Differential metabolism of exogenous platelet-activating factor by glandular epithelial and stromal cells of rabbit endometrium

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Significant changes in platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine) concentration have been observed in rabbit endometrium during the preimplantation period, but, under *in vitro* conditions, constitutive PAF biosynthesis by isolated endometrial tissues was not easily demonstrable. Relative changes in enzymes involved in the synthesis and metabolism of PAF in the tissues may account for this disparity. In addition, during this period of preimplantation, marked changes in PAF receptor concentration have been noted. The present study examines the factors that may modulate the metabolism of exogenous [³H]PAF in the endometrium of rabbits on day 6 of pregnancy. Since preferential [³H]PAF binding *in situ* by the glandular epithelial, but not by the stromal, cells was demonstrated, their cell-specific metabolism of exogenous [³H]PAF was also examined. After entry into the endometrial cell, [³H]PAF was rapidly metabolized by the sequential action of cytosolic Ca²⁺-independent acetylhydrolase to [³H]lyso-PAF and this was in turn acylated by membrane-associated transacylase to [³H]alkylacyl-glycerolphosphorylcholine. PAF resynthesis was not observed and, in stromal cells, there was a significant build-up of [³H]lyso-PAF, suggesting that lyso-PAF:acetyl-CoA acetyltransferase may be a limiting factor. In the glandular epithelial cells, however, there was a significant accumulation of a neutral lipid without a significant build-up of [³H]lyso-PAF or [³H]PAF. The neutral lipid co-migrated with the product of phospholipase C-catalysed metabolism of PAF and authentic 1-O-hexadecyl-2-acetyl-glycerol. In addition, the elution times of phospholipase C digestion of C18 PAF and the neutral lipid produced by cellular metabolism of [³H]PAF, determined by gas chromatography/flame ionization detection, were similar. It seems that it is the synthesis of the neutral lipid from reacylated [³H]lyso-PAF that prevented [³H]PAF accumulation under *in vitro* conditions. This is the first documentation of the synthesis of this lipid in the mammalian uterus. The lipid may serve as the precursor for *de novo* PAF synthesis in the glandular epithelial cells during endometrial proliferation.

Introduction

Platelet-activating factor (PAF), chemically identified as 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine, is a very potent ether-linked phospholipid with a wide spectrum of biological and pathophysiological activities (Snyder *et al.*, 1989; Snyder, 1990). A variety of cells can synthesize PAF upon stimulation, and PAF has been implicated in a number of reproductive

phenomena (Harper, 1989), including sperm motility (Ricker *et al.*, 1989), ovulation (Abisogun *et al.*, 1989), fertilization (O'Neill, 1987), implantation and early development of the embryo (O'Neill *et al.*, 1987, 1988). Platelet-activating factor has been detected in the uterus of rats (Yasuda *et al.*, 1986) and rabbits (Angle *et al.*, 1988), and separated human endometrial cells (Alecozay *et al.*, 1989). It may be biosynthesized via either the *de novo* pathway (Lee *et al.*, 1986, 1988; Blank *et al.*, 1988) from the immediate precursor 1-O-alkyl-2-acetyl-glycerol via a dithiothreitol (DTT)-insensitive cholinephosphotransferase, which is thought to be responsible for maintaining endogenous

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physiological concentrations of PAF (constitutive expression), or by the 'remodelling' pathway (Wykle *et al.*, 1980; Uemura *et al.*, 1991) in which it is activated in inflammatory states by deacylation of pre-existing 1-O-alkyl-2-acyl-*sn*-glycero-3-phosphorylcholine (alkylacyl-GPC) by phospholipase A₂, which produces lyso-PAF, which in turn is rapidly acetylated to PAF. In many circumstances PAF is not released from the cells but remains cell-associated.

