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Characterization of platelet-activating factor binding to human airway epithelial cells: modulation by fatty acids and ion-channel blockers

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Radioligand-binding studies were performed in primary cultured human airway epithelial cells with [^{3}H]PAF to determine whether these cells express platelet-activating factor (PAF) receptors. Scatchard analysis of PAF binding data revealed a single class of PAF binding sites with K_d 1.8 ± 0.2 nM and $B_{max.}$ 21.0 ± 2.1 fmol/10^6 cells (13000 receptors/cell). PAF binding increased the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), indicating functional PAF receptors. Palmitate ($C_{16:0}$), linoleic acid ($C_{18:2\omega6}$) or eicosapentaenoic acid ($C_{20:5\omega3}$) was incubated with the cells to test the effect on PAF binding. Incorporation of each fatty acid into cellular phospholipid occurred. [^{3}H]PAF (1 nM) binding decreased in cells supplemented with $C_{20:5\omega3}$, but increased in the cells supplemented with $C_{16:0}$. Scatchard analysis revealed that the inhibition of PAF binding by supplementation with $C_{20:5\omega3}$ was due to a decrease in both affinity and number of PAF receptors. PAF-stimulated increase in $[Ca^{2+}]_i$ was also decreased by 60 % in cells supplemented with $C_{20:5\omega3}$. Verapamil, a Ca^{2+}-channel blocker, and amiloride, a Na^+-channel blocker, inhibited specific binding of [^{3}H]PAF to the cells, with IC_{50} 4–5 μM and 0.2 mM respectively. Diphenylamine-2-carboxylate (DPC), a Cl^--channel blocker, dramatically increased PAF binding to the cell in a dose-dependent manner. Scatchard analysis revealed that verapamil and amiloride decreased both binding affinity and number of PAF receptors, whereas DPC increased PAF binding sites without affecting binding affinity. These results demonstrate that human airway epithelial cells have a functional receptor for PAF and that PAF receptor binding can be modulated by exogenous fatty acids and by ion-channel blockers.

INTRODUCTION

Platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF) is a potent phospholipid mediator involved in a variety of pathophysiological events, e.g. inflammation, pulmonary and cardiovascular disorders [1–3]. PAF is produced endogenously in pathological conditions and also upon stimulation of various cells and tissues [2–4]. The effects of extracellular PAF are mediated by interaction of PAF with specific cell membrane receptors [5,6]. Radioligand-binding studies using [^{3}H]PAF have demonstrated specific receptors for PAF in platelets [7,8], neutrophils [9,10], lymphocytes [11,12], monocytes [13], macrophages [14], human lung tissues [15], rat liver tissues [16] and rat brain tissues [17]. The PAF receptor from guinea-pig lung was recently cloned by functional expression and showed homology to the G-protein-coupled receptors with seven transmembrane-spanning segments [18]. The binding of PAF to its receptor initiates a cascade of biochemical events, including phospholipid turnover, activation of protein kinase C, increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and generation and release of other mediators (e.g. eicosanoids) [3,5,19]. Specific PAF receptor antagonists inhibit PAF-induced responses [20,21].

PAF has been implicated in the pathogenesis of several pulmonary diseases and in inflammatory disorders such as asthma [22]. Pulmonary airway epithelium has been reported to synthesize PAF [23] as well as eicosanoids (e.g. prostaglandins, leukotrienes etc.) upon stimulation [24], suggesting involvement of the epithelial cells in pathological processes of inflammatory lung disease. Whether or not airway epithelial cells respond to PAF via specific receptors may impact on development of airway inflammation, because the responses of airway epithelial cells to PAF could amplify the injury effects of PAF on the inflamed airway via the generation of more mediators. Although a previous study has demonstrated specific binding sites for PAF in human lung tissue homogenates [15], it is unknown if airway epithelial cells express PAF receptors.

Since the effects of extracellular PAF are mediated by specific cell membrane receptors [5], understanding of regulation of PAF receptor and the PAF-receptor-mediated signalling mechanism would provide insight into the pathogenesis and therapy of PAF-involved inflammatory diseases such as asthma. However, knowledge of regulatory factors that affect specific PAF receptors and subsequent cellular responses is very limited. Generally, any factor decreasing PAF binding to its receptor or subsequent PAF-receptor-mediated signal transduction would play a role in anti-inflammation. Fish oil enriched with eicosapentaenoic acid ($C_{20:5\omega3}$) has apparent anti-inflammatory effects [25,26]. Although the major mechanism for the effect of $C_{20:5\omega3}$ is thought to be a decrease in the production of inflammatory mediators, including arachidonate metabolites, PAF and cytokines [26,27], the exact mechanism of action of $C_{20:5\omega3}$ is not completely understood. Previous studies found that dietary supplementation with ω3 polyunsaturated fatty acid decreased PAF-induced Ca^{2+} mobilization and InsP_3 formation [28,29], suggesting an interaction between the fatty acids and PAF's signal transduction. However, whether $C_{20:5\omega3}$ and other fatty acids can affect PAF binding to its receptors remains to be examined.

Pulmonary inflammatory disease is a main manifestation of cystic fibrosis, whose basic defect is impermeability of epithelial

Abbreviations used: $[Ca^{2+}]_i$, free intracellular Ca^{2+} concentration; DPC, diphenylamine-2-carboxylate; HBSS, Hanks balanced salt solution; IC_{50}, concentration giving 50 % inhibition; lyso-PAF, 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor (1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine).

