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IDENTIFICATION OF PHOSPHOLIPID PLATELET-ACTIVATING FACTOR (1-O-ALKYL-2-ACETYL-SN-GLYCERO-3-PHOSPHOCHOLINE) IN HUMAN AMNIOTIC FLUID AND URINE

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SUMMARY. Human amniotic fluid and fetal urine were examined for the presence of phospholipid platelet-activating factor (PAF). PAF was detected in lipid extracts of some samples of amniotic fluid obtained from women in labor but it was undetectable in samples of amniotic fluid obtained before the onset of labor. PAF was identified by chromatographic mobility, platelet aggregation and chemical modifications. LysoPAF was also present in amniotic fluid at higher concentrations than those of PAF. Both PAF and lysoPAF were identified also in newborn and adult urine.

Prostanoids are involved in human parturition and appear in increased amounts in amniotic fluid during early labor (1). In fetal membranes free arachidonic acid, the obligate precursor of prostanoids of the 2-series, is released from phosphatidylethanolamine and phosphatidylinositol during early labor (2). The enzymes involved in this process include phospholipase A_2 (3) and phospholipase C (4). These activities of phospholipase A_2 and phospholipase C are both Ca^{2+}-dependent (3,4). Ca^{2+} also inhibits the recycling of diacylglycerols by inhibiting diacylglycerol kinase (5). It has been suggested that the liberation of arachidonic acid in amnion cells may be regulated by Ca^{2+} (2-4). PAF[1] (6-9), induces a rapid increase of Ca^{2+} into the cytosol of platelets (10) and neutrophils (11) resulting in activation of phospholipases and the liberation of arachidonic acid for the formation of eicosanoids (12-14). The objective of this investigation, was to identify, and

Abbreviations: PAF, Platelet-activating factor, (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine); LysoPAF, deacetylated platelet-activating factor, 1-O-alkyl-sn-glycero-3-phosphocholine; BSA, bovine serum albumin; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.

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if present, to characterize PAF and its deacetylated derivative, lysoPAF in human amniotic fluid and in human newborn and adult urine samples.

MATERIALS AND METHODS

Materials: Synthetic PAF and [^{3}H]PAF (1-[1,2-^{3}H]-alkyl-2-acetyl-sn-glycero-3-phosphocholine, 48 Ci/mmol) were kindly provided by Dr. Fred Snyder, Oak Ridge, TN. PAF was also obtained from Calbiochem, La Jolla, CA. [^{3}H]LysoPAF was prepared by deacetylation of [^{3}H]PAF with methanolic NaOH. Silica gel G or F thin-layer plates (0.25 mm) were purchased from Brinkman, Westbury, NY. BSA (essentially fatty acid-free) and indomethacin were obtained from Sigma, St. Louis, MO.

Buffers: (A) HEPES-Tyrode's-BSA: HEPES (10 mM), NaCl (120 mM), KCl (5 mM), $NaHCO_3$ (10 mM), glucose (10 mM), BSA (0.35%), pH 7.4. (B) Calcium-free HEPES-Tyrode's-BSA; same as Buffer A but with ethyleneglycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (0.2 mM).

Collection and processing of amniotic fluid and fetal urine samples. Amniotic fluid samples were obtained from patients by transuterine amniocentesis at the time of cesarean section or by direct needle aspiration through the fetal membranes per vagina during labor. Samples of amniotic fluid were centrifuged at 600 x g at 4°C for 20 min. The resulting supernatant fluid was centrifuged at 35,000 x g for 30 min at 4°C to obtain a pellet fraction that was rich in lamellar bodies (15) and a supernatant fraction. Urine samples from newborn infants were collected immediately after birth and centrifuged at 600 x g for 20 min to remove cell-debris. Adult urine was collected over a 24 hr period and aliquots of this were employed for lipid extraction.

