Vol. 122, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 18, 1984 Pages 325-332

MOLECULAR HETEROGENEITY OF PLATELET-ACTIVATING FACTOR PRODUCED BY STIMULATED HUMAN POLYMORPHONUCLEAR LEUKOCYTES

R. Neal Pinckard[1,2,4], Evelyn M. Jackson[4], Carol Hoppens[1], Susan T. Weintraub[3], Janet C. Ludwig[1], Linda M. McManus[1,4], and Glen E. Mott[1,4]

Departments of Pathology[1], Medicine[2] and Pharmacology[3],
The University of Texas Health Science Center and
Department of Cardiopulmonary Diseases[4]
Southwest Foundation for Biomedical Research, San Antonio, Texas 78284

Received May 21, 1984

The molecular heterogeneity of platelet-activating factor (PAF) produced by stimulated human neutrophilic polymorphonuclear leukocytes (PMN) was assessed by both normal and reverse phase high performance liquid chromatography (HPLC). As detected by rabbit platelet stimulation, at least 5 PAF molecules were separated by HPLC. Fast atom bombardment (FAB) mass spectrometry revealed one of these PAFs was acetyl glyceryl ether phosphorylcholine (AGEPC) with a $C_{16:0}$ alkyl chain in the sn-1 position. Although the structures of the remaining PAFs are unknown, two of the peaks of PAF activity had the same retention times on reverse phase HPLC as the C_{15}- and C_{18}-saturated alkyl chain AGEPC homologues. These studies indicate that the human PMN produces multiple molecular species of PAF.

The structure of rabbit basophil-derived platelet-activating factor PAF was identified nearly five years ago as acetyl glyceryl ether phosphorylcholine (1-O-hexadecyl- and 1-O-octadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine, C_{16}- and C_{18}-AGEPC) (1,2). Since then, immense interest in this acetylated alkyl phosphoglyceride has developed because of its remarkable spectrum of potent physiologic and inflammatory properties (3,4). Further, stimulated inflammatory cells actively synthesize and release PAFs with physicochemical properties similar to those of AGEPC (3). However, their chemical structures have not been determined and the question of possible structure/function heterogeneity of naturally occurring PAF has not been addressed. This may be of some consequence since several studies of synthe-

Abbreviations: PAF, platelet-activating factor; AGEPC, acetyl glyceryl ether phosphorylcholine; PMN, neutrophilic polymorphonuclear leukocytes; FMLP, N-formyl-methionyl-leucyl-phenylalanine; HPLC, high performance liquid chromotography; HSA, human serum albumin; C, chloroform; M, methanol; TLC, thin layer chromatography; RRT, relative retention time; FAB, fast atom bombardment mass spectrometry.

0006-291X/84 $1.50

tic molecules have demonstrated that even minor structural alterations of AGEPC have profound effects upon its biological properties and potency (1, 5-7). Therefore, it is important to isolate homogeneous populations of PAF from various stimulated cells to determine their chemical structures and elucidate their functional activities. We have addressed these issues by separating molecular species of PAF with sequential use of normal and reverse phase HPLC (8). The present report demonstrates that stimulated human PMN release not only C_{16}-AGEPC, but also several other PAF molecules.

METHODS

Chemicals and reagents. All organic solvents were HPLC grade and were obtained from Fisher Scientific Co. (Pittsburg, PA). Choline chloride, beef heart-derived PAF (i.e., semi-synthetic AGEPC), cytochalasin B, and FMLP were purchased from Sigma Chemical Co. (St. Louis, MO). C_{16}-AGEPC was purchased from Bachem Fine Chemicals (Torrance, CA), C_{18}-AGEPC was a gift from Dr. Donald Ayer of the Upjohn Company (Kalamazoo, MI), C_{12}-AGEPC was prepared as described (6) and C_{14}- and C_{15}-AGEPC were purchased from Calbiochem-Behring (San Diego, CA). The ^3H-C_{16}-AGEPC marker for HPLC was purified from beef heart-derived, semi-synthetic ^3H-AGEPC (New England Nuclear, Boston, MA; 30-60 Ci/mmole) by reverse phase HPLC as described (8). Crystallized HSA was purchased from Miles Laboratory (Elkhart, IN).