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cells to Cl^- [30,31]. The relationship between the Cl^- impermeability and the inflammatory process in the epithelial cell is not known. Whether or not changes in ion conductance across the cell membrane can alter inflammatory reactions by affecting the action of inflammatory mediators such as PAF is also unknown. Various univalent and bivalent cations have been found to exert regulatory effects on PAF receptor binding [32]. Moreover, cation-channel blockers, such as Ca^{2+}-channel blockers [33–35] and Na^+-channel blockers [36], have been shown to inhibit PAF binding to platelets. However, it is unknown whether Cl^- channel blockers affect PAF receptor binding.

The objective of this study is to characterize [^{3}H]PAF binding to human airway epithelial cells and to determine if supplementation of the cell with different fatty acids or ion-channel blockers affects PAF binding and action.

MATERIALS AND METHODS

Materials

[^{3}H]C_{16}-PAF (1-*O*-[1,2-^{3}H(n)]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; sp. radioactivity of 60.0 Ci/mmol) was purchased from NEN, Canada. Unlabelled C_{16}-PAF, lyso-PAF, BSA, Hepes, probenecid, digitonin and fatty acids were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). WEB 2086 was obtained from Boehringer Ingelheim (Ridgefield, CT, U.S.A.). Fluo-3 acetoxymethyl ester (AM) was obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.).

Cell isolation and culture

Isolation and primary culture of human airway epithelial cells were carried out as previously described [37]. Epithelial cells were isolated from fresh human nasal turbinates with protease (0.1 %, Type 14) and then plated on collagen-coated plates with culture medium (Dulbecco's modified Eagle's/F12, hormones, antibiotics, 1 % fetal-bovine serum). Cells were cultured at 37 °C in a 5 %-CO_2 atmosphere and 98 % relative humidity in a tissue-culture incubator (model 3173, Forma Scientific) until the cells grew to confluence (usually 5–7 days). Cultures with an epithelial-cell purity of more than 90 % were used for binding assay. To modify the membrane fatty acid profile, 100 μM fatty acid bound to BSA was added to serum-free culture medium as described previously [28]. After 6 h of incubation with different fatty acids, the cells were harvested and subjected to binding assay, $[Ca^{2+}]_i$ measurement and fatty acid analysis. Cell viability was not affected by this concentration of fatty acid.

[^{3}H]PAF binding assay

PAF binding was performed by using cell suspensions, as most receptors on the epithelial cell are situated in the basolateral membrane [38]. Cells were detached with 0.05 % trypsin/EGTA, washed twice with 10 % (v/v) fetal-bovine serum culture medium (as above) and incubated at 37 °C for at least 1 h before they were used for binding. Cell viability was more than 95 %, as measured by Trypan Blue exclusion. PAF binding in the cell suspension was more reproducible with smaller variations and showed higher levels of binding, when compared with the binding measured initially in monolayer, as well as in the cells harvested by scraping, suggesting that mild trypsin treatment did not affect PAF binding activity.

Cells [$(1–2) \times 10^6$] were resuspended in 1 ml of HBSS (Hanks balanced salt solution) containing 10 mM Hepes (pH 7.4) and 0.25 % (w/v) BSA and placed in 1.5 ml microcentrifuge tubes. Cell suspensions were incubated in duplicate with [^{3}H]PAF with or without unlabelled PAF or WEB 2086 at 4 °C for appropriate times. This temperature (4 °C) was used to minimize both the incorporation into cells and the metabolism of [^{3}H]PAF. The cells were harvested by centrifugation for 1 min in a Beckman Microfuge. The supernatant was carefully aspirated and the cell pellet washed with 2×1 ml of cold HBSS. The resulting cell pellet was resuspended in 300 μl of 10 % (w/v) SDS, mixed with 10 ml of scintillation cocktail (ScintiVerse, Fisher) and assessed for cell-bound radioactivity in a Beckman LS5801 liquid-scintillation counter. Under these conditions the counting efficiency was 54 %. Non-specific binding was defined as total binding in the presence of 1000-fold excess unlabelled PAF. Specific binding was calculated as the difference between total and non-specific binding. The dissociation constant (K_d) and maximal binding ($B_{max.}$) were determined by Scatchard analysis [39].

Measurement of $[Ca^{2+}]_i$

This was done by methods similar to those described by Merritt et al. [40]. Isolated epithelial cells were loaded with Fluo-3 by incubating the cell suspension in buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose and 2.5 mM probenecid, pH 7.4) with 5 μM Fluo-3/AM at 37 °C for 40 min. Cells (1×10^6/ml) were then washed once with buffer and resuspended in buffer supplemented with 1 mM $CaCl_2$ and 0.5 % BSA. A portion (0.5 ml) of cells was transferred to a 1 ml fluorimeter cuvette and prewarmed to 37 °C. Cell fluorescence was monitored at 37 °C with constant mixing in a CAF-100 Ca^{++} Analyzer (Jasco Inc.) at 490 nm excitation, 530 nm emission. WEB 2086 was introduced with (< 0.5 %) ethanol as the solvent, which did not affect baseline $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ was calculated from the equation: $[Ca^{2+}]_i = K \times [(F - F_{min.})/(F_{max.} - F)]$, where K is the dissociation constant of Fluo-3–Ca^{2+}, 864 nM at 37 °C [31], and F is the Fluo-3 fluorescence measured during the experiments. $F_{max.}$ and $F_{min.}$ are the fluorescence values when Fluo-3 is Ca^{2+}-saturated and Ca^{2+}-free, respectively. $F_{max.}$ was determined by lysis of the cells with digitonin at the end of each experiment. $F_{min.}$ was obtained by addition of sufficient EGTA after lysis of the cells [40].

