Lyso-PAF acetyltransferase activity in neutrophils of patients during acute asthma and after recovery


ABSTRACT: The production of platelet-activating factor (PAF) by inflammatory cells is regulated by lyso-PAF acetyltransferase, and the activity of this enzyme is increased in neutrophils of stable asthmatic patients. The aim of this investigation was to determine whether acetyltransferase activity is further upregulated in asthmatic patients experiencing acute symptoms.

A radioenzymatic assay was used to measure the enzymatic affinity constant (Km) and maximal enzymatic activity (Vmax) for acetyltransferase from unstimulated and Ca2+ ionophore (A23187)-stimulated neutrophils from 16 patients with acute asthma, and the measurement was repeated at the time of discharge (n=9) and after recovery from the acute episode (n=13).

During acute asthma, Km (median 93.8 (interquartile range 64.1–109.7) µM) was lower than that measured in nonasthmatic subjects in a previous study using identical methods (155.1 (122.2–179.9) µM; p=0.0001), and in 10 out of 13 acute patients Km for unstimulated neutrophils increased following recovery. In A23187-stimulated neutrophils, Km during acute asthma (84.3 (73.6–100.2) µM) and at discharge (83.9 (83.1–94.8) µM) were similar, but Km after recovery was increased (115.0 (95.6–119.5) µM; p=0.02). The change in Km following stimulation with A23187 was also significantly less during acute asthma than previously measured in nonasthmatic subjects (p=0.003). Although Vmax during acute asthma (12.9 (interquartile range 10.5–22.5) nmol·min-1·mg-1 protein) did not differ significantly from that at discharge (14.4 (12.3–20.4) nmol·min-1·mg-1) or after recovery (17.3 (12.3–18.4) nmol·min-1·mg-1), both median Km and Vmax tended to be lowest during acute asthma and increase at discharge and after recovery.

An increase in lyso-PAF acetyltransferase activity alone may not account for increased systemic PAF concentrations during acute asthma. However, the reduction in the enzymatic affinity constant and its smaller change following in vitro stimulation suggest that alterations in the affinity of acetyltransferase for acetyl-coenzyme A (CoA) and in the regulation of enzyme activity may be occurring during acute asthma.


Platelet-activating factor (PAF) is an ether-linked phospholipid with numerous biological activities which suggest that it plays a significant role in the pathogenesis of asthma [1]. PAF causes bronchoconstriction and, in some studies, has been shown to induce a persistent increase in bronchial hyperresponsiveness in humans [2, 3]. This may relate to the potency of PAF as a chemoattractant for eosinophils [4], since these cells appear to play a significant role in the development of airway hyperresponsiveness [5, 6]. However, other studies, both in normal and asthmatic subjects [7, 8], have failed to demonstrate any increase in airway responsiveness after PAF inhalation. PAF also causes airway microvascular leakage and oedema [9], stimulates tracheal mucus secretion [10], and inhibits mucociliary clearance in normal subjects [11].

There is much indirect evidence implicating PAF in the pathogenesis of asthma, but it has been more difficult to obtain direct evidence based on measurements of this mediator and its precursor, lyso-PAF, in biological fluids [12]. However, PAF has been detected in bronchoalveolar lavage fluid (BAL) from asthmatics [13], and allergen challenge has resulted in high levels of lyso-PAF in nasal fluids [14], and increased plasma PAF concentrations [15]. A number of recent studies have also suggested that systemic PAF concentrations may be increased in symptomatic asthmatic subjects [16–18]. Despite these observations, the interpretation of such measurements is complicated by factors such as the short half-life of PAF, the fact that lyso-PAF is both a precursor and metabolite of PAF, the likelihood that PAF acts mainly at localized sites of inflammation and that it may be rapidly metabolized and reincorporated by surrounding inflammatory cells [19].

