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SYNTHESIS OF THE 1-O-HEXADECYL MOLECULAR SPECIES OF PLATELET-ACTIVATING FACTOR BY AIRWAY EPITHELIAL AND VASCULAR ENDOTHELIAL CELLS

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Epithelial and endothelial cells may regulate leukocyte adherence and influx into underlying tissue, and this regulatory function may be based on the synthesis of leukocyte chemotaxins by these cells. We have measured the production of the potent lipid autocoid, platelet-activating factor (PAF) by airway epithelial and vascular endothelial cells using stable isotope dilution negative-ion chemical-ionization mass spectrometry. Both primary cultures of airway epithelial cells isolated from human and ovine tracheal mucosa and cultures of human umbilical vein endothelial cells generated measurable amounts of PAF under basal culture conditions and significantly increased amounts upon stimulation with ionophore A23187. The 1-O-hexadecyl molecular species of PAF was much more abundant than the 1-O-octadecyl species in each of these cell populations. The results suggest a possible common biochemical mechanism for regulation of inflammatory cell influx into tissues by barrier cells in epithelium and endothelium.

Epithelial tissues in general (and lung epithelium in particular) maintain and regulate the boundary between host and environment. The tissue protects against injury by acting as a barrier and modulates the inflammatory response by regulating leukocyte influx into the underlying tissue (1). We have proposed that epithelial cell injury is an initial event during inflammation and that the injured cells serve as a target of leukocyte influx and a source of leukocyte chemotaxins. This hypothesis led to characterization of the capacity of isolated airway epithelial cells to release lipid mediators derived from arachidonic acid oxygenation. Studies of airway epithelial cells have subsequently yielded evidence of an intricate enzymatic network for release and oxygenation of arachidonic acid, and these biochemical pathways generate products implicated in the regulation of a broad range of physiologic and pathophysiologic processes including inflammation (1). The absence of significant arachidonate 5-lipoxygenase activity in human airway epithelial cells (2) and the relative lack of chemotactic activity of the predominant 15- and 12-lipoxygenase products

[1]The abbreviations used are: PAF, platelet-activating factor; lysoPAF: 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBSS, Hanks' balanced salt solution; TLC, thin-layer liquid chromatography; NICI GC-MS, negative-ion chemical-ionization gas chromatography-mass spectrometry; PFB, pentafluorobenzoate; PMSF, phenylmethylsulfonyl fluoride.

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generated by human and animal cells (3) suggests that an alternative class of inflammatory mediators may be a more likely source of chemotactic activity.

One such mediator is platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF) . Nanomolar concentrations of PAF activate leukocytes and platelets via specific cell surface receptors and induce leukocyte chemotaxis (4). Observations consistent with a role for PAF in the development of airway inflammation and hypersensitivity include the ability of PAF to stimulate preferential migration of eosinophils into the airway (5) and to induce airway smooth muscle contraction and hyperreactivity in otherwise healthy subjects (6). In addition, PAF-receptor antagonists effectively inhibit airway obstruction and hyperreactivity in animal models of pulmonary airway inflammation (7, 8). Airway epithelial cells and vascular endothelial cells both selectively express leukocyte adhesion molecules (9, 10), and the receptors for these ligands are regulated by PAF (11). These findings also support a potential role for PAF as a trigger for leukocyte influx into the epithelium.

Based on the hypotheses that airway epithelial cells are a likely source of inflammatory mediators and that PAF may be a critical mediator in the development of airway inflammation and hypersensitivity, we have determined whether airway epithelial cells synthesize PAF using stable isotope dilution GC-MS. In addition, we have compared the characteristics of PAF production by airway epithelial cells to that by vascular endothelial cells. To our knowledge, the findings provide the first evidence that airway epithelial cells synthesize PAF and the first identification of the molecular species of PAF generated by vascular endothelial cells.