Lipid analysis

The fatty acid composition of membrane phospholipids was analysed as described previously [41]. Cell total lipid was extracted with chloroform:methanol (2:1, v/v) and separated into phospholipid and neutral lipid by t.l.c. Fatty acids of the phospholipid fraction were methylated and fatty acid methyl esters were analysed by g.l.c. using a fully automated Varian Vista 6000 GLC apparatus equipped with a flame-ionization detector. This procedure quantified all fatty acids. The chromatography utilized a fused-silica BP_{20} capillary column (25 M × 0.25 mm internal diam.). Helium was used as the carrier gas at a flow rate of 1.8 ml/min, by using a splitless injection. The initial oven temperature was 150 °C, increased to 190 °C at 20 °C/min and held for 23 min, then increased to 220 °C at 2 °C/min for a total analysis time of 40 min. These analytical conditions separated all saturated, mono-, di- and poly-unsaturated fatty acids from C_{14} to C_{25} carbon atoms in chain length. A Varian Vista 402 data system was used to analyse area percentage for all resolved peaks.

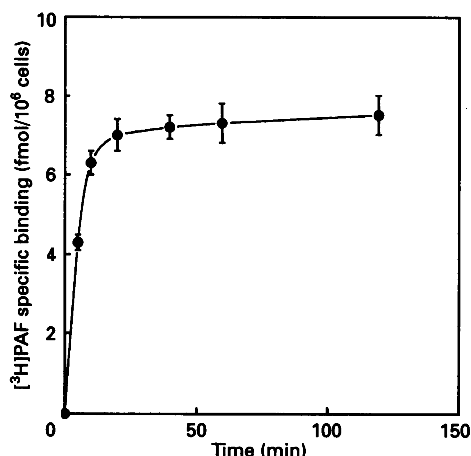

Figure 1 Time course of [³H]PAF (1 nM) binding to human airway epithelial cells at 4 °C

Specific binding was the difference between PAF binding in the absence and presence of excess unlabelled PAF (1–2 μM). Each value is the mean ± S.D. of three separate experiments performed in duplicate.

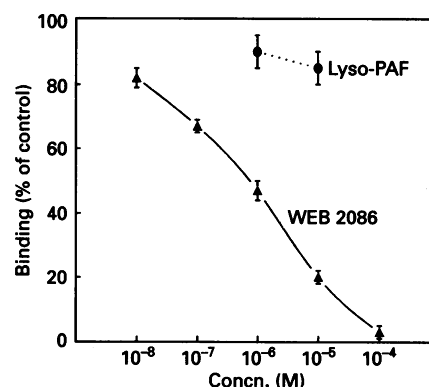

Figure 3 Competition of [³H]PAF binding to human airway epithelial cells by PAF-receptor antagonist WEB 2086 (▲——▲) and lyso-PAF (●····●)

The cells were incubated with 1 nM [³H]PAF and different concentrations of WEB 2086 and lyso-PAF at 4 °C for 60 min. Results are expressed as means ± S.D. of the binding (percentage of control) from three separate experiments.

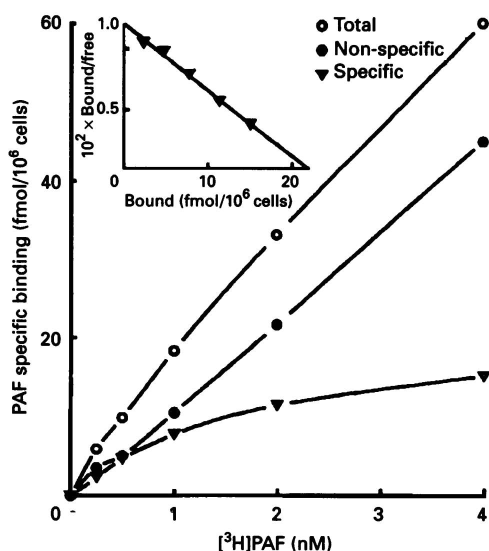

Figure 2 Binding isotherm of [³H]PAF to human airway epithelial cells

The cells were incubated at 4 °C for 60 min with increasing concentrations of [³H]PAF (0.25–4.0 nM). Specific binding (▼) was calculated as the difference between total (○) and non-specific (●) binding determined in the presence of excess of unlabelled PAF. Inset: Scatchard analysis of the specific binding of [³H]PAF to the epithelial cells. The data are representative of five separate experiments for cells derived from different individuals. The K_d was 1.8 ± 0.2 nM and $B_{max.}$ was 21.0 ± 2.1 fmol/10⁶ cells.

RESULTS

Characterization of [³H]PAF binding to human airway epithelial cells

To examine the kinetics of PAF binding, epithelial cells (1×10^6/ml) were incubated in duplicate with 1 nM [³H]PAF, with or without 1 μM unlabelled PAF for 5, 10, 20, 40, 60 and 120 min. Specific binding reached saturation by 30–40 min (Figure 1). The kinetics of PAF binding is similar to that found in monocytes [13] and lymphoblastoid cells [12]. Non-specific binding at this concentration (1 nM [³H]PAF) was 50–60 % of total binding.