Knowledge of the PAF metabolic pathways is derived from studies with different cells from various species (Robinson *et al.*, 1985; Touqui *et al.*, 1985). For a target cell responding to an exogenous PAF molecule, the following would appear to be the sequence of events: PAF penetration into the outer plasma membrane, upon which (i) complexing with specific membrane receptors may promote cell activation to perform diverse receptor-mediated biological responses (Hwang *et al.*, 1989), and (ii) PAF may be subject to membrane-bound PAF-metabolizing enzymes that degrade the phospholipid (Lachachi *et al.*, 1985; Snyder *et al.*, 1986; Kudolo and Harper, 1989, 1990). The PAF molecule, which is internalized intact, may also be transported into the cell, traversing the cytosol where PAF may be metabolized. During passage through the cell membrane, PAF may be converted to lyso-PAF before reacylation to alkylacyl-GPC (see Kudolo and Harper, 1989, 1990 for references). These two metabolites are important precursors for PAF biosynthesis. Other than our studies with purified rabbit endometrial membranes (Kudolo and Harper, 1989, 1990), in which the attempts made were solely to control exogenous PAF metabolism during measurement of PAF receptors, there are no reports of the fate of exogenous PAF in rabbit uterus. This information is important because significant concentrations of uterine PAF are demonstrable in the rabbit, reaching a peak (a more than 15-fold increase over oestrous concentrations) on day 5 of pregnancy (Angle *et al.*, 1988). Unlike human endometrial cells, however, constitutive PAF synthesis in separated rabbit endometrial cells *in vitro* is not easily detectable; significant synthesis is observed only after cell activation by pulsing with the calcium ionophore A23187 (Kasamo *et al.*, 1992). Relative changes in enzymes in the synthesis and metabolism of PAF may account for the changes in endogenous uterine PAF during the preimplantation period of pregnancy and the inability to observe significant PAF accumulation in endometrial cells during *in vitro* incubation. The object of the present study was to examine some of the factors controlling the metabolism of exogenous [³H]PAF in endometrial cells of rabbits on day 6 of pregnancy, since this was the day on which PAF concentrations decrease markedly.

Materials and Methods

Materials

The radioligands, 1-O-[³H]octadecyl-2-acetyl-*sn*-glycero-3-phosphorylcholine (114–184 Ci per mmol) and 1-O-[³H]hexadecyl-2-lyso-*sn*-glycero-3-phosphorylcholine (110 Ci per mmol) were obtained from Amersham International (Arlington Heights, IL) (purity > 97%) and were used without further purification. Carbamyl-PAF (1-O-alkyl-2-N-methylcarbamyl-*sn*-glycero-3-phosphocholine) was purchased

from Calbiochem (La Jolla, CA). Sigma Chemical Co. (St Louis, MO) supplied reagent grade of most of the chemicals used, including PAF (octadecyl; C18 PAF), lyso-PAF (hexadecyl; C16 lyso-PAF), 1-O-hexadecyl-*sn*-glycerol (chimy alcohol), 1-O-hexadecyl-2-acetyl-*rac*-glycerol, sphingomyelin, egg yolk phosphatidylcholine, fraction V and fatty-acid-free bovine serum albumin and phospholipase C (from *C. welchii*). Tert-butyltrimethylchlorosilyl/imidazole (TBDMSI) reagent was from Alltech Applied Science (Deerfield, IL). Diisopropyl fluorophosphate (DFP) was from Aldrich Chemical Company (Milwaukee, WI). Silica Gel G plates (250 µm; 20 cm × 20 cm) were obtained from Analtech (Newark, DE).

Animals

Mature New Zealand White–Cambridge female rabbits (*Oryctolagus cuniculus*) (body mass > 3.0 kg; Penn Acres, Wimberley, TX) were caged individually in a controlled environment under a photoperiod of 14 h light:10 h dark, fed 170 g rabbit pellets per day and individually provided with water *ad libitum*. Rabbits in oestrus were inseminated with 0.5 ml of fresh mixed sperm suspension (collected via an artificial vagina from fertile bucks) followed by i.v. injection of 50 iu of hCG (Ayerst Laboratories, New York, NY) and designated as at day 0 of pregnancy. On day 6 of pregnancy, the animals were killed with an i.v. overdose of sodium pentobarbital. The uterine tissues were quickly removed (extraneous fat and mesenteries were excised and excess blood was blotted off), and then washed in ice-cold Tris–HCl buffer (50 mmol l⁻¹, pH 7, 4°C). The uterine horns were opened at the mesometrial junction and the endometrial tissues were harvested into fresh Tris buffer by scraping with a glass microscope slide. Contaminating blood and its constituents were removed by allowing the endometrial cells to sediment at unit gravity. This washing procedure was repeated twice before resuspension in 10 volumes of Tris buffer. Portions of the cell suspension were added to 16 ml polypropylene tubes (with snap caps) and sedimented by centrifugation at 800 g for 10 min at 4°C. Each tube contained approximately 30 mg of endometrial tissues and the cellular content of the preparations was computed by estimating the total DNA per tube subsequent to the extraction of lipids as described below.