METHODS

Sources of materials—Authentic reference compounds included 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C16:0-PAF); 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C18:0-PAF); 1-*O*-oleoyl-2-acetyl-*sn*-glycero-3-phosphocholine (C18:1-PAF); and 1-*O*-hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine (C16:0-lysoPAF) obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). Deuterium-labeled PAF ([$^2H_3$]PAF) was prepared by treatment of C16:0-lysoPAF with trideuteroacetyl chloride in perdeuteroacetic acid as described previously (12).

Cell purification and culture—Human umbilical vein endothelial cells were obtained from Clonetics Corp. (San Diego, CA) and cultured up to passage 6 in Endothelial Cell Basal Medium (EBM) supplemented with epidermal growth factor, bovine brain extract, gentamicin, amphotericin B, and 2% fetal bovine serum. Human and ovine tracheal epithelial cells were isolated from tracheal mucosa and cultured in LHC-8e medium as described previously (13).

Cell incubation and labeling conditions—Several characteristics of cellular PAF generation were determined: (i) constitutive release was assessed by assaying PAF levels in aliquots of media and in cells at various times (0, 8 h, and 24 h) after changing media (LHC-8e or LHC basal); (ii) stimulated release was assessed by assaying aliquots of media and cells after the addition of 5 μM ionophore A23187 dissolved in dimethylsulfoxide for 10 min at 37° C. In radiochemical experiments designed to examine PAF biosynthesis by TLC, incubation mixtures included 25 μCi/ml carrier-free [^{3}H]acetate. For GC-MS determinations of PAF, reagents incubated with cell-free media were processed in parallel with cell-derived and standard curve materials as an analytic blank.

TLC isolation of [^{3}H]PAF—For radiochemical analysis of [^{3}H]PAF biosynthesis, incubations were terminated with acidified methanol. Cell supernatants and monolayers were extracted separately as described previously (14). Sample extracts (containing 10 ng of unlabeled carrier PAF) were reconstituted in chloroform/methanol (9:1, vol/vol), and an aliquot was used to determine total radioactivity. The remainder of the sample was applied to silica gel 60 plates (E. Merck, Darmstadt, FDR), which were developed in a solvent system of chloroform/methanol/acetic acid/water (50:25:8:4, vol/vol) as described previously (15). Samples were visualized under ultraviolet light after spraying with 0.1% 8-anilino-1-

naphthalene sulfonic acid. Both the band corresponding to PAF and the remainder of the lane were scraped separately and subjected to liquid scintillation counting. Incorporation of [^{3}H]acetate into PAF was estimated by multiplying the fraction of ^{3}H dpm in the PAF band by the total dpm in the sample lane.

GC-MS measurement of PAF—Extractions and all subsequent analyses were carried out in silanized glassware. Incubations were terminated by the addition of two volumes of methanol, and samples were extracted by the method of Bligh and Dyer (16) after addition of 10 ng of [$^2H_8$]PAF as an internal standard. Extracts were partially purified by solid phase extraction with disposable silicic acid columns (J. T. Baker Chemical. Co., Philipsburg, NJ) and were then digested to the diglyceride with phospholipase C, converted to the PFB derivative with pentafluorobenzoyl chloride, and purified by TLC as described previously (17). Derivatives were analyzed on a Hewlett-Packard 5890 Series II gas chromatograph interfaced with a Hewlett-Packard 5988 mass spectrometer. Samples were introduced into the chromatograph via a Grob-type injector operated in the splitless mode with helium as the carrier gas (head pressure 2 lb/in^2; total flow 10 ml/min; injector temperature 290°C) and analyzed on an 8 m x 0.32 mm (i.d.), cross-linked methylsilicone capillary GC column (Ultraperformance, Hewlett-Packard). GC oven temperature was programmed from 85°C to 285°C at 40°C/min starting 0.5 min after injection and then held at 285°C. The mass spectrometer was operated in the negative ion chemical ionization (NICI) mode (ionization voltage 230 eV; source temperature 100°C) with methane (source pressure 1.5 torr) as reagent gas. The major ion in the methane NICI mass spectrum of the PFB derivatives of PAF was the molecular ion: m/z 552 (C16:0-PAF), m/z 578 (C18:1-PAF), and m/z 580 (C18:0-PAF). The molecular ion in the methane NICI mass spectrum of the PFB derivative of [$^2H_3$]PAF was m/z 555.