The affinity and number of PAF binding sites in airway epithelial cells were determined by saturation binding assays in which the cells were incubated with various concentrations of [³H]PAF with or without unlabelled PAF for 60 min. Specific binding of PAF to the cells increased with concentration of labelled ligand, and the binding was saturable (Figure 2). Data from saturation binding was subjected to Scatchard analysis [39]. Five separate experiments yielded linear plots (a representative is shown in Figure 2 inset), indicating the presence of a single class of binding sites. The equilibrium dissociation constant representing the affinity (K_d) and the total number of receptor sites ($B_{max.}$) are 1.8 ± 0.2 nM and 21 ± 2.1 fmol/10⁶ cells ($n = 5$) respectively. Assuming an equimolar ligand–receptor complex, the $B_{max.}$ corresponds to 12600 ± 1260 binding sites per epithelial cell. This number of receptors is similar to that found in human monocytes [13] and human Raji lymphoblasts [11], but greater than that found in neutrophils [9] and platelets [7]. The K_d for PAF observed in the epithelial cell is very close to that found in human lymphoblasts [11,12], higher than that found in human neutrophils [9] and human lung homogenates [15], but lower than that found in human monocytes [13] and human platelets [7].

The specificity of PAF binding was established by competition studies utilizing WEB2086, a known potent PAF-receptor antagonist [22], and lyso-PAF. WEB2086 inhibited the specific binding of [³H]PAF to the cells in a dose-dependent manner, with $IC_{50} = 0.3$–0.5 μM, whereas lyso-PAF at a concentration of 10 μM did not apparently inhibit the binding (Figure 3), suggesting that the binding sites are specific for PAF.

To assess the functional capability of PAF binding sites, the epithelial cell was loaded with the Ca^{2+} indicator dye, Fluo-3 AM and $[Ca^{2+}]_i$ was monitored after stimulation of the cell with PAF. PAF at 100 nM induced a rapid rise in $[Ca^{2+}]_i$ in epithelial cells (Figure 4a). Addition of the PAF antagonist WEB 2086 to the cell suspension 5 min before PAF stimulation resulted in attenuation of the peak response (Figure 4b), indicating that the specific sites for PAF are functional receptors. These results are

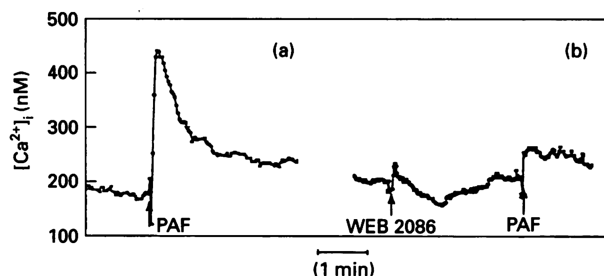

Figure 4 Changes in $[Ca^{2+}]_i$ induced by PAF (100 nM) in human airway epithelial cells pretreated with solvent vehicle (control, a) or 2 μM WEB 2086 (b) 5 min before PAF stimulation

Arrows indicate the addition of WEB 2086 and PAF. Results are representative of three separate experiments that produced a change in Ca^{2+} concentration of 215±32 nM by 100 nM PAF.

Table 1 Effects of fatty acid supplementation on the fatty acyl composition of epithelial phospholipids

Human airway epithelial cells were cultured for 6 h in medium containing 100 μM exogenous fatty acid. The cells were then harvested, cellular lipids were extracted and fatty acid methyl esters were analysed by g.l.c. as described in the Materials and methods section. Values are means±S.D. of three individual cultures: *$P < 0.05$.

		Composition (%, w/w)			
Fatty acid	Fatty acid supplemented...	None	$C_{16:0}$	$C_{18:2\omega6}$	$C_{20:5\omega3}$
$C_{16:0}$		19.0±0.5	27.0±0.5*	16.4±0.2	17.8±0.2
$C_{16:1\omega7}$		4.0±0.4	5.7±0.3*	2.8±0.2	3.6±0.4
$C_{18:2\omega6}$		4.5±0.3	4.3±0.1	21.0±0.5*	4.2±0.2
$C_{20:3\omega6}$		1.8±0.3	1.9±0.2	2.5±0.3	1.9±0.2
$C_{20:4\omega6}$		5.1±0.4	5.5±0.3	6.5±0.3*	4.5±0.3
$C_{20:5\omega3}$		0.8±0.2	0.8±0.2	–	8.9±0.3*
$C_{22:5\omega3}$		0.7±0.2	0.6±0.1	0.7±0.2	3.1±0.3*

Table 2 Effect of fatty acid supplementation on PAF binding

Epithelial cells were cultured in medium containing 100 μM of different fatty acids for 6 h. The cells were harvested and resuspended in binding buffer and incubated with 1 nM [^{3}H]PAF in the presence or absence of 1 μM unlabelled PAF for 40 min at 4 °C. Specific binding was determined as described in the Materials and methods section. Values are means±S.D. of six independent experiments: *$P < 0.05$.

Fatty acid treatment	PAF specific binding (fmol/10^6 cells)
Control	7.80±0.5
$C_{16:0}$	11.80±1.5*
$C_{18:2\omega6}$	8.25±1.1
$C_{20:5\omega6}$	2.10±1.1*

consistent with previous findings in human monocytes [13], human lymphoblasts [11,12] and macrophages [14]. A phenomenon of desensitization of $[Ca^{2+}]_i$ response to PAF was observed (results not shown), suggesting that PAF down-regulates its own receptors [5].