Whole cell incubations

Endometrial tissues were suspended in 50 mmol Tris–HCl buffer l⁻¹ (pH 7.4) and incubated at 37°C for 60 min without any additions (control) or with various concentrations of carbamyl-PAF. After the incubations, the cell suspensions were centrifuged for 10 min at 500 g and the cells resuspended in the original volume of buffer. The cell suspensions were used for whole cell incubation with tritiated ligands: 0.5 ml aliquots of the endometrial tissue suspensions were added to 0.5 ml solution containing various additives (as described in the legends and figures) and 0.5 ml [³H]PAF (5 µCi) or [³H]lyso-PAF (5 µCi) and incubated for 15 min at 37°C. Incubations were carried out in snap-capped polypropylene tubes. The lipids were extracted by a modification of the procedure of Bligh and Dyer (1959). Briefly, the reactions were terminated

by addition of 6 ml of a mixture of chloroform and 2% (v/v) acetic acid in methanol (1:2) after adjusting the aqueous volume to 1.6 ml. The monophasic solution was vortexed at 15 min intervals for 1 h at room temperature. Thereafter, 2 ml chloroform and 2 ml water were added to each tube, vortexed and centrifuged at 500 *g* for 10 min to effect separation of the aqueous and organic phases. The chloroform phase was removed and the aqueous layer washed twice, each time with 4 ml chloroform, pooling all the chloroform extracts. The aqueous layer was made up to 0.2 mol perchloric acid (PCA) l^{-1} , vortexed and centrifuged at 1000 *g* for 10 min at 4°C to remove the cellular pellets. The cellular pellets were washed again with 0.2 mol PCA l^{-1} before extraction with 1 ml 0.5 mol PCA l^{-1} at 70°C for 60 min for measurement of total cellular DNA, according to the procedure of Burton (1956). The DNA content of a known number of endometrial cells (isolated as described below) was determined. Each cell was estimated to contain approximately 10 pg DNA and was used to estimate the number of cells in tissue suspensions.

[³H]Platelet-activating factor uptake into the endometrial cell

To examine the time course of [³H]PAF entry into the endometrial cells, the endometrial cells were isolated as described above and resuspended in 20 ml Tris-HCl buffer, pH 7.4, and incubated with 50 μCi [³H]PAF. After 5, 15, 30, 45 and 60 min, 2 ml aliquots were taken in duplicate and centrifuged at 1000 *g* for 10 min at 4°C. The supernatants were removed and 500 μl aliquots taken in triplicate for measurement of radioactivity. The pellets were washed with Tris-HCl buffer containing 0.25% (w/v) BSA twice and finally with Tris-HCl buffer, each time taking 500 μl aliquots in triplicate for liquid scintillation counting. The cell pellets were then suspended in 2 ml Tris-HCl buffer and homogenized in a glass-to-glass Dounce tissue grinder with four strokes. The homogenate was centrifuged at 1000 *g* for 10 min. The pellet was resuspended in 2 ml buffer and 500 μl aliquots in triplicate were taken for liquid scintillation counting. The supernatant was removed and centrifuged at 30 000 *g* for 30 min to isolate the plasma membranes and the cytoplasmic fractions. The membrane pellet was resuspended in 2 ml Tris-HCl buffer and 500 μl aliquots of this suspension and the cytoplasmic fraction (supernatant) were taken in triplicate for liquid scintillation counting.

Cell separation and culture

The isolation of stromal and glandular cells for culture was achieved by aseptic digestion of uterine tissues from animals at day 6 of pregnancy according to a modified procedure of Ricketts *et al.* (1983). Briefly, each uterine horn was washed four times with 10 ml incomplete (without Ca^{2+} and Mg^{2+}) Dulbecco's PBS before it was slit open at the longitudinal margin. After one additional washing with 10 ml PBS, each uterine horn was incubated in 6.25 ml PBS containing 2% (w/v) pancreatin and 0.5% (w/v) trypsin initially for 2 h at 4°C, and then for 1 h at 21°C. The incubate was vortexed briefly and the cell supernatant transferred to a conical flask containing 0.6 ml of heat inactivated (56°C, a temperature insufficient to inactivate acetylhydrolase, 30 min) fetal calf serum (FCS) and 1.6 ml Medium 199 with Hank's balanced salts (M199 + HBSS)