RESULTS AND DISCUSSION

Validation of PAF identification and quantification—Quantitation of the various molecular species of PAF was performed by stable isotope dilution GC-MS under conditions in which the

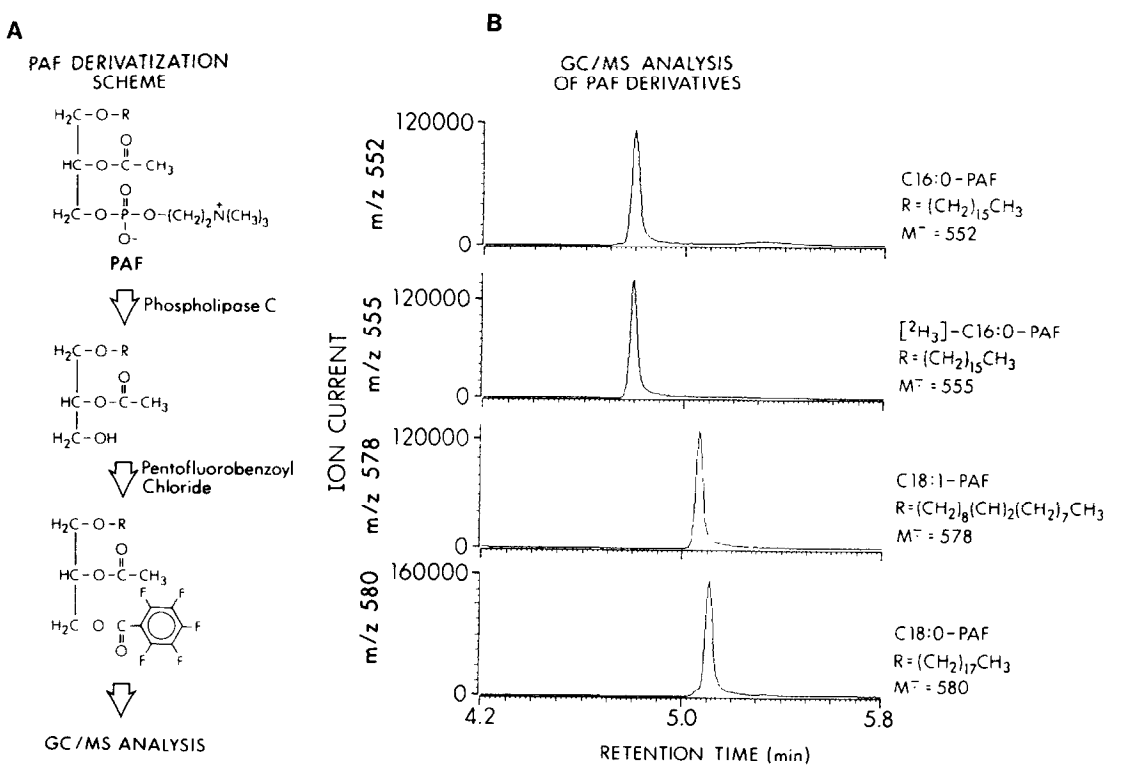

Fig. 1. Quantitative analysis of different molecular species of PAF standards by capillary column GC and NICI MS. Each species of native PAF (C16:0-, C18:0-, and C18:1-PAF) and [$^2H_8$]PAF (C16:0 species) was converted to the corresponding PFB derivative and then purified by TLC before analysis by GC-MS. Ions monitored were: 552 m/z for C16:0-PAF, 555 m/z for the internal standard C16:0-[$^2H_8$]PAF, 578 m/z for C18:1-PAF, and 580 m/z for C18:0-PAF.