Effect of fatty acid supplementation on PAF binding

To examine the effect of modification of membrane fatty acid composition on PAF binding, $C_{16:0}$ (palmitate) and $C_{18:2\omega6}$ (linoleic acid) or $C_{20:5\omega3}$ (100 μM) was added to the culture medium and incubated with the cells for 6 h. Supplementation of the cell with individual fatty acids resulted in substantial modification of cellular phospholipid fatty acid composition (Table 1). Exogenous $C_{20:5\omega3}$ resulted in an approx. 10-fold increase in total $C_{20:5\omega3}$ content of phospholipid and a 4-fold increase in membrane content of $C_{22:5\omega3}$. Supplementation of the medium with $C_{16:0}$ also markedly increased cellular $C_{16:0}$ and $C_{16:1\omega7}$ content in membrane phospholipid. Consistent with our previous observations [37], exogenous $C_{18:2\omega6}$ resulted in a significant increase in the $C_{18:2\omega6}$, $C_{20:3\omega6}$ and $C_{20:4\omega6}$ content in membrane phospholipid.

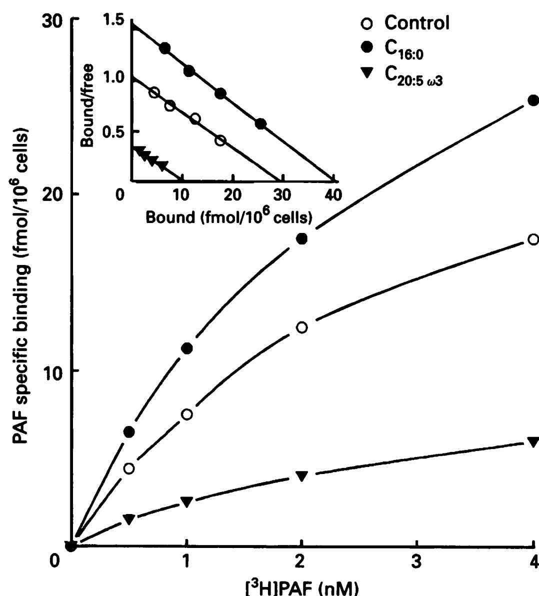

Figure 5 Saturation course and Scatchard analysis of [^{3}H]PAF binding to cells supplemented with $C_{16:0}$ or $C_{20:5\omega3}$

Airway epithelial cells were cultured in medium containing 100 μM $C_{16:0}$ or $C_{20:5\omega3}$ for 6 h. The cells were then incubated with different concentrations of [^{3}H]PAF in the presence or absence of 1 μM unlabelled PAF. Specific binding of [^{3}H]PAF was determined as described in the Materials and methods section. Values are means of two separate experiments (in duplicate). Inset is Scatchard analysis of the binding data.

After incubation with different fatty acids, the cells enriched in each fatty acid were used for assay of PAF binding. At 1 nM [^{3}H]PAF, specific binding increased by 50% (from 7.8±0.6 to 11.8±2.5 fmol/10^6 cells) in cells supplemented with $C_{16:0}$, but decreased by 65% (from 7.8±0.6 to 2.2±1.1 fmol/10^6 cells) in the cells supplemented with $C_{20:5\omega3}$. Supplementation with $C_{18:2\omega6}$ did not significantly change PAF binding (Table 2). Scatchard analysis revealed that supplementation with $C_{16:0}$ increased the number of PAF receptors ($B_{max.}$: from 26.5 to 42.5 fmol/10^6 cells), but did not significantly affect affinity (K_d: from 2.2 to 2.7 nM); supplementation with $C_{20:5\omega3}$ decreased both affinity (K_d: from 2.2 to 2.9 nM) and number ($B_{max.}$: from 26.5 to 10.2 fmol/10^6 cells) of PAF receptors (Figure 5).

To test if the effect of $C_{20:5\omega3}$ is associated with metabolism of the fatty acid, indomethacin, an inhibitor of cyclo-oxygenase and 5-lipoxygenase [42], was added to the medium when cells were incubated with $C_{20:5\omega3}$. Indomethacin (100 μM) did not change the effect of $C_{20:5\omega3}$ on PAF binding, suggesting that the effect of $C_{20:5\omega3}$ is not due to its metabolites. Addition of $C_{20:5\omega3}$ to the cells

Table 3 Effect of supplementation with $C_{20:5\omega3}$ on response of $[Ca^{2+}]_i$ to different concentrations of PAF

Human airway epithelial cells were incubated with or without 100 μM $C_{20:5\omega3}$ for 6 h. The cells were loaded with 5 μM Fluo-3 for 40 min and $[Ca^{2+}]_i$ was measured as described in the Materials and methods section, after stimulation of the cell with 1 nM, 10 nM or 100 nM PAF. Values are means $\pm$ S.D. of four experiments: $^*P < 0.05$.

[PAF] (nM)	$[Ca^{2+}]_i$ (nM)	
	Control	$C_{20:5\omega3}$
2	55.0 ± 7.0	25.0 ± 6.0*
10	95.0 ± 15.0	45.0 ± 8.0*
100	210.0 ± 32.0	76.0 ± 20.0*

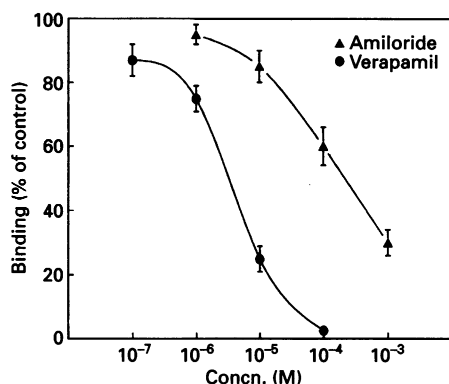

Figure 6 Inhibition of specific [³H]PAF binding to human airway epithelial cells by different concentrations of verapamil and amiloride

Cultured epithelial cells (1×10^6 cells) were preincubated with different concentrations of verapamil (dissolved in methanol) or amiloride for 15 min at 37 °C, then cooled rapidly to 4 °C. Specific binding of [³H]PAF (1 nM) to the cells was assayed at 4 °C as described in the Materials and methods section. Values are means $\pm$ S.D. of three separate experiments with duplicate determinations.

a few minutes before assay of binding had similar but less pronounced effects on PAF-binding, indicating that the effect of $C_{20:5\omega3}$ may be partly derived from a direct interaction between unesterified $C_{20:5\omega3}$ and the PAF receptor. The direct interaction between fatty acid and receptor has been previously suggested for steroid receptors [43].