PAF is synthesized by a variety of cell types, including neutrophils, eosinophils, alveolar macrophages, monocytes and endothelial cells, in response to stimuli, such as the calcium ionophore A23187, opsonized zymosan...
[19], granulocyte/macrophage colony-stimulating factor (GM-CSF) [20], bradykinin, tumour-necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) [21, 22]. Stimulated inflammatory cells synthesize PAF in a two-step process catalysed by the enzymes phospholipase A2 and acetylcoenzyme-A (CoA):lyso-PAF acetyltransferase [23]. Acetyltransferase activity can be increased in vitro by A23187, TNF-α and IL-1α, and in vivo activation of this enzyme could result in the production of excessive amounts of PAF [19]. Thus, upregulation of acetyltransferase activity may possibly explain the increased PAF concentrations reported in some studies of symptomatic asthmatics.

In a previous study from our laboratory, increased acetyltransferase activity was observed in neutrophils from clinically stable atopic asthmatic subjects compared with nonasthmatic control subjects, suggesting that in these nonsymptomatic asthmatics, neutrophils may be subject to chronic priming [24]. It is not clear, however, whether disease exacerbation further modulates acetyltransferase activity. Thus, the aim of the present investigation was to measure neutrophil acetyltransferase activity in a group of asthmatic subjects experiencing acute disease exacerbations.

Methods

Subjects

Sixteen subjects presenting to the Emergency Department with a clinically diagnosed acute exacerbation of asthma were recruited for the study, which was approved by the Committee for Human Rights of the University of Western Australia. Informed consent was obtained from all patients in the study group which comprised 13 females and 3 males (mean age 39±15 yrs, range 19–69 yrs). The clinical details and drug treatments were noted for all patients, and spirometry and oximetry data were obtained for most patients (table 1). Patients were bled prior to receiving treatment and a second blood sample was obtained at the time of discharge from those patients admitted to hospital (n=9). A further blood sample was obtained from patients (n=13) approximately 8 weeks after the acute exacerbation, when their asthma was considered to be stable and they had not been taking oral corticosteroids for at least 2 weeks. Three subjects could not be contacted after discharge and it was, therefore, not possible to obtain a follow-up blood sample from these subjects.

Isolation of neutrophils

Whole blood, anticoagulated with ethylenediamine tetra-acetic acid (EDTA), was layered on a discontinuous Percoll gradient (densities 1.082, 1.094) and centrifuged (500×g for 30 min). The neutrophil band was recovered and contaminating erythrocytes were lysed in 0.2% (w/v) saline for 30 s. An equal volume of 1.6% (w/v) saline was added and the cells were again centrifuged (500×g for 10 min). Neutrophils were washed in hydroxyethylpiperazine ethanesulphonic acid (HEPES)-buffered Hank's balanced salt solution (HBSS) (HEPES 4.2 mM, NaCl 137 mM, KCl 2.6 mM, glucose 5.6 mM, pH 7.4) and resuspended in HBSS supplemented with CaCl2 (1.3 mM) and MgCl2 (1 mM). Neutrophils were counted and diluted to 11×10⁶ cells·mL⁻¹. Purity and viability were consistently >95%.

Assay of acetyltransferase activity

Acetyltransferase activity of neutrophils was assayed as described previously [24]. Briefly, unstimulated neutrophils, and neutrophils stimulated with 5 µM Ca²⁺ ionophore (A23187) to induce maximum acetyltransferase activity, were incubated at 37°C for 10 min. Neutrophils were centrifuged (500×g for 10 min). Neutrophils were counted and diluted to 11×10⁶ cells·mL⁻¹. Purity and viability were consistently >95%.

Table 1. – Lung function, oximetry and drug treatment for patients with acute asthma