predominant ion was the molecular ion. The structures of the various species of PAF are illustrated in Fig. 1. The derivatives of C16:0-PAF (m/z 552), C18:1-PAF (m/z 578), and C18:0-PAF (m/z 580) were distinguished both by GC retention time and by molecular ion m/z ratio and were quantified relative to the [$^2H_3$]PAF derivative (m/z 555). Identification of molecular species of PAF by this method was sufficient to exclude the 1-acyl-2-acetyl-glycerophosphocholine species which may be synthesized concomitantly with PAF by some cells (18). A 5-point standard curve was run with authentic C16:0-PAF and C18:0-PAF in each experiment and was linear in the range of 0.2 to 20 ng.

PAF biosynthesis in vascular endothelial cells—PAF biosynthesis by human umbilical vein endothelial cells has previously been reported based on radiochemical data (14). Radiolabeling techniques do not permit identification of the molecular species of PAF or precise quantification of PAF amounts. In the present experiments, PAF production by endothelial cells was also clearly demonstrable by GC-MS, and C16:0-PAF was the predominant molecular species. Identification of the molecular species of PAF was based on co-elution with synthetic standards on GC-MS (Fig. 2). PAF generation by the cells was stimulated by ionophore A23187, and most of this PAF remained cell-associated with little released into the supernatant. The amounts of PAF (C16:0-plus C18:0-species) in the cell pellet increased from 2.07 pmol up to 9.62 pmol/10^6 cells upon stimulation with ionophore A23187 with a ratio of C16:0/C18:0-PAF of 15:1. The corresponding amounts of PAF in the cell supernatants were 0.54 and 0.56 pmol/10^6 cells, respectively. A similar pattern of cell-association and A23187-stimulation of PAF was obtained using [^{3}H]acetate-

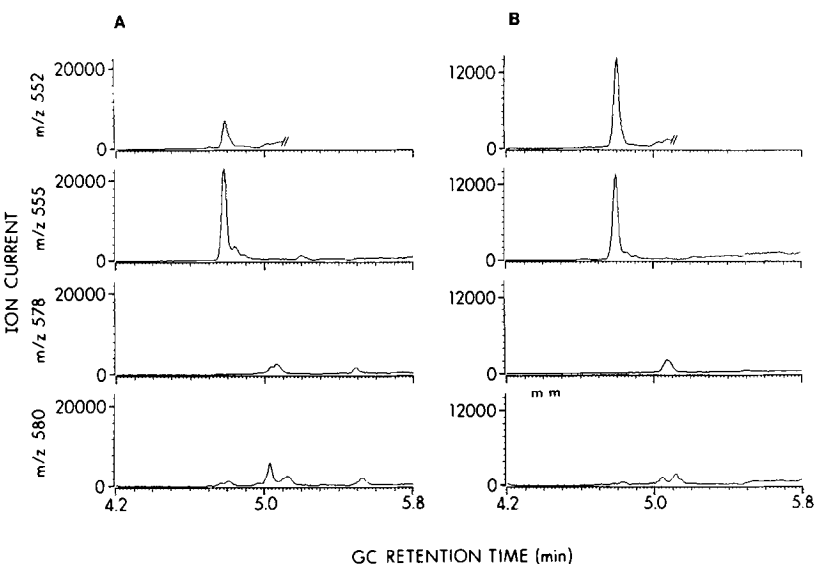

Fig. 2. NICI GC-MS analysis of PAF molecular species in vascular endothelial cells without (A) and with (B) stimulation by ionophore A23187. Cells were incubated with or without 5 μM A23187 in HBSS for 10 min at 37° C and then were extracted with chloroform/methanol using 10 ng of [$^2H_3$]PAF added as an internal standard. PAF was purified by silicic acid chromatography, derivatized, purified by TLC, and analyzed by GC-MS as in Fig. 1.

incorporation and TLC separation of phospholipids as an indication of PAF biosynthesis (not shown) in agreement with previous reports (14). The levels of PAF detected with A23187-stimulation also are in general agreement with recent reports of PAF levels based on assay of biologic activity (19).