Our preliminary data show that $C_{22:6\omega3}$ and $C_{18:3\omega3}$ also decreased PAF binding, by approx. 60 % and 25 % respectively. It appears that the effect of $C_{18:3\omega3}$ was less potent compared with $C_{20:5\omega3}$ and $C_{22:6\omega3}$.

To test whether $C_{20:5\omega3}$ can suppress PAF-induced Ca^{2+} mobilization as a result of decreased PAF binding, the change in $[Ca^{2+}]_i$ in response to different concentrations of PAF was measured after the cells were incubated with or without $C_{20:5\omega3}$ for 5–6 h. Additions of PAF induced a typical $[Ca^{2+}]_i$ response in control cells. Cells supplemented with $C_{20:5\omega3}$ showed a decrease in PAF-stimulated increase in $[Ca^{2+}]_i$ at all concentrations, by 50–60 % [at 2 nM, from 55 ± 7 to 25 ± 6 nM; at 10 nM, from 95 ± 15 to 45 ± 8 nM; at 100 nM, from 215 ± 32 to 76 ± 20 nM ($n = 4$)] (Table 3). It seems that the increased $[Ca^{2+}]_i$ in $C_{20:5\omega3}$-supplemented cells returned to the basal level quickly, whereas the increased $[Ca^{2+}]_i$ in control cells were sustained above basal

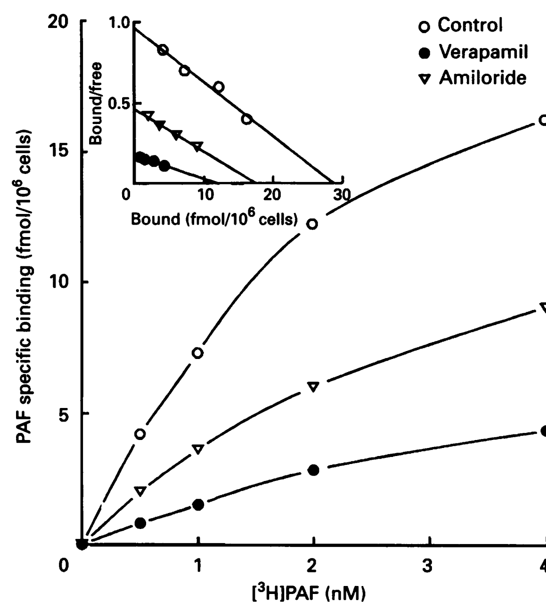

Figure 7 Binding isotherm of [³H]PAF to human airway epithelial cells in the presence or absence (control) of ion-channel blockers

Cells (1×10^6) were incubated at 4 °C for 60 min with increasing concentrations of [³H]PAF (0.5–4 nM) in the presence of verapamil (25 μM) (●) or amiloride (500 μM) (▽), or in the absence of ion-channel blockers (○). Specific binding was determined as described in the Materials and methods section. The data are means of the two separate experiments with two determinations. Insert is Scatchard analysis of the binding data.

levels for a longer time period (Table 3). It is notable there is an apparent relationship between the decrease in PAF binding (65 %) and the decrease in $[Ca^{2+}]_i$ response (60 %), suggesting that both effects may be related events. It is logical to speculate that inhibition of the PAF-induced increase in $[Ca^{2+}]_i$ by $C_{20:5\omega3}$ may be the consequence of decreased PAF binding to the cell.

Effects of ion-channel blockers on PAF binding

To assess the effect of Ca^{2+}- and Na^+-channel blockers on PAF binding to human airway epithelial cell, different concentrations of verapamil, a Ca^{2+}-channel blocker, or amiloride, a Na^+-channel blocker, were added to the medium and incubated with cells for 15 min at 37 °C, and then specific binding of PAF to the cells was assayed at 4 °C. It was observed that both verapamil and amiloride inhibited PAF binding, but verapamil was much more potent than amiloride (Figure 6). The inhibition of PAF binding by verapamil and amiloride was dose-dependent, with a IC_{50} of approx. 4–5 μM and 0.2 mM respectively (Figure 6). Scatchard analysis revealed that verapamil and amiloride decreased PAF-receptor binding affinity and the receptor number (Figure 7). These results are similar to those found in previous studies with platelets [33,35].