Production and partial purification of PAF from human PMN. Human PMN were isolated as described (9) from blood obtained from the antecubital vein of human subjects who had not taken any medication for at least two weeks. These cell suspensions consistently were comprised of 95-98% viable PMN. To effect the synthesis and release of PAF (9), the PMN (5×10^6/ml) were incubated at 37°C for 10 minutes in the presence of 2.5 mg/ml HSA and 1.4 mM Ca^{++} prior to the addition of cytochalasin B (5 µg/ml, final concentration). Two minutes later FMLP was added (10^{-6} M, final concentration) and the PMN were incubated for an additional 10 minutes. The PMN then were sedimented at 2,400 x g for 10 minutes at 4°C and the resulting cell-free supernatants and cell pellets were separately extracted. Following addition of chloroform and methanol to the PMN supernatant or resuspended cell pellets in proportions of C:M:H_2O, 1:2:0.8 (v/v/v), the samples were incubated at room temperature with mixing for at least 60 minutes. The mixture was then centrifuged at 2,400 x g for 10 minutes to remove precipitated proteins and cell debri; additional chloroform and water then were added to the supernatant to effect phase separation (C:M:H_2O, 1:1:0.9, v/v/v). The lower, chloroform-rich phase contained all PAF activity.

Lipid extracts obtained from the PMN supernatants from 20 donors were pooled and subjected to TLC as described (10) on 1 mm, preparative Silica Gel G plates (Analtech, Inc., Newark, DE) using a solvent system of C:M:H_2O, 65:35:6 (v/v/v). The area of the plates containing PAF (from R_f 0.1 to 0.3) was removed and lipid extracted.

Normal and reverse phase HPLC. A 10 micron Radial-Pak silica cartridge (8 mm x 10 cm) and a 10 micron Radial-Pak C18 cartridge (8 mm x 10 cm) were used in a Z-Module or a RCM-100 radial compression module (Waters Assoc., Milford, MA) for normal and reverse phase HPLC, respectively, as described (8). The normal phase elution solvent was acetonitrile:methanol:85% phosphoric acid, 130:5:1.5 (v/v/v). Samples were loaded on the column using a 100 µl sample loop of a Rheodyne 7125 injector and were eluted at a flow rate of 2.0 ml/

min. ^{3}H-C_{16}-AGEPC was added to the samples in trace amounts (less than 2 pmoles) as an internal marker for the calculation of the RRT (see Results). For reverse phase, the elution solvent was methanol:water:acetonitrile, 85: 10:5 (v/v/v) that contained 20 mM choline chloride. Samples were dissolved in the elution solvent without choline chloride, loaded on the column with a 100 µl loop, and were eluted at a flow rate of 1.0 ml/min. The retention times are expressed as time after elution of the column void volume.

Mass Spectral Analysis. Mass spectra were obtained by FAB using a Finnigan MAT 212/INCOS 2200 system equipped with an Ion Tech saddle field atom gun operating at 8 kV with either argon or xenon. The accelerating voltage in the mass spectrometer was 3 kV and the ion source temperature was 70°C. Following application of 1 - 2 µl of glycerol to the copper probetip, a background spectrum was acquired. The probe then was removed from the mass spectrometer and the sample of interest was added in 2 - 3 µl of methanol and mixed thoroughly with the glycerol. Sample spectra were evaluated after subtraction of contributions from glycerol and from the HPLC mobile phase.

Functional PAF assay. PAF activity was estimated by the capacity of 4 µl of a given sample to effect the release of ^{3}H-serotonin from 200 µl of washed rabbit platelets (250,000 platelets/µl) as described (1). One unit of PAF was defined as the amount required to induce 50% secretion of ^{3}H-serotonin within 60 seconds at 37°C. If a sample failed to effect ^{3}H-serotonin secretion, it was assessed further by the more sensitive evaluation of platelet aggregation in the presence of 10 µM indomethacin and creatine kinase-creatine phosphate as described (10).