containing 400 U collagenase. The residual uterine tissue was washed twice with 10 ml PBS and all the supernatants pooled and incubated for 30 min at 37°C with shaking. The cell suspension was then filtered through a 350 μm nylon mesh to obtain the epithelial cell fraction. The stromal cells were obtained by subjecting the residual uterine tissue to additional enzymatic digestion for 2 h at 21°C in 5 ml PBS containing 0.05% (w/v) trypsin, 0.02% (w/v) EDTA and 400 U deoxyribonuclease 1 (DNase 1), and then incubating for 15 min at 37°C with continuous shaking. The stromal cell suspension was obtained essentially as described for the epithelial cell fraction, except that the cell suspension was filtered through a 35 μm nylon mesh. Both the epithelial and stromal cell fractions were washed twice in 25 ml PBS by centrifugation at 500 *g* for 10 min and resuspended in 15 ml basal medium, which consisted of M199 with Earle's salts, penicillin (100 000 U l^{-1}), streptomycin (100 mg l^{-1}), sodium pyruvate (1 mmol l^{-1}), insulin (5 mg l^{-1}), transferrin (5 mg l^{-1}), selenite (5 μg l^{-1}) and epidermal growth factor (10 μg l^{-1}). Each cell suspension was incubated in Petri dishes in humidified air with 5% CO_2 at 37°C. After 45 min, the floating cells from the epithelial cell fraction and the sedimented cells from the stromal cell fraction were isolated, and resuspended in 10% heat-inactivated FCS-supplemented basal medium. The number and viability (> 95% at time of plating) of the cells were estimated using a haemocytometer and the trypan blue exclusion test. Viable cell concentration was adjusted to approximately 0.5×10^6 cells ml^{-1} for epithelial and 0.3×10^6 cells ml^{-1} for stromal cell preparations and 2 ml of the cell suspensions plated per well (35 mm), using 6-well plastic tissue culture plates (Corning Glass Works, Corning, NY). The cells were incubated under standard culture conditions and the culture medium was changed daily. On the second day of culture, after two washes of the cells with warm PBS, the culture medium was changed to serum-free basal medium, but containing 0.25% (w/v) BSA (Fraction V). Next, [³H]PAF was added to a final concentration of 4 nmol l^{-1} and the incubation continued for 15, 30 and 60 min (tritiated substrates were first solubilized in minimal volume of 70% (v/v) ethanol, as described by Kudolo and Harper, 1990, when over 95% of [³H]PAF remains in aqueous solution). The medium was removed and 1.5 ml aliquots used for extraction of lipids as described above. The lipids associated with the glandular epithelial cells were also extracted, both before and after incubation with phospholipase C.

Catabolism of platelet-activating factor by phospholipase C

The [³H]PAF and [³H]lyso-PAF (5 μCi) were added to 200 μg of cold PAF and lyso-PAF, dried under a stream of nitrogen gas and solubilized in 0.5 ml 0.125% (w/v) BSA in Tris-HCl buffer (containing 25 mmol Ca^{2+} l^{-1}) and incubated with 10 μg phospholipase C for 1 h at 37°C (Benveniste *et al.*, 1977). The reaction product was extracted by the method of Bligh and Dyer (1959) and resuspended in 0.5 ml chloroform.

Identification of lipids

Thin layer chromatography. The chloroform lipid extracts, dried under nitrogen gas, were suspended in 0.5 ml chloroform.

Duplicate 0.05 ml aliquots were quickly taken for liquid scintillation counting for eventual estimation of the recovery rate of the radioactivity, and duplicate 0.2 ml aliquots were taken and redried under nitrogen for chromatography: one was used for the chromatography of phospholipids and the other for neutral lipids, all on Silica Gel G plates. The plates were washed in chloroform:methanol:water (65:35:6) and heat activated at 120°C, for 3 h before use. A solvent system consisting of chloroform:methanol:acetic acid:water (50:25:8:4) was used to separate the phospholipids, and at least two of the following authentic compounds were added to the lipid extracts as internal standards: octadecyl-PAF, sphingomyelin, lysophosphatidylcholine, and egg yolk phosphatidylcholine. This solvent system resolved the glycerophosphatides as follows: lysophosphatidylcholine, which co-migrated with hexadecyl- $[^3\text{H}]$ lyso-PAF (relative motility $R_f = 0.12\text{--}0.14$), octadecyl-PAF ($R_f = 0.22\text{--}0.27$) and phosphatidylcholine ($R_f = 0.57\text{--}0.60$). In all cases, the TLC plates were exposed to iodine vapour; the separation zones on the plates were divided into 15 1 cm zones, noting the fraction numbers of the authentic standards; and each fraction was scraped into 7 ml minivials for liquid scintillation spectrometry after addition of 4 ml of Ready-Protein[®] scintillation cocktail (Beckman Instruments Inc., Fullerton, CA). For correction of radioactivity counts, 100 000 d.p.m. of $[^3\text{H}]$ PAF was added to silica gel scrapings (the same amount as the developed plates) and counted under the same conditions. Neutral lipid products were identified by chromatography of the lipid extracts in a solvent system consisting of chloroform and methanol (98:2) with 1-O-hexadecyl-*sn*-glycerol ($R_f = 0.25\text{--}0.30$) and 1-O-hexadecyl-2-acetyl-*rac*-glycerol ($R_f = 0.46\text{--}0.55$) as standards. These C16 compounds were used as standards, since the analogous C18 1-O-octadecyl-2-acetyl-glycerol is not available.