Diphenylamine-2-carboxylate (DPC) has been reported to be a Cl^--channel blocker in human airway epithelial cells [44]. To test if DPC affects PAF binding, DPC was added to the cell 15 min before binding assay. DPC increase specific [³H]PAF binding in a dose-dependent manner (Figure 8). PAF binding to the cells increased dramatically when DPC concentration was over 100 μM. At 1 mM DPC, specific binding of PAF was about 3–4 times that in the absence of DPC. Scatchard analysis of PAF binding in the presence of different fixed concentrations of DPC

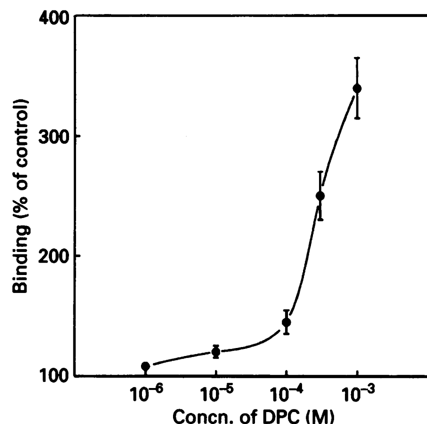

Figure 8 Effect of different concentrations of DPC on specific [^{3}H]PAF (1 nM) binding to human airway epithelial cells

Cells (1×10^6) were preincubated with DPC (dissolved in dimethyl sulphoxide) for 15 min at 37 °C, then cooled rapidly to 4 °C. Specific binding of [^{3}H]PAF (1 nM) to the cells was assayed as described in the Materials and methods section. Values are means ± S.D. of three experiments with duplicate determinations.

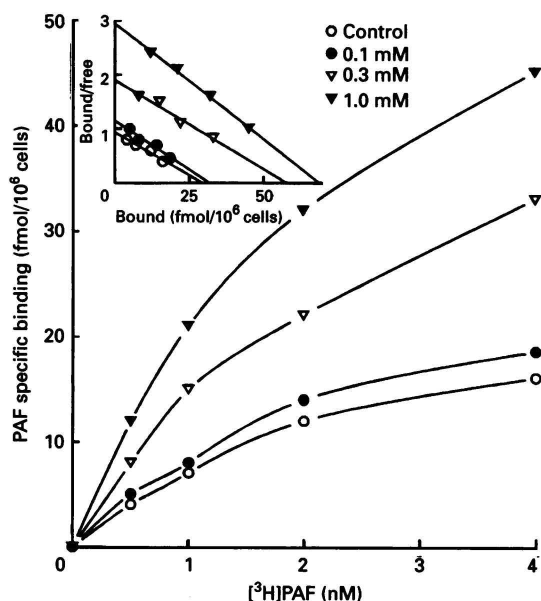

Figure 9 Saturation isotherms and Scatchard plots (insert) of [^{3}H]PAF binding to human airway epithelial cells without (○) and with DPC at concentrations of 0.1 mM (●), 0.3 mM (▽) and 1 mM (▼)

Cells (1×10^6) were preincubated with different concentrations of DPC at 37 °C for 15 min, then specific binding of increasing concentrations (0.5–4 nM) of [^{3}H]PAF was assayed at 4 °C. The data are means of two experiments with duplicate determinations.

revealed that this agent increased PAF receptor number, but did not affect binding affinity, apparently (Figure 9).

DISCUSSION

Characterization of PAF binding

The present study demonstrates that human airway epithelial cells have functional receptors for PAF. It appears that the cell suspension used in the present study should be an appropriate preparation for study of PAF binding in epithelial cells, because it allows exposure of the receptors located in both the apical and basolateral membranes to ligand. Although we cannot exclude the possibility that detachment of the cell by trypsin treatment might damage the receptor, it appears unlikely that the treatment affects binding properties. Previous studies indicate that the ligand-binding domain of G-protein-coupled receptors is located within the hydrophobic transmembrane core of the receptor protein, and that deletion of most of the extra-membrane hydrophilic regions of the protein does not affect ligand-binding properties, suggesting that the extra-membrane regions of the receptor are not required for ligand binding to occur [45]. Thus, even if the trypsin treatment has destroyed some of the extra-membrane regions, it would not be expected to change PAF binding activity. In the present study greater levels and higher affinity of PAF binding sites were observed in the epithelial cells compared with some other human cells.

Existence of functional PAF receptors in human airway epithelial cells suggests that the airway epithelium may play a direct role in the inflammatory process of pulmonary diseases such as asthma and cystic fibrosis. Thus control of PAF receptor expression and/or modulation of PAF binding activity in airway epithelial cells is a novel approach to manipulate pathological process of these pulmonary diseases.

Effects of fatty acid supplementation

It is evident that PAF binding to its membrane receptor can be altered by modifying the membrane fatty acid composition. Enrichment of the cell with $C_{20:5\omega3}$ results in a decrease in specific PAF binding, and consequently the PAF-stimulated increase in $[Ca^{2+}]_i$ is decreased. These results indicate an interaction between $C_{20:5\omega3}$ and the PAF signal-transduction pathway.

Previous studies have demonstrated a close association between fatty acids and cell signalling [46]. Non-esterified fatty acids have been shown to act as modulators and/or second messengers of signal transduction [46]. Fatty acids can directly interact with steroid hormones [43]. $C_{20:5\omega3}$ can decrease binding of the angiotensin II receptor [47], the low-density lipoprotein receptor [48], the thromboxane A_2 receptor [49] and the leukotriene B_4 receptor [50], as well as related receptor-mediated responses. In terms of an effect of lipid on the PAF receptor, a previous study [51] showed that incubation of monocytic U937 cells with low-density lipoprotein for 24 h increased the number of PAF receptors. In the same cell type, another study showed that $C_{22:6\omega3}$ (10 μM) decreased the PAF-induced increase in $[Ca^{2+}]_i$ [52]. Dietary ω3 polyunsaturated fatty acid was found to inhibit PAF-mediated formation of InsP_3 in human neutrophils [29]. Similarly, supplementation with fish oil significantly inhibited both the $[Ca^{2+}]_i$ response and InsP_3 formation in response to PAF in rat Kupffer cells [28]. Together, these observations and our present findings strongly suggest that there may be an interaction between fatty acid and PAF-mediated signal-transduction pathways. Our results further suggest that the effect of ω3 fatty acid on PAF-mediated signal transduction may occur at the ligand-receptor level.

