells *in vivo*. In fact, its precise mechanism has not yet been elucidated although diverse hypotheses have been proposed [11]. Moreover, the authors have shown a lack of bronchial response to solutions of ClNa such that vehicle inhalation cannot modulate pulmonary function [15].

McFADDEN *et al.* [16] showed that, in exercise-induced asthma, airway obstruction is related to the thermal gradient that develops between the airway cooling of hyperpnoea and the airway rewarming. When this gradient is lessened, the severity of the airway obstruction response is reduced. They also demonstrated that changes in airway blood flow significantly alter the gradient for intrathoracic heat exchange. GILBERT *et al.* [17] observed that inhaled Fur reduced the thermal gradient for rewarming of the airways after hyperpnoea and proposed that the protective action of this agent was due to the increased blood flow to the airways. CORBOZ *et al.* [3] confirmed that Fur, when applied topically in a rat model, dilates tracheal arterioles and venules by cyclo-oxygenase- and nitric oxide-independent mechanisms. In addition, Fur is known to be an effective vasodilator of the pulmonary circulation [12].

Based on previous work, in both healthy individuals [8, 18, 19] and in patients with mild asthma [6, 9, 10], the authors postulate that PAF-induced pulmonary gas exchange abnormalities and increases of R_{rs} are related to narrowing of airway lumenal calibre secondary to abnormally increased microvascular leakage rather than to a primary constrictor effect on airway smooth muscle by itself. Mediators that increase abnormal vascular permeability on bronchial circulation operate directly on the post-capillary venular wall endothelium, possibly by altering normal cell-to-cell contact and inducing the presence of intercellular gaps [1, 20]. The mechanism of these gaps has been accepted as a contractile phenomenon provoking wide clefts intercellularly. As a result, non-sieved plasma and cells escape *via* these venular holes into the interstitial airway wall, possibly under the influence of the hydrostatic pressure gradient [1, 20]. Both the postcapillary venular endothelium and the mucosal epithelium seem to harbour anti-leakage mediating β$_2$ receptors.

β_2-receptor agonists were among the first drugs that were demonstrated to exhibit anti-oedema effects in a variety of systemic vascular beds [20]. In this regard, it has been shown that inhaled salbutamol (300 µg), but not ipratropium bromide (80 µg), was efficacious in completely antagonizing PAF-inducing effects in a laboratory-induced model developed in control subjects [18] and in patients with asthma [10]. Conceivably, the vasodilatory effect on bronchial circulation provoked by Fur [3] may interact with the constrictive effects of PAF on endothelial cells, hence ultimately modulating the subsequent lung function abnormalities on both airway tone and pulmonary gas exchange induced by bronchoconstriction and abnormally increased airway permeability. Moreover, this vasodilatory effect of Fur can also be active in the pulmonary circulation [12], thus modulating the tone of the vessels and consequently not affecting the transitory sequestration of neutrophils provoked by PAF. Fur has been shown to be active as a vasodilator at concentrations of 10^{-4} M [3], but this concentration could have been achieved in the airways submucosa since a concentration of 3.0×10^{-2} M was aerosolized and since even allowing for a dilution factor of 100 for this diffusible molecule, such active concentrations of Fur would be achieved at the assumed sites of action. However, direct measurements are not available and therefore the hypothesis of the airway vascular mechanisms of action for Fur relies on the likelihood of achieving these concentrations.

Akin to former studies [4, 5], $uLTE_4$ was observed not to increase significantly after PAF when Fur was pre-administered. Leukotrienes may be involved as secondary mediators in the production of the systemic and pulmonary effects caused by PAF in asthmatic patients. It has been recently proven that PAF can increase the subsequent release of chemotactic mediator LTB_4, thereby suggesting that it may prime the constitutive cells of the lung to augment inflammatory effects relevant to the pathogenesis of asthma [21]. The administration of PAF in humans is associated with an increase in $uLTE_4$ and these augmented levels reflect an integrated form of endogenous whole body LTC_4 and LTD_4 release during a specific period of time [22]. The author's group recently observed that oral pretreatment with zileuton (600 mg), a selective 5-lipoxygenase inhibitor, partially abolished PAF-induced pulmonary effects in patients with mild asthma by a slightly lower order of magnitude than in the current study [6], therefore suggesting that LT can mediate the latter effects. Although in the current study LTE_4 urinary excretion was inhibited, there was no prevention of PAF-induced bronchoconstriction. This could be due to the possibility that inhibition of the 5-lipoxygenase enzyme needs to be complete before functional abolition of bronchoconstriction is observed. A reduction by about half LTE_4 urinary excretion by zileuton in a previous study was not associated with any modulation of the allergen-induced late phase response [23].

In summary, one plausible explanation for the lack of action of furosemide on platelet-activating factor-induced systemic and pulmonary effects in this study, is that platelet-activating factor can be an inflammatory mediator that may exert its effects through different pathways, involving not only the release of leukotriene but also that of other putative inflammatory mediators. In this sense, the vasodilatory activity of furosemide could enhance vascular leakage thus facilitating both the systemic and pulmonary effects induced by platelet-activating factor inhalation, a response that cannot be offset even with the partial inhibition of leukotriene. This is reflected by reducing the increased urinary excretion of its stable metabolite, urinary cysteinyl leukotriene E_4.

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