Gas chromatography-flame ionization detection. Gas chromatography-flame ionization detection (GC/FID) analysis was performed using a Hewlett Packard (HP) 5830A gas chromatograph with FID. The capillary column was an Alltech Econocap Carbowax, 30 cm $\times$ 0.53 mm. The standard operating conditions were a starting temperature of 150°C held for 4 min, increased by 10°C min⁻¹ up to 240°C and held at this temperature for 10 min. The injection and FID temperatures were 250°C. The lipid samples (including the standard C18 PAF – phospholipase C digestion product) were extracted and chromatographed on TLC to separate the lipids co-migrating with authentic PAF and the neutral lipid, 1-O-hexadecyl-2-acetyl-*rac*-glycerol. The lipids were derivatized with TBDMSI, according to the methods of Triolo *et al.* (1991) and GC performed according to the method of Maggi *et al.* (1994). Owing to unavailability, authentic 1-O-octadecyl-2-acetyl-glycerol could not be used as a standard; however, the elution patterns of the phospholipase C digestion of C18 PAF (which gives rise to 1-O-octadecyl-2-acetyl-glycerol) was compared with that of the neutral lipid derived from metabolism of $[^3\text{H}]$ PAF by the glandular epithelial cells.

Statistical analysis

Each value is the mean $\pm$ SEM, usually from the three independent experiments. The ANOVA and Student-

Fig. 1. Factors affecting the metabolism of exogenous $[^3\text{H}]$ platelet-activating factor (PAF) by rabbit endometrial tissue. Endometrial tissue was isolated on day 6 of pregnancy and incubated at 37°C for 60 min before approximately 30 mg (wet mass) of the cell suspension was taken for incubation with 5 μCi $[^3\text{H}]$ PAF for 15 min at 37°C. Incubation was carried out either in Tris-HCl buffer (pH 7) without any additives or in the presence of EDTA (10 mmol l⁻¹), diisopropyl-fluorophosphate (DFP; 10 mmol l⁻¹) or dithiothreitol (DTT; 10 mmol l⁻¹). Extraction of total lipids, detection of lipids by TLC, and estimation of cellular DNA content (normalized to number of cells; each cell contains approximately 10 pg DNA) were performed. The experiments expressed in this figure were conducted at the same time as those described in Figs 2 and 3. They have been presented separately for simplicity. The control treatment groups are identical. (■), Lyso-PAF; (□), Alkylacyl-GPC.

Newman-Keuls' tests of significance were performed using the SAS computer program (SAS Institute Inc., Cary, NC). Differences of $P < 0.05$ were considered significant.

Results

$[^3\text{H}]$ Platelet-activating factor uptake by endometrial tissues

As early as 1 min after incubation of the endometrial tissues with $[^3\text{H}]$ PAF, only 40% of the radioactivity could be recovered in the supernatant after three washes with 0.25% BSA in Tris-HCl buffer. Of the 60% associated with the cells, 54% was found in the cytosol and only 4% associated with the plasma membranes that were recovered after centrifugation at 30 000 g for 30 min. The amount of radioactivity associated with the cells increased to 74% after incubation for 60 min, and only 6% was bound to the plasma membranes. This observation confirmed that exogenous $[^3\text{H}]$ PAF was transported into the cells during incubation.

Platelet-activating factor metabolism by unprimed endometrial tissues

In the present series of experiments, 5 μCi $[^3\text{H}]$ PAF was used to provide excess substrate for about 30 mg endometrial tissue per tube and control untreated endometrial cells metabolized the exogenous $[^3\text{H}]$ PAF first into $[^3\text{H}]$ lyso-PAF and subsequently $[^3\text{H}]$ alkylacyl-GPC (Fig. 1): the amount of $[^3\text{H}]$ lyso-PAF build-up was about 2.5-fold the quantity of $[^3\text{H}]$ alkylacyl-GPC. In the presence of 0.25% BSA, $[^3\text{H}]$ PAF metabolism was

completely abolished (data not shown) as we have observed using purified endometrial membranes (Kudolo and Harper, 1990). Therefore, all ligands were solubilized in 70% ethanol and the ethanol final concentration in the reaction mixture did not exceed 0.875%. Endometrial [^3H]PAF deacetylation was apparently catalysed mainly by cytosolic acetylhydrolase because pretreatment of the cells with 10 mmol DFP l^{-1} , a protease inhibitor of serine hydrolases, abrogated over 98% of [^3H]PAF metabolism. In the presence of the calcium-chelating agent, EDTA, the amount of [^3H]lyso-PAF formed was significantly reduced by 70% without any significant change in accumulation of [^3H]alkylacyl-GPC. The presence of DTT also caused a significant reduction in lyso-PAF concentration but, unlike treatment with EDTA, this was accompanied by an increased accumulation of [^3H]alkylacyl-GPC, 118% greater than in the control. There was no apparent reconversion of the metabolic products back to [^3H]PAF. Instead, there was an apparent loss of radioactivity not accountable for when the radioactivity co-migrating with PAF, lyso-PAF and alkylacyl-GPC was combined. This residual radioactivity was detected in conjunction with a lipid product migrating to the solvent front when chromatographed in chloroform:methanol:acetic acid:water (50:25:8:4). The lipid co-migrated with the product of phospholipase C digestion of [^3H]PAF when chromatographed in a solvent system consisting of chloroform and methanol (98:2) and with authentic 1-*O*-hexadecyl-2-acetyl-*rac*-glycerol. This neutral lipid accounted for up to 30% of the radioactivity added. Phospholipase C digestion of [^3H]lyso-PAF did not yield a neutral lipid and the radioactivity remained at the origin.