RESULTS

The PAF obtained either from cell-free supernatants or cell pellets of stimulated PMN eluted from the normal phase silica column as a single, homogeneous peak of PAF activity (Fig. 1). The retention time of the PMN-derived PAF was identical to ^{3}H-C_{16}-AGEPC and beef heart-derived PAF activity. The PMN-derived PAF was well separated from other classes of phospholipids including lyso-lecithin and ^{3}H-lyso-GEPC (8).

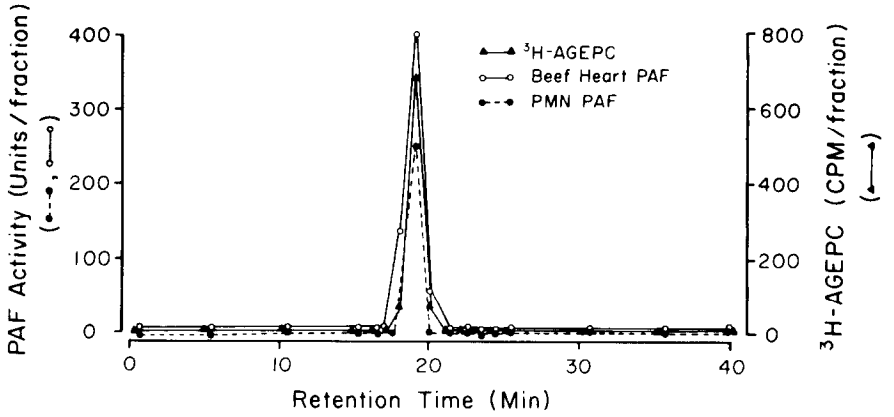

Figure 1: Composite of normal phase HPLC chromatograms of released PMN PAF, beef heart-derived PAF and the ^{3}H-C_{16}-AGEPC marker. PAF obtained from stimulated PMN cell pellets and the 5 AGEPC alkyl homologues had identical elution profiles.

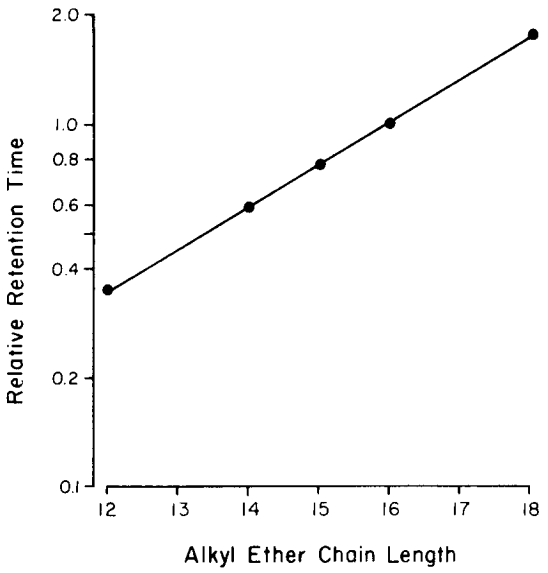

Figure 2: Relationship of saturated, alkyl chain length to the RRT of AGEPC homologues fractionated by reverse phase HPLC. The peak elution time of each homologue as determined by its PAF activity was divided by the elution time of C_{16}-AGEPC to calculate RRT.

For standard AGEPC samples, reverse phase HPLC revealed that a linear relationship existed between the length of the saturated, alkyl chain and the $\log_{10}$ of the retention time relative to C_{16}-AGEPC (Fig. 2). Although the absolute retention times of the various AGEPC alkyl chain homologues varied by as much as 2 minutes from run to run, their RRT were reproducible. Therefore, in subsequent studies, the retention times of PMN-derived PAF components were calculated relative to the internal ^{3}H-C_{16}-AGEPC marker.