Lipid extraction and thin-layer chromatography. Four vol of sample were mixed with 6 vol of methanol for 60 min at room temperature. Protein was removed by centrifugation at 10,000 x g for 10 min. To the supernatant fraction was added 6 vol of chloroform and 1.4 vol of water. The resulting chloroform phase was transferred and dried under nitrogen. The pellet fraction of amniotic fluid was extracted by the procedure of Bligh and Dyer (16). Phospholipids were separated by one-dimensional thin-layer chromatography (17) on silica gel plates using chloroform/methanol/acetic acid/water (50:30:8:6, by vol, solvent A). In this solvent system, PAF was separated from other glycerophospholipids. Two additional solvent systems (18) were used: (B) methanol/water (2:1, v/v) in which PAF and lysophosphatidylcholine streaked (Rf 0-0.5) whereas other phospholipids remained at the origin, and (C) chloroform/methanol/H_2O (65:35:6 by vol) where sphingomyelin and PAF had a similar Rf (0.58 and 0.55), respectively.

The silica gel area between phosphatidylcholine and lysophosphatidylcholine (Solvent A) was collected and lipids were extracted from the silica using 2x5 ml of $CHCl_3$/methanol/water (1:2:0.8, by vol). Chloroform (2.5 ml) and water (2.5 ml) were then added to separate the phases (16). The lower chloroform phase was transferred to polypropylene tubes and evaporated under nitrogen and the residue resuspended in a known volume of Buffer B by sonication. Aliquots of this suspension were used for platelet aggregation assays.

Quantification of PAF. Washed rabbit and horse platelets were prepared as described previously (13,14). The aggregation assay for PAF was performed employing 5 x 10^7 platelets in Buffer B supplemented with Ca^{2+} (1 mM) plus Mg^{2+} (1 mM) in a total vol of 0.5 ml and were conducted in a dual channel, platelet aggregometer (Sienco Inc., Morrison, CO) at 37°C with

constant stirring. The assay samples were added in a vol ranging between 5-50 µl. In some experiments, indomethacin (20 µM) was added to platelet suspension 10 min before the addition of the sample. Indomethacin inhibits cyclooxygenase activity and thus inhibits arachidonate-induced platelet aggregation (13). A standard curve of aggregation versus PAF concentration was prepared for each assay.

Quantification of lysoPAF and 1-0-alkyl-2-acyl-sn-glycero-3-phosphocholine. The alkyl ether bond is stable to both acidic and alkali treatments (18,19). Phospholipids which were extracted from the appropriate areas of thin-layer chromatograms were treated with methanolic NaOH (0.5 N, 8 min at 25°C) to hydrolyse the acetyl and long chain acyl ester bonds (18,19). Subsequent treatment with HCl (3N, 20 min at 25°C) results in the hydrolysis of the 1-0-alkenyl bond (plasmalogens) (18). The residual phospholipids were extracted (16) and the fraction was eluted with methanol, dissolved in chloroform (200 µl), and acetic anhydride (20 µl) in the presence of pyridine (40 µl) was added. The samples were incubated for 60 min at 60°C in capped tubes. The samples were re-extracted (16). The lower layer was taken to dryness under nitrogen. The samples were resuspended in buffer B. PAF activity was quantified by platelet aggregation assays and the concentration of lysoPAF or phosphatidylcholine containing the 1-0-alkyl ether bond computed.

[^{3}H]PAF and [^{3}H]lysoPAF of high specific activity (48 Ci/mmol, less than 0.02 pmol) were added to the samples before starting lipid extraction. The recovery of radioactivity was determined following resuspension of PAF in Buffer B for aggregation assays. The recoveries were between 35-50%. Values were corrected for the percent recovery and expressed as the mean ± S.E.

RESULTS

Identification of PAF in amniotic fluid. In Fig. 1 is illustrated the platelet aggregation that was initiated by a sample prepared from amniotic fluid obtained from a woman in active labor. The sample was prepared by extraction of lipids from the amniotic fluid followed by thin-layer chromatography and elution of the silica gel area corresponding to the area of migration of authentic PAF. No platelet-aggregating activity was found when other areas of the thin-layer chromatograms were extracted. The platelet aggregating factor obtained from amniotic fluid migrated with synthetic PAF in three different solvent systems (solvents A, B and C). The aggregation of platelets as initiated by the amniotic fluid factor was dose-dependent (Fig. 1). The platelet-aggregation induced by PAF or by amniotic fluid factor were not affected by prior treatment of the platelets with indomethacin (20 µM). This concentration of indomethacin inhibited completely platelet aggregation induced by arachidonic acid (500 µM).