Effect of *N*-carbamyl-platelet-activating factor on platelet-activating factor metabolism

The possible effect of high PAF concentration on the endometrial handling of exogenous [^3H]PAF at a time when the endogenous concentrations are falling was examined by treating endometrial tissues from day 6 of pregnancy with graded doses of *N*-carbamyl-PAF for 1 h at 37°C before addition of exogenous [^3H]PAF. Increasing concentration of carbamyl-PAF from 10 nmol l^{-1} to 10 $\mu\text{mol l}^{-1}$ was accompanied by decreased [^3H]lyso-PAF accumulation, so that at 10 $\mu\text{mol l}^{-1}$, only 14% of the amount found in the control cells remained (Fig. 2a). The concentration of [^3H]alkylacyl-GPC was also reduced in parallel with [^3H]lyso-PAF up to 1 $\mu\text{mol l}^{-1}$. At 10 $\mu\text{mol l}^{-1}$, however, a significantly higher concentration of [^3H]alkylacyl-GPC accumulation was observed, and this increase was accompanied by a reduced [^3H]lyso-PAF build-up. Increasing the concentration of *N*-carbamyl-PAF resulted in a corresponding increase in the radioactivity of the lipid that co-migrated with authentic PAF (Fig. 2b).

Effect of calcium on platelet-activating factor metabolism

The role of both intracellular and extracellular calcium on PAF metabolism was examined, since the presence of the calcium chelator, EDTA, caused a 70% reduction in the accumulation of [^3H]lyso-PAF (Fig. 1). The presence of

Fig. 2. Effect of carbamyl-platelet activating factor (PAF) on rabbit endometrial tissue metabolism of exogenous [^3H]PAF. Endometrial tissue was incubated in plain Tris-HCl buffer (pH 7; control) or different concentrations of carbamyl-PAF (c-PAF; solubilized in 70% ethanol; final concentration < 1% ethanol per tube) for 60 min at 37°C. Duplicate aliquots of the cell suspensions with approximately 30 mg (wet mass) of cells were incubated with 5 μCi [^3H]PAF for 15 min at 37°C. Total lipids were extracted and chromatographed, and the DNA content of the cells per tube estimated. The experiments were repeated three times. (a) The amounts of [^3H]lyso-PAF (■) and [^3H]alkylacyl-GPC (□) detected. (b) The percentages of unmetabolized [^3H]PAF (defined as the ratio of radioactivity co-migrating with authentic C18 PAF to the total radioactivity ([^3H]PAF) added $\times 100$).

10 mmol extracellular CaCl_2 l^{-1} ($[\text{Ca}^{2+}]_o$) produced a modest reduction in [^3H]lyso-PAF accumulation (30%), accompanied by 170% increase in [^3H]alkylacyl-GPC production over values obtained in the absence of Ca^{2+} (Fig. 3). In the presence of EGTA, another calcium chelator, however, there was more than a threefold increase in the build-up of [^3H]lyso-PAF, and about a twofold increase in [^3H]alkylacyl-GPC accumulation, an indication of a pronounced calcium-dependent [^3H]PAF metabolism by the cells. This is confirmed by the fact that cell treatment with calcium ionophore A23187, in the absence of extracellular Ca^{2+} in the buffer, did not produce significant changes in the accumulation of the metabolic products.

Fig. 3. The role of calcium in the metabolism of exogenous [³H] platelet-activating factor (PAF). Rabbit endometrial tissue was pre-incubated in Tris-HCl buffer containing CaCl₂ (10 mmol l⁻¹), EGTA (10 mmol l⁻¹) or calcium ionophore A23187 (10 μmol l⁻¹), or plain Tris-HCl buffer (control) for 60 min before addition of 5 μCi [³H]PAF and incubation for a further 15 min at 37°C. Total lipids were extracted and chromatographed. The DNA content of the cells per tube was estimated. The experiments were conducted three times in duplicate. (■), Lyso-PAF; (□), Alkylacyl-GPC.