Following purification of the PMN PAF by normal phase HPLC, further fractionation was accomplished by reverse phase HPLC. The PAF activity released from the stimulated PMN eluted from reverse phase HPLC in a similar fashion to the PMN cell pellet PAF activity. PMN-derived PAF activity, as detected by rabbit platelet ^{3}H-serotonin secretion, was heterogeneous, i.e., it was comprised of several HPLC peaks of biologically active material (Fig. 3). Although the RRT of some of the peaks of biological activity corresponded to those of authentic AGEPC alkyl homologues (i.e., C_{15}-, and C_{18}-AGEPC, RRT of 0.78 and 1.78, respectively), other regions of PAF activity did not

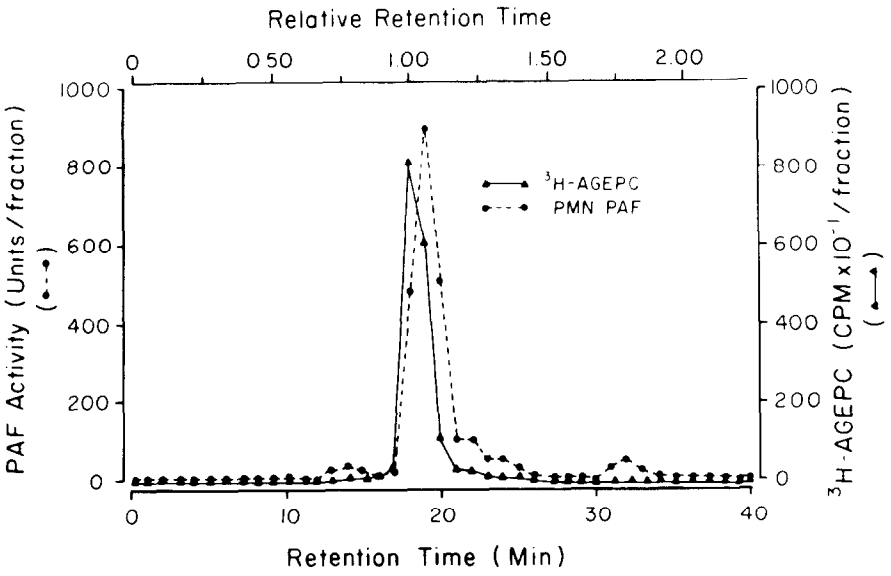

Figure 3: Reverse phase HPLC separation of PAF isolated from the cell pellet of stimulated PMN from one donor.

correlate with any of the standard AGEPC alkyl homologues available. Specifically, a somewhat broad undefined peak of PAF activity with a RRT in a range from 0.52 to 0.66 was detected by platelet shape change/aggregation. Also, the principal peak of PAF activity did not co-elute precisely with the ^{3}H-C_{16}-AGEPC marker even though the PAF activity of synthetic C_{16}-AGEPC did consistently co-elute with the ^{3}H-C_{16}-AGEPC marker. Finally, a prominent region of PAF activity was observed in between the C_{16}- and C_{17}-AGEPC regions of the chromatogram.

The reverse phase chromatogram shown in Figure 3 is representative of the supernatant and cell pellet PAF activities isolated from two separate PMN donors. Similar heterogeneity was observed when the PAFs released from the PMN isolated from 20 donors were pooled prior to TLC and subsequently fractionated by normal and reverse phase HPLC. In the latter case, sufficient PAF with a RRT of 1.0, i.e., 19 minutes (Fig. 4), was recovered to obtain mass spectral analysis. After background subtraction, ions at m/z 524 and 482 were observed at a ratio of 6.5 to 1; the m/z 524 ion had a relative intensity of 30% of the m/z 184 base peak. When this sample was subjected to