<table>
<thead>
<tr>
<th>Pt No.</th>
<th>Sex</th>
<th>Age yrs</th>
<th>Blood taken</th>
<th>FEV₁ acute</th>
<th>S₂O₂</th>
<th>FEV₁ recovery</th>
<th>Drug treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>% pred</td>
<td></td>
<td>% pred</td>
<td>Preadmision</td>
</tr>
<tr>
<td>1</td>
<td>F</td>
<td>19</td>
<td>a, r</td>
<td>62</td>
<td>98</td>
<td>102</td>
<td>ba</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>32</td>
<td>a, d, r</td>
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<td>93</td>
<td>122</td>
<td>ba, Bc</td>
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<tr>
<td>3</td>
<td>F</td>
<td>19</td>
<td>a</td>
<td>58</td>
<td>95</td>
<td>ND</td>
<td>ba, ip, Bf</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>22</td>
<td>a, r</td>
<td>41</td>
<td>98</td>
<td>93</td>
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<td>F</td>
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<td>a, d, r</td>
<td>53</td>
<td>91</td>
<td>90</td>
<td>ba, th</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>31</td>
<td>a, d, r</td>
<td>22</td>
<td>90</td>
<td>78</td>
<td>ba, Bc</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>25</td>
<td>a</td>
<td>16</td>
<td>94</td>
<td>ND</td>
<td>ba</td>
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<td>69</td>
<td>a, d, r</td>
<td>53</td>
<td>99</td>
<td>ND</td>
<td>ba, pn, bu</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>58</td>
<td>a, d, r</td>
<td>22</td>
<td>ND</td>
<td>95</td>
<td>ba, pn, th</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>28</td>
<td>a</td>
<td>ND</td>
<td>94</td>
<td>ND</td>
<td>ba</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>29</td>
<td>a, d, r</td>
<td>38</td>
<td>92</td>
<td>92</td>
<td>ba</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>47</td>
<td>a, r</td>
<td>63</td>
<td>97</td>
<td>72</td>
<td>ba, pn, th</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>52</td>
<td>a, d, r</td>
<td>82</td>
<td>ND</td>
<td>78</td>
<td>ba, pn, Bf</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>47</td>
<td>a, d, r</td>
<td>31</td>
<td>98</td>
<td>49</td>
<td>ba, Bf</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>48</td>
<td>a, r</td>
<td>54</td>
<td>ND</td>
<td>91</td>
<td>ba, ip, Bf, cr, th</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>41</td>
<td>a, d, r</td>
<td>54</td>
<td>ND</td>
<td>87</td>
<td>ba, sm, fp</td>
</tr>
</tbody>
</table>

Pt: patient; F: female; M: male; a: acute; d: discharge; r: recovery; ND: not determined; ba: β₂-adrenoceptor agonists; Bc: Becotide; Bf: Becloforte; bu: budesonide; pn: prednisolone; fp: fluticasone propionate; th: theophylline; cr: cromoglycate; sm: salmeterol; ip: ipratropium bromide; FEV₁: forced expiratory volume in one second; % pred: percentage of predicted value; S₂O₂: arterial oxygen saturation. Subjects from whom a blood sample was taken after recovery had ceased prednisolone for at least 2 weeks.
by sonication for 20 s on ice, and the neutrophil lysates were assayed in duplicate for acetyltransferase activity in a reaction mixture containing lyso-PAF (40 µM) and [3H]acetyl-CoA (50–500 µM) in a final volume of 0.5 mL HEPES-HBSS containing 0.25% (w/v) bovine serum albumin (BSA). After incubation at 37°C for 10 min, the reaction was stopped by addition of methanol-chloroform-methanol-chloroform-acetic acid (2.1:0.04, by volume). Lipo-PAF was extracted into chloroform [25], the extract was washed twice with 2 mL of 0.9% (w/v) saline-methanol-chloroform-0.1 M sodium acetate (1.0:2.5:3.75:1.0, by volume) and [acetyl-3H]PAF was measured by liquid scintillation counting. Losses during lipid extraction were corrected for by extracting a control sample, to which [alkyl-3H]PAF was added. The mean recovery was 84±4%. Acetyltransferase activities were expressed as [acetyl-3H]PAF produced (nmol·min⁻¹·mg⁻¹ protein) after subtraction of the radioactivity in blank incubations which contained no lyso-PAF. Protein concentrations of neutrophil lysates were determined using the Coomassie blue protein assay (Bio-rad, Hercules, CA, USA).

Acetyltransferase activity, as measured in this assay, has previously been shown to increase linearly over the range of protein concentrations used and also over the incubation period of 10 min [24]. In addition [acetyl-3H]PAF produced in this assay has been characterized on the basis of co-chromatography with authentic PAF standards on thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) and by stimulation of human platelet aggregation, which was inhibited by the specific PAF receptor antagonists WEB 2086 and STY 2108 [24].