Synthesis and identity of neutral lipid product

A neutral lipid product was synthesized during the metabolism of exogenous [³H]PAF. It appeared initially that the neutral lipid was synthesized subsequent to [³H]PAF deacetylation and not directly from the added [³H]PAF, and yet phospholipase C digestion of [³H]PAF, but not [³H]lyso-PAF, produced the neutral lipid, which co-migrated with 1-O-hexadecyl-acetyl-glycerol (Fig. 4). Therefore, the neutral lipid produced when endometrial cells were incubated with [³H]lyso-PAF (5 μCi) for 15 min, and enhanced when a microsomal preparation (from endometrial tissues) was added, must have been produced after [³H]lyso-PAF reacylation to [³H]PAF. We have shown that this neutral lipid was identical to the lipid product of PAF incubation with phospholipase C (Kudolo and Harper, 1995). In addition to this, after TLC and using GC/FID, the neutral lipid extracted from the glandular epithelial cell incubation medium was found to be eluted at 11.7 min (*n* = 2), which was not different from that for the lipid produced by

phospholipase C digestion of PAF, which was 11.5 ± 1.6 min (mean ± SD, *n* = 3).

Platelet-activating factor metabolism by subcellular fractions of endometrial cells

After demonstrating that an appreciable amount of [³H]PAF was transported into the endometrial cells within 5 min of

Fig. 4. Chromatographic profile of phospholipase C-catalysed neutral lipid synthesis from platelet-activating factor (PAF). [³H]PAF and [³H]lyso-PAF (5 μCi) were added to 200 μg of cold PAF and lyso-PAF, dried under a stream of nitrogen gas and solubilized in 0.5 ml 0.125% (w/v) BSA in Tris-HCl buffer (containing 25 mmol Ca²⁺ l⁻¹) and incubated with 10 μg phospholipase C for 1 h at 37°C. The reaction product was extracted by the method of Bligh and Dyer (1959). (a) and (b) When chromatographed in chloroform:methanol:acetic acid:water (50:25:8:4), a lipid product migrating to the solvent front was produced from [³H]PAF, but not from [³H]lyso-PAF, and also from extracts of [³H]PAF metabolism by rabbit glandular epithelial but not by stromal cells (see Fig. 7). (c) and (d) The lipid co-migrated with the product of phospholipase C digestion of [³H]PAF and authentic 1-O-hexadecyl-2-acetyl-glycerol when chromatographed in a solvent system consisting of chloroform and methanol (98:2). (a) and (c) (■), PAF alone; (□), PAF + phospholipase C; (b) and (d) (▨), lyso-PAF; (▩), lyso-PAF + phospholipase C.

incubation, and that DFP (10 mmol l^{-1}) abolished its metabolism, indicative of acetylhydrolase activity, it remained to confirm these observations with isolated subcellular fractions. Only the cytosolic fraction avidly metabolized [^3H]PAF, with less than 10% of the native [^3H]PAF remaining after 30 min of incubation (Fig. 5). The formation of alkylacyl-GPC in the cytosol, also observed in the human platelet cytosol by Kramer *et al.* (1984), may be due to the leakage of membrane-associated transacylase enzymes into the cytosol during tissue homogenization. Neither the membranes nor the microsomes metabolized [^3H]PAF, but both avidly metabolized [^3H]lyso-PAF, over 80% by the membranes and 40% by the microsomes within 5 min (Fig. 6).

Platelet-activating factor metabolism by cultured endometrial cells

Separated endometrial cells were cultured to examine whether the different cell types metabolized exogenous [^3H]PAF similarly. Addition of $4 \text{ nmol } [^3\text{H}]\text{PAF l}^{-1}$ on day 2 of culture produced the results shown in Fig. 7. Within 5 min, [^3H]PAF was metabolized via distinct pathways: there was significantly more [^3H]lyso-PAF accumulation in stromal cells than in epithelial cells. The radioactivity that could not be accounted for by the sum of [^3H]lyso-PAF and [^3H]alkylacyl-GPC in the epithelial cells was again found to be converted to a neutral lipid.

Discussion

A principal observation from these experiments was that the metabolism of exogenous [^3H]PAF took place after it had traversed the cell membrane and come into contact with cytosolic acetylhydrolase. Almost 60% of added [^3H]PAF was in the cytosolic fraction of the cell within 5 min. Once inside the cell, [^3H]PAF metabolism appears to have been accomplished by the sequential action of two enzymes. The first step was catalysed by acetylhydrolase in the cytosolic fraction that deacetylates [^3H]PAF forming [^3H]lyso-PAF. This step was independent of calcium stimulation. Calcium appeared to be inhibitory because [^3H]PAF metabolism was enhanced over three times in the presence of EGTA. The second step was catalysed by a membrane-associated transacylase enzyme that incorporates a long-chain fatty acid into [^3H]lyso-PAF to produce [^3H]alkylacyl-GPC, in agreement with other cell systems (Blank *et al.*, 1981; Albert and Snyder, 1983; Kramer *et al.*, 1984). However, in addition to [^3H]alkylacyl-GPC formation, there was also the formation of a neutral lipid, the synthesis of

which was catalysed by a microsomal enzyme found in the glandular epithelial, but not in the stromal, cells. We have provided the first documentation of the cell-specific synthesis