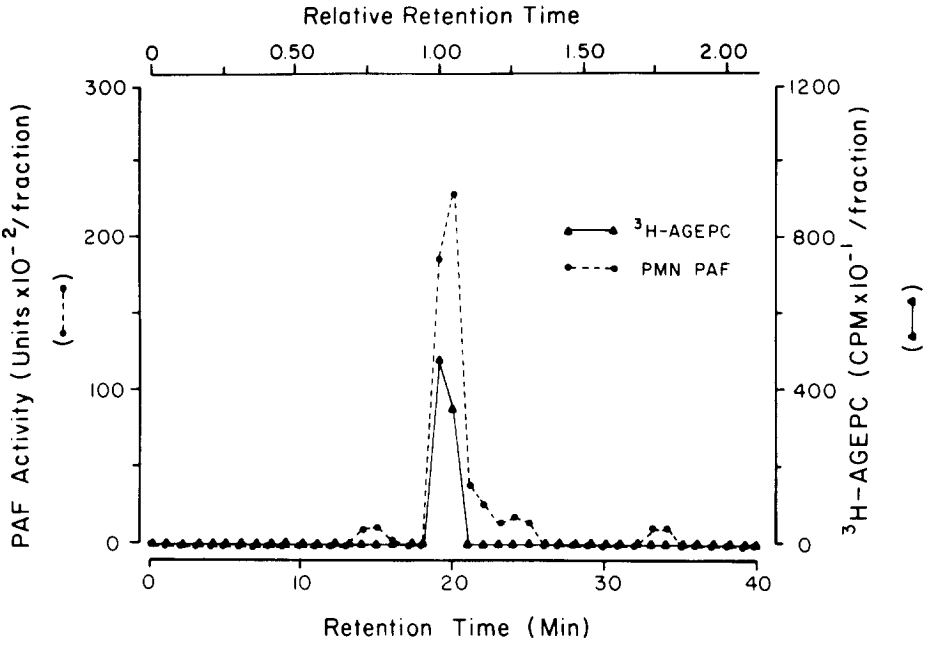

Figure 4: Reverse phase HPLC separation of PAF isolated from the pooled cell-free supernatant of stimulated PMN from 20 donors.

base-catalyzed methanolysis (10) and analyzed by FAB mass spectrometry, the m/z 524 ion decreased to a level of 5% of the m/z 482 ion which became the predominent ion in the range of 400-600 amu; this base-treated sample had no detectable PAF activity. The hydrolyzed material then was acetylated with acetic anhydride (11). Subsequent FAB mass spectral analysis revealed that the acetylated sample again had prominent m/z 524 and 184 ions and full PAF activity was restored. The FAB mass spectral results of this PMN PAF fraction were identical to those obtained from synthetic C_{16}-AGEPC treated in a similar fashion. There was insufficient material present in the other reverse phase HPLC fractions with biologic activity to permit unambiguous interpretation of their mass spectra.

DISCUSSION

Normal and reverse phase HPLC have been used to demonstrate that the PAF released by stimulated human PMN is comprised of at least five or six different molecules (Fig. 4). The mass spectrum of one of these PAF molecules (RRT of 1.0, Fig. 4) is consistent with that of C_{16}-AGEPC. This

conclusion is further supported by the observation that base treatment of this molecule resulted in a loss of all PAF activity and a decrease of 42 mass units which correlates with the hydrolysis of an acetyl residue.

The extent of the molecular heterogeneity of PAF derived from stimulated human PMN documented in the present study was greater than that reported for stimulated rabbit basophils in which C_{16}- and C_{18}-AGEPC were detected (2) and may be contrasted with recent evidence given for the presence of C_{16}-, C_{18}- and a trace of $C_{18:1}$-AGEPC in ionophore-challenged human PMN (12). These apparent differences could reflect the different purification procedures used, i.e., high resolution HPLC as opposed to thin layer chromatography, or to differing sensitivites of the techniques employed.In addition, more diverse molecular heterogeneity may exist in the PAFs synthesized by human PMN than by the rabbit basophil.

The detection of PAF in most investigations, including the present study, has been based upon platelet stimulation,i.e., platelet aggregation and/or secretion. Therefore, several cautionary comments are warranted. If stimulated cells produce structurally related, biologically active molecules with little or no platelet stimulating activity, they would remain undetected. Indeed, AGEPC has a spectrum of biological activities in addition to its PAF activity (3,4). Moreover, since there is considerable variability in the PAF activity of AGEPC homologues and analogues (1,6), the profiles of PMN PAF activities (Figs. 1, 3, 4) do not reflect their relative molar amounts. Therefore, additional studies are clearly required to establish the extent of the apparent structure/function heterogeneity of this class of potent lipid autacoids. Such information will be invaluable for elucidating the role of these novel lipids in mediating both normal and abnormal acute allergic and inflammatory reactions.

ACKNOWLEDGEMENTS

We thank Richard Castillo, Pat Padilla, Dawn Hass and Cynthia Lear for their technical assistance and Laida Garcia for typing this manuscript. This work was supported in part by USPHS Grant HL22555, HL-29121 and Contract NO1-8V-53030.

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