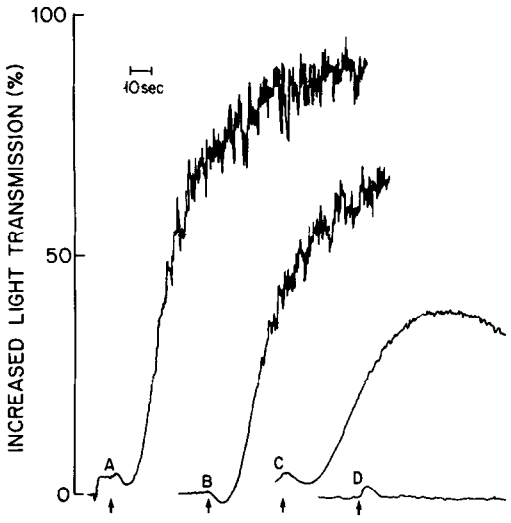

Fig. 1 PAF in Amniotic Fluid.

The phospholipids in the lipid extract of amniotic fluid obtained from women in labor were separated by thin-layer chromatography using solvent A. Thin layer areas corresponding to PAF were eluted and the extracts were tested for platelet-aggregating activity as described. A, B, and C represent lipid extracts from 10 ml, 5 ml and 2 ml of amniotic fluid, respectively, and (D) a lipid extract from 50 ml of amniotic fluid obtained from a woman at term but not in labor.

In Fig. 2A is illustrated the platelet aggregation induced by synthetic PAF (a) and a purified sample obtained from amniotic fluid of women in labor (b). The acetyl group at sn-2 position of PAF is essential for its biologic activity (7-9,18,19). When PAF and the amniotic fluid factor were subjected to alkaline hydrolysis (0.5 N NaOH in methanol, 5 min at 25°C), a complete loss of the aggregation activities of the amniotic fluid sample and authentic PAF was observed (Fig. 2B). Subsequent treatment of these biologically inactive samples with acetic anhydride resulted in the restoration of biologic activity (Fig. 2C). Both PAF and the amniotic fluid factor were stable to acidic conditions (4 N HCl, 2 h) excluding the possibility of the presence of a 1-O-alkenyl bond. (Data not shown.) The prior treatment of platelets with PAF in the absence of Ca^{2+} has been reported to desensitize the platelets to subsequent addition of PAF in the presence of Ca^{2+} (18,20). Similar desensitization of platelets to PAF was observed when the platelets were exposed to the factor obtained from amniotic fluid in the absence of Ca^{2+}.

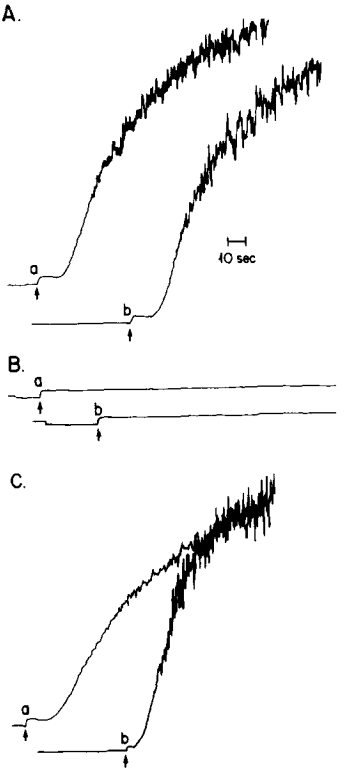

Fig. 2 Identification of PAF in Human Amniotic Fluid.

A. Platelet-aggregation induced by authentic PAF (10^{-9} M) (a) and by the factor obtained from 5 ml of amniotic fluid of a woman in labor (b). B. Both PAF (a) and amniotic fluid factor (b) were treated with 0.5 ml of 0.5 N NaOH in methanol for 5 min at 25°C. After neutralization with 0.5 ml of 0.5 N HCl, the lipids were extracted and tested for platelet-aggregation. C. Samples obtained after alkali treatment were dissolved in $CHCl_3$ and treated with 20 µl acetic anhydride plus 40 µl pyridine for 60 min at 60°C.