Analysis of data and statistics

Acetyltransferase activity was measured over a range of acetyl-CoA substrate concentrations both for unstimulated and A23187-stimulated neutrophil lysates. For each subject, the maximal enzymatic activity (Vmax) and the enzymatic affinity constant (Km) for unstimulated and A23187-stimulated neutrophils were determined from double reciprocal plots of acetyltransferase activity (1/V) against acetyl-CoA concentration (1/[S]). Vmax and Km values during the acute episode, at the time of discharge and following recovery are presented as medians and interquartile range (25th to 75th percentile). The degree of stimulation of acetyltransferase activity induced by A23187 was calculated as the ratio Vmax-stimulated/Vmax-unstimulated, and the change in Km following stimulation with A23187 was calculated as the ratio Km-unstimulated/Km-stimulated. Differences in median Vmax and Km values and ratios for the 16 patients with acute asthma, the nine patients at discharge and the 13 patients at recovery were evaluated for statistical significance by nonparametric analysis of variance (Kruskal-Wallis test) using the InStat computer program (GraphPad Software, San Diego, CA, USA). The Mann-Whitney test was used to compare Vmax and Km values and ratios with data obtained previously using identical methodology, for 20 nonasthmatic subjects [24]. For statistically significant differences (p<0.05), the differences in median values and the associated 95% confidence intervals (95% CI) are presented.

Results

Acetyltransferase activity in unstimulated and A23187-stimulated neutrophils increased with increasing acetyl-CoA substrate concentration, reaching a plateau at 200 µM acetyl-CoA (fig. 1). For each subject, Vmax and Km values were determined from double reciprocal plots, which did not deviate significantly from linearity, indicating that the enzyme followed Michaelis-Menten kinetics both in unstimulated and A23187-stimulated neutrophils for acetyl-CoA concentrations of 50–500 µM and a fixed lyso-PAF concentration (40 µM). In the acute asthma group, the median Km for acetyltransferase in unstimulated neutrophils (93.8 (64.1–109.7) µM; n=16) was not significantly different to the value measured at discharge (104.6 (95.4–115.1) µM; n=9) or after recovery (107.2 (96.7–126.9) µM; n=13) (fig. 2). However, an increase in Km was observed in 10 of the...
Median Km ratios did not differ significantly during acute asthma, at discharge or after recovery, but the ratios both during acute asthma and after recovery were significantly lower than the median Km ratio measured previously in nonasthmatic subjects [24] (table 2).

Table 2. – Ratios Km-unstimulated/Km-stimulated and Vmax-stimulated/Vmax-unstimulated in patients with acute asthma, at discharge and following recovery

<table>
<thead>
<tr>
<th>Group</th>
<th>Pt</th>
<th>Median Km ratio</th>
<th>IQR</th>
<th>Median Vmax ratio</th>
<th>IQR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute asthma</td>
<td>16</td>
<td>0.94*</td>
<td>0.80–1.08</td>
<td>1.59†</td>
<td>1.16–2.11</td>
</tr>
<tr>
<td>Discharge</td>
<td>9</td>
<td>1.06</td>
<td>1.01–1.35</td>
<td>1.61</td>
<td>1.10–1.95</td>
</tr>
<tr>
<td>Recovery</td>
<td>13</td>
<td>0.95†</td>
<td>0.88–1.15</td>
<td>1.68</td>
<td>1.50–1.91</td>
</tr>
<tr>
<td>Nonasthmatic</td>
<td>20</td>
<td>1.31</td>
<td>1.12–1.61</td>
<td>2.07</td>
<td>1.60–2.59</td>
</tr>
</tbody>
</table>

Values significantly different to corresponding value previously measured in nonasthmatic subjects [24]: *: median difference -0.39 (95% CI -0.62 to -0.19); p=0.003; †: median difference -0.33 (95% CI -0.53 to -0.1), p=0.006; ‡: median difference -0.46 (95% CI -0.88 to -0.06), p=0.029. Km: enzymatic affinity constant; Vmax: maximal enzymatic activity; IQR: interquartile range; 95% CI: 95% confidence interval.