Tracheal epithelial cell production of PAF—Tracheal epithelial cells were also found to produce PAF based on GC-MS analysis. Under most conditions, C16:0-PAF was the predominant molecular species, although detectable amounts of C18:0-PAF were sometimes observed. Primary cultures of human tracheal epithelial cells generated PAF under basal conditions in the absence of stimulation and most of this material was located in the cell pellet. Assay of PAF performed immediately after changing culture medium and at various times for up to 24 h later yielded a mean level of PAF (C16:0- plus C18:0-species) of 1.95 pmol/10^6 cells in the cell pellet (ratio of C16:0/C18:0-PAF of 12:1) and an undetectable amount of PAF (<0.01 pmol/10^6 cells) in the cell supernatant. Cell-association of PAF has been reported previously for neutrophils and endothelial cells (14, 20).

Ovine tracheal epithelial cells cultured under conditions identical to those for human cells also contained stable levels of cell-associated C16:0-PAF. The mean levels of PAF (C16:0- plus C18:0-species) in the cell pellet increased from 1.03 pmol up to 2.07 pmol/10^6 cells with stimulation by ionophore A23187 (ratio of C16:0/C18:0-PAF was 8:1). PAF release from these cells into the cell supernatant was also observed: PAF accumulated in a time-dependent manner during incubation in basal media (Fig. 3), and accumulation could be stimulated by ionophore A23187 (5 μM for 10 min at 37° C) (Fig. 4). The level of PAF (C16:0 plus C18:0-species)

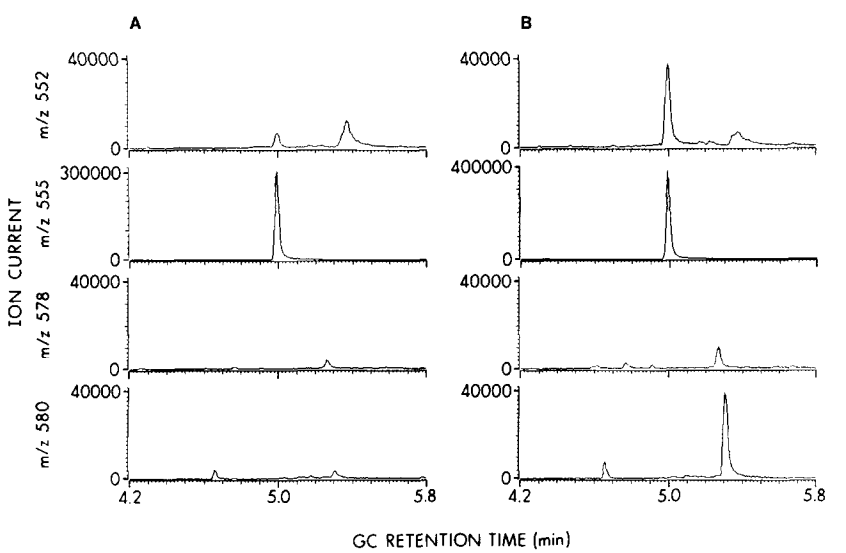

Fig. 3. NICI GC-MS analysis of PAF present in the supernatant from cultured ovine tracheal epithelial cells immediately after changing culture media (A) and 18 h later (B). Cells were incubated in LHC basal medium at 37° C, and PAF analysis was performed as in Fig. 2. GC retention times differ slightly from those in Fig. 1 and 2 because a slightly longer capillary GC column was used.