On the basis of its chemical and biological properties, we suggest that the factor purified from the amniotic fluid of women in labor is identical to PAF. Final proof, however, must await mass spectrometric analysis when adequate amounts of PAF can be isolated.

Contents of PAF in amniotic fluid and in fetal urine. We have analyzed the 35,000 x g supernatant and pellet fractions of amniotic fluid for PAF activity. Of the 24 samples obtained from women in labor, 14 of these samples contained detectable amounts of PAF activity in both the supernatant and pellet fractions. The concentration of PAF in the supernatant fractions was 107 ± 36 pM (n=14). In the remaining 10 samples no PAF activity was

demonstrable in either fraction. PAF activity was not detectable in 14 samples of amniotic fluid obtained from women at term but not in labor. PAF activity was detected in 6 of 10 samples of first voided urine of newborns.

Amount of LysoPAF in amniotic fluid and urine. The 35,000 x g supernatant and pellet fractions of amniotic fluid samples were also analysed for lysoPAF employing sequential treatment of lysophosphatidylcholine fraction with NaOH, HCl and acetic anhydride. LysoPAF was found consistently in both fractions of all amniotic fluid samples irrespective of whether the amniotic fluid was obtained before or after labor. The lysoPAF concentrations in the supernatant (9 ± 0.9 nM, n=11) and pellet (8.6 ± 1.8 nM, n=10) fractions of amniotic fluid samples obtained from women in labor were not significantly different from those found in the supernatant (7.8 ± 1.3 nM, n=5) and pellet (8.6 ± 2.0 nM, n=5) fractions of amniotic fluid samples obtained from women at term but not in labor. We have also detected lysoPAF in the first voided urine of newborns (3.7 ± 1.2 nM, n=7) and in the 24 h urine sample obtained from several women (2.8 ± 1.3, n=3).

1-O-Alkyl-2-acyl-sn-glycero-3-phosphocholine in amniotic fluid. Amniotic fluid samples obtained before and after labor also contained substantial amounts of 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine. This phospholipid was present in much higher concentrations in the pellet fractions (4.52 ± 1.73 μM ± SE, n=3) than in the supernatant fractions (200 ± 23 nM, ± SE, n=6).

DISCUSSION

We have detected and quantified both PAF and lysoPAF in human amniotic fluid. These two lipids in amniotic fluid most likely are of fetal origin. The amniotic fluid is composed primarily of fetal urine and fetal lung secretions. In rat, kidney and lung were the richest sources of the enzymes involved in the formation of PAF and lysoPAF (21). We have found PAF and lysoPAF in fetal urine and in the pellet fraction of amniotic fluid which is rich in the lamellar bodies secreted by the fetal lung (15). These observations were suggestive that fetal kidney and lung are potential sources of PAF and

lysoPAF in human amniotic fluid. The presence of lysoPAF in amniotic fluid may also be explained by the action of acetyl hydrolase activity which is present in amniotic fluid (Billah and Johnston, unpublished observation).

Although PAF was initially recognized for its ability to activate platelets, it was subsequently found to stimulate other cell-types such as monocytes and neutrophils (11). It also exerts a platelet-independent antihypertensive action (9). In spite of the diversified function of PAF in various cell types, it appears that all of its actions could be closely associated with the rapid influx of Ca^{2+} (10) possibly by a receptor mediated event (20) or as a Ca^{2+} ionophore (10). We have proposed a central role for Ca^{2+} (5) in the regulation of arachidonic acid release from diacylphosphatidylethanolamine and phosphatidylinositol (2) for prostaglandin E_2 formation in amnion cells (22). The presence of PAF in amniotic fluid during labor is indicative that it is potentially available to stimulate amnion cells during labor and may be important in the Ca^{2+}-dependent mobilization of arachidonic acid for the formation of eicosanoids involved in human parturition. Furthermore, it may be speculated that an overproduction (or increased release) of this potent platelet-activating agent during pregnancy may be associated with certain disorders of pregnancy involving abnormal platelet function e.g., pre-eclampsia (23).

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