Median Km ratios did not differ significantly during acute asthma, at discharge or after recovery, but the ratios both during acute asthma and after recovery were significantly lower than the median Km ratio measured previously in nonasthmatic subjects [24] (table 2).

In acute asthma, at discharge and after recovery, median Km values for A23187-stimulated neutrophils did not differ significantly from the values for unstimulated neutrophils (fig. 2), although A23187 stimulation was previously shown to cause a significant reduction in Km in nonasthmatic subjects [24]. In A23187-stimulated neutrophils, the median Km values in acute asthma (84.3 (73.6–100.2) µM) and at discharge (83.9 (83.1–94.8) µM) were similar, although the median Km after recovery was increased (115.0 (95.6–119.5) µM; p=0.02, analysis of variance. The Km for A23187-stimulated cells in acute asthma was also significantly lower than the value measured previously in nonasthmatic subjects (106.6 (98.1–148.7) µM; p=0.003; median difference -27.8 (95% CI -48.4 to -13.6) µM) (fig. 2).

The change in Km following in vitro stimulation was measured as the ratio Km unstimulated/Km-stimulated.
**LYSO-PAF ACETYLTRANSFERASE IN ACUTE ASTHMA**

V\textsubscript{\text{max}} value measured in acute asthmatic patients in this study was not significantly different to that measured previously [24] in nonasthmatic subjects (14.7 (10.9–17.4) nmol·min\(^{-1}\)·mg\(^{-1}\) protein). There were no significant differences in the median V\textsubscript{\text{max}} values for A23187-stimulated neutrophils during acute asthma, at discharge or after recovery, and the values were also not significantly different to those measured previously in nonasthmatic subjects.

The degree of stimulation of acetyltransferase activity induced by A23187 was calculated for each subject as the ratio V\textsubscript{\text{max}}-stimulated/V\textsubscript{\text{max}}-unstimulated. The median V\textsubscript{\text{max}} ratios during acute asthma, at discharge and after recovery did not differ significantly but the V\textsubscript{\text{max}} ratio during acute asthma was significantly lower than that measured previously in nonasthmatic subjects (table 2). The V\textsubscript{\text{max}} ratio after recovery also differed from that of nonasthmatic subjects, although the difference did not quite reach statistical significance (p=0.06).

**Discussion**

Acetyl-CoA:lyso-PAF acetyltransferase activity was previously shown to be significantly increased in neutrophils from atopic, asthmatic subjects compared with a control group of nonasthmatic subjects [24]. The present study sought to extend these observations by measuring acetyltransferase activity of neutrophils obtained from patients experiencing symptoms of acute asthma. For most of these patients, it was possible to repeat the measurement of acetyltransferase activity after recovery, contrasting with the previously measured [24] in nonasthmatic subjects. Thus, neutrophils from asthmatic subjects appear to be inherently less sensitive to in vitro stimulation with A23187, supporting our previous findings [24].

It was possible that oral medications, such as corticosteroids and theophylline, administered either prior to or during the hospital admission may have influenced neutrophil acetyltransferase activity and the measurement of the enzyme activity at discharge was primarily performed in order to assess any such influence. The results indicating no significant differences in the Km and V\textsubscript{\text{max}} values at discharge compared with those measured during the acute stage and/or after recovery would suggest that the treatments received by these patients in hospital had little or no effect on acetyltransferase activity.

Studies with the oral PAF antagonists, WEB 2086 and modipafant, have shown little or no beneficial effect on lung function or asthma symptoms [30, 31], although a recent trial of another oral PAF antagonist, Y-24180 showed a significant reduction in bronchial hyperresponsiveness in asthmatic patients [32]. The importance of PAF as a mediator in asthma, therefore, remains controversial. While PAF may not be the primary effector in the asthmatic response, it may act as a priming agent in a network of lipid mediators and cytokines involved in the chronic inflammatory process [5, 19], and there is also evidence that systemic PAF concentrations are increased during acute asthma. A23187-stimulated granulocyte PAF production and plasma PAF levels are reported to be higher in children with asthma symptoms compared with asymptomatic or control children [16, 17], and in patients with mild asthma, blood PAF levels were increased following allergen-induced bronchoconstriction [15] and during spontaneous exacerbations [18].