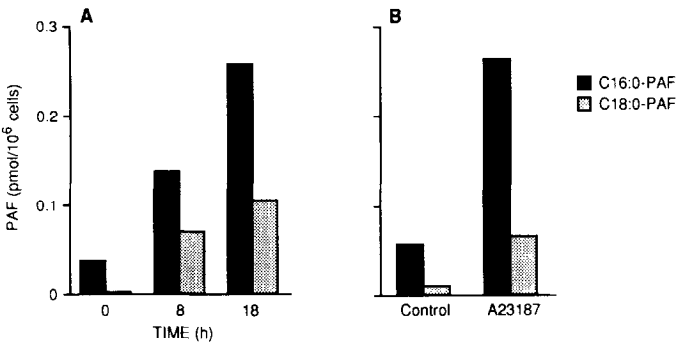

Fig. 4. Dependence of PAF release on time (A) and on A23187-stimulation (B) in cultured ovine tracheal cells. In (A), aliquots of the culture media were collected immediately and at 8 and 18 h after change of medium and then were subjected to analysis. In (B), cell supernatants were collected from unstimulated control cells and from cells stimulated with 5 μM ionophore A23187 in HBSS for 10 min at 37° C. PAF analysis by GC-MS was performed as in Fig. 2.

reached a maximum of 0.36 pmol/10^6 cells under basal conditions by 18 h after changing the culture medium and increased from 0.07 pmol up to 0.33 pmol/10^6 cells upon stimulation with ionophore A23187 (Fig. 4). Release of lysoPAF was not detected under basal or stimulated conditions.

The molecular species of PAF produced by epithelial and endothelial cells may differ significantly from PAF species in some types of leukocytes. For example, the initial structural characterization of PAF from antigen-stimulated rabbit basophils demonstrated that the alkyl side chains were >90% C18:0-PAF and <10% C16:0-PAF (21). Others claim a higher percentage of PAF is composed of the C16:0 molecular species (22). The molecular species of PAF from A23187-stimulated human, rabbit, and rat neutrophils exhibits a predominance of the C16:0 molecular species varying from 40 to 96% of total PAF production (15, 23, 24).

The amounts of PAF produced by epithelial and endothelial cells also differs from levels detected in stimulated leukocytes. The level of total PAF species increases from 0.2 up to 8.0 pmol/10^6 human neutrophils upon stimulation with A23187 (23) and up to 59.1 pmol/10^6 in A23187-stimulated polymorphonuclear leukocytes isolated from rats (24). Thus, the levels of PAF produced either by tracheal epithelial or vascular endothelial cells may be lower than for neutrophils on a per cell basis. However, metabolism of PAF to long-chain acylphosphatidylcholine molecular species may be rapid in lung epithelial cells (25) and rates of PAF biosynthesis may therefore be underestimated in this cell type. We observed that treatment of ovine tracheal epithelial cells with the acetylhydrolase inhibitor PMSF (2 mM for 15 min at 37° C) caused a 4-fold increase in amounts of PAF over basal conditions. This finding suggests that metabolism of PAF may also occur to a significant degree in tracheal epithelial cells.

In summary, the present results demonstrate that biosynthesis of PAF occurs in both airway epithelial cells and in vascular endothelial cells and that the predominant molecular species from both cells is C16:0-PAF. The facts that tracheal epithelial cells and vascular endothelial cells

generate PAF under basal culture conditions and in response to A23187 suggest that both the de novo pathway for PAF biosynthesis via CDP-choline:alkyl-acetyl-glycerol cholinephosphotransferase activity (26) and the stimulatable pathway for biosynthesis via phospholipase A_2 and acetyltransferase activity (27) may be active in these cells. Our findings further suggest a biochemical mechanism for sentinel epithelial (and endothelial) surfaces to modulate leukocyte adherence and influx. The same access of the airway epithelial cells (and other epithelial cell surfaces) to environmental agents will permit lipid-modifying drugs to be delivered to them, and much of this therapeutic potential is still unexplored. Therefore, determining the factors that regulate PAF metabolism in airway epithelial cells is a fundamental goal for determining the role of the compound in airway inflammation.

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