The mechanism by which ω3 fatty acid affects PAF binding activity in the cell is not known. It is possible that the mechanism involves direct interaction between $C_{20:5\omega3}$ and the receptor; or $C_{20:5\omega3}$ may bind to the receptor and change receptor conformation, decreasing the availability of binding sites; or the mechanism may involve change in properties of membrane lipid by modifying membrane lipid-bilayer structure, altering receptor conformation or turnover. It is also conceivable that $C_{20:5\omega3}$

suppresses expression of a gene encoding a receptor component. In addition, it is possible that the decreased PAF binding observed in the cells supplemented with $C_{20:5\omega3}$ is due to blocking Ca^{2+} influx, because a Ca^{2+}-channel blocker was found to decrease PAF binding.

We propose that suppression of signal transduction at the level of PAF-receptor binding and the consequent decrease in cellular responsiveness to inflammatory stimuli may provide a novel mechanism by which $C_{20:5\omega3}$ produces an anti-inflammatory effect, before its suppression of inflammatory mediator generation.

Effects of ion-channel blockers

In the present study a Ca^{2+}-channel blocker (verapamil) and a Na^{+}-channel blocker (amiloride) inhibited PAF binding to human airway epithelial cells, whereas a Cl^{-}-channel blocker (DPC) increased PAF binding to the cells. Previous studies have demonstrated that verapamil inhibited PAF binding to human platelets [33,35] and human neutrophils [34]. Valone [33] reported that verapamil inhibited PAF binding to human platelets in a dose-dependent manner, with 50% inhibition (IC_{50}) at 63 ± 12 μM. Scatchard analysis revealed that verapamil decreased PAF-receptor binding affinity, but increased the receptor number in platelets, suggesting that this agent acts in competitive and non-competitive mechanisms. Similar results have been found in human neutrophils, except that the IC_{50} is higher (95 ± 14 μM) [34]. On the other hand, Wade et al. [35] reported that verapamil inhibited PAF binding to human platelets, with IC_{50} 32 ± 7 μM and, based on the observation that the agent decreased PAF-receptor binding affinity without affecting receptor number, suggested that the effect is competitive. In the present study, we found that verapamil inhibited PAF binding to human airway epithelial cells, with IC_{50} 4–5 μM; Scatchard analysis of PAF binding in the presence of verapamil indicated that this agent decreased both PAF-receptor binding affinity and PAF receptor number. This discrepancy in potency and mechanism of action of verapamil observed in different studies may be attributed to differences in receptors in different cell types and/or differences in the experimental conditions. Our findings that both affinity and binding site were altered by verapamil support the suggestion that the mechanism by which Ca^{2+}-channel blockers inhibit PAF binding is probably more complex than simple competition for receptor binding. It is possible that blocking Ca^{2+} influx and subsequently changing $[Ca^{2+}]_i$ by a Ca^{2+}-channel blocker contribute, at least in part, to its effect on PAF binding. PAF binding to its receptor may be dependent on $[Ca^{2+}]_i$, since an increase in extracellular Ca^{2+} concentration enhances PAF binding [32]. It is also possible that a Ca^{2+} channel is coupled with the PAF receptor and regulates the receptor binding activity. However, the exact mechanism by which Ca^{2+}-channel blockers inhibit PAF binding remains to be studied.

Similarly to the Ca^{2+}-channel blocker, amiloride, a Na^{+}-channel blocker, also has inhibitory effects on PAF binding, but it was less effective than verapamil. This result is consistent with previous findings that amiloride and its analogues inhibit the specific PAF binding to rabbit platelet membrane [36]. Amiloride has been proved to be an effective drug in improving the pulmonary conditions in cystic fibrosis [53]. The effect of amiloride may be in part due to its inhibitory effect on PAF binding, in addition to inhibition of Na^{+} and water absorption.

In terms of the effect of the Cl^{-} channel blocker on PAF binding, there is no relevant information available to date. In the present study, it was observed that 1 mM DPC, which under the conditions tested inhibits Cl^{-} conductance across membrane in airway epithelial cells at this concentration [44], increased specific binding of PAF to the epithelial cells by 2–3-fold. It is not known how blocking the Cl^{-} channel increases PAF binding in the epithelial cell. We speculate that blocking Cl^{-} channels leads to change in intracellular Cl^{-} concentration, which may cause alteration of cellular volume, cellular pH and membrane potential. These changes may activate gene expression and protein modification, perhaps stimulating cellular protein translocation between cellular compartments, for example, stimulating transfer of protein from the endoplasmic reticulum to the plasma membrane, consequently resulting in higher density of PAF receptors in the cell membrane. However, it cannot be excluded that the effect of DPC is due to direct interaction between DPC and the membrane lipid and/or membrane protein (PAF receptor) rather than blocking Cl^{-} conductance.

In summary, these results imply that ion-channel function may modify the action of PAF at the receptor level. The present finding, that blocking the Cl^{-} channel increases PAF binding, may have physiological relevance to pathological conditions such as cystic fibrosis in which both Cl^{-} impermeability and inflammation occur. It is possible that the defect in Cl^{-} transport potentiates the inflammatory reaction in cystic fibrosis. Furthermore, it is suggestive that, since Ca^{2+}- and Na^{+}-channel blockers decrease PAF binding to epithelial cells, they may be beneficial drugs for treatment of PAF-involved inflammatory diseases.

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