Since in vitro studies indicate that acetyl-CoA:lyso-PAF acetyltransferase regulates PAF production in human neutrophils and endothelial cells [33, 34], it might be predicted that increased systemic PAF concentrations...
in symptomatic asthma would result from upregulation of neutrophil acetyltransferase activity. However, the present study suggests that acetyltransferase activity is not increased, and may actually be reduced, during the acute episode compared with the activity in stable asthma. It is possible that increased inflammatory mediator or cytokine production consequent to the acute episode may initiate a negative feedback mechanism that reduces acetyltransferase activity as a means of controlling excessive PAF production. Following recovery, activity appeared to increase towards the chronically stimulated level previously observed in stable asthmatic subjects [24].

While upregulation of neutrophil acetyltransferase may, therefore, be involved in chronic PAF production in stable asthma, it may not be the major source of increased systemic PAF concentrations in acute asthma. It is possible that activated eosinophils in peripheral blood contribute significantly to systemic PAF production in acute asthma. Although the neutrophil preparations may have contained a few eosinophils, the present study does not permit any comment on whether acetyltransferase activity was increased in eosinophils alone, during acute asthma. Overall regulation of PAF biosynthesis in neutrophils may also depend on the activity of a CoA-independent transacylase in addition to lyso-PAF acetyltransferase [35, 36]. Increased PAF production during acute asthma may, therefore, result from increased CoA-independent transacylase activity, and an increased supply of lyso-PAF substrate for acetyltransferase, the activity of which is already increased in stable asthma. Alternatively, increased systemic PAF concentrations in asthma may reflect a reduced rate of PAF degradation due to decreased PAF acetylhydrolase activity in stable asthma. Several methods to increase the rate of PAF degradation have been reported [24]. The activity of platelet PAF acetylhydrolase was increased in eosinophils during acute asthma. Although the neutrophil preparations may have contained a few eosinophils, the present study does not indicate any involvement of eosinophils in peripheral blood in acute asthma. Increased PAF production during acute asthma may, therefore, be involved in chronic PAF production in stable asthma. Although the present study did not indicate any increase in lyso-PAF acetyltransferase activity during acute asthma, Km was significantly reduced with a trend for Km and Vmax to increase on recovery. The change in Km and Vmax may reflect a reduced rate of PAF degradation due to decreased PAF acetylhydrolase activity, decreased PAF acetyltransferase activity, or decreased acetyl-CoA availability in neutrophils. The activity of platelet PAF acetylhydrolase was increased during acute asthmatic attacks and decreased in stable asthmatics [24]. Platelet acetyltransferase activity may, therefore, result from increased CoA-independent transacylase activity, and an increased supply of lyso-PAF substrate for acetyltransferase, the activity of which is already increased in stable asthma. Alternatively, increased systemic PAF concentrations in asthma may reflect a reduced rate of PAF degradation due to decreased PAF acetylhydrolase activity, decreased PAF acetyltransferase activity, or decreased acetyl-CoA availability in neutrophils. The activity of platelet PAF acetylhydrolase was increased during acute asthmatic attacks and decreased in stable asthmatics [24]. Platelet acetyltransferase activity may, therefore, result from increased CoA-independent transacylase activity, and an increased supply of lyso-PAF substrate for acetyltransferase, the activity of which is already increased in stable asthma. Alternatively, increased systemic PAF concentrations in asthma may reflect a reduced rate of PAF degradation due to decreased PAF acetylhydrolase activity, decreased PAF acetyltransferase activity, or decreased acetyl-CoA availability in neutrophils.

Acknowledgements: The authors thank T. Hamilton and the staff of the Department of Emergency Medicine at Sir Charles Gardner Hospital, Perth, Western Australia for their assistance in performing this study.

References

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