Fig. 5. [^3H] platelet-activating factor (PAF) metabolism by subcellular fractions of rabbit endometrial tissue. Endometrial tissues were fractionated into plasma membranes, cytosol and microsomes as described in the Materials and Methods. Portions ($50 \mu\text{g}$ protein) were incubated with $4 \text{ nmol } [^3\text{H}]\text{PAF l}^{-1}$ at 37°C and the reaction terminated after 1, 5, 15 and 30 min. The lipids were extracted by the Bligh and Dyer (1959) procedure and chromatographed. The experiments, in triplicate, were performed only twice, but with less than 10% variation between the means. (a) Membrane; (b) Microsomes; (c) Buffer; and (d) Cytosol. (■), PAF; (□), Lyso-PAF; (○), Alkylacyl-GPC.

Fig. 6. [^3H]lyso-PAF metabolism by (a) membrane and (b) microsomal fractions of rabbit endometrial tissue. Membrane and microsomal fractions were prepared and incubated with 4 nmol [^3H]lyso-PAF 1^{-1} . The lipids were extracted by the Bligh and Dyer (1959) procedure and chromatographed. The experiments, in triplicate, were performed only twice, but with less than 10% variation between the means. (■), platelet-activating factor (PAF); (□), Lyso-PAF; (○), Alkylacyl-GPC.

Fig. 7. Metabolism of exogenous [^3H]PAF by separated rabbit (a) endometrial stromal and (b) epithelial cell fractions. Endometrial cells were enzymatically separated into glandular epithelial and stromal cells and on the second day of culture, exogenous [^3H]PAF was added to the cells in 2 ml culture medium (in the absence of calf serum) and incubation continued for (■), 15 (□), 30 and (▨), 60 min. Total lipids were extracted from the cells and medium and chromatographed in a solvent system consisting of chloroform:methanol:acetic acid:water (50:25:8:4). The neutral lipid that migrated to the solvent front in this system, co-migrated with 1-O-hexadecyl-2-acetyl-glycerol when chromatographed in chloroform:methanol (98:2). Experiments were performed at least three times, in triplicate wells.

of this lipid in the mammalian endometrium (Kudolo and Harper, 1995). The present study demonstrated the ability of intact endometrial cells to metabolize exogenous [^3H]PAF and [^3H]lyso-PAF, and in this regard rabbit endometrium may differ from other cell systems: for example [^3H]lyso-PAF is poorly metabolized by intact rabbit platelets (Lachachi *et al.*, 1985; Touqui *et al.*, 1987) and by cultured rat Kupffer cells (Chao *et al.*, 1989).

Our inability to observe significant PAF accumulation in rabbit endometrium was initially intriguing because similar incubation conditions had promoted PAF synthesis in other cells, and because it could have been produced merely from reacylation of [^3H]lyso-PAF. There are two different enzymes that can catalyse PAF synthesis: lyso-PAF:acetyl-CoA acetyltransferase (Wykle *et al.*, 1980) and CDP-choline:1-alkylacetyl-glycerol-3-phosphate:cholinephosphotransferase (Renooij and Snyder, 1981). Biosynthesis of PAF is calcium dependent (Ninio *et al.*, 1983); both EDTA and EGTA inhibit acetyltransferase activity (Wykle *et al.*, 1980). However, treatment of mouse peritoneal cells with DTT results in a two-

threefold increase of acetyltransferase activity and corresponding PAF synthesis (Ninio, 1987). Lyso-PAF:acetyltransferase activity could have been activated in the endometrial cells without activation of PAF synthesis, as seen in cultured mast cells (Joly *et al.*, 1990). Alternatively, PAF could have been produced and rapidly metabolized to lyso-PAF or the neutral

lipid, depending on the cell type. We have demonstrated the incorporation of [3 H]acetyl-CoA into exogenous lyso-PAF catalysed by a microsomal preparation from endometrial tissues (Kudolo and Harper, 1995). Therefore, the synthesis of the neutral lipid must have taken place subsequent to the reacylation of lyso-PAF to PAF and its conversion via a phospholipase C-catalysed reaction, in agreement with observations made in cultured rat hepatocytes (Okayasu *et al.*, 1986).

In rabbit endometrial cells in culture, PAF synthesis is essentially via the remodelling pathway (Kasamo *et al.*, 1992). However, this may not be true for constitutive synthesis *in vivo*. In the stromal cells where no neutral lipid synthesis was observed, lyso-PAF:acetyl CoA:acetyltransferase activity, but not phospholipase C, may be the limiting factor, hence the build-up of lyso-PAF. In the *de novo* pathway, neutral lipids may serve as precursors for the synthesis of PAF (Wykle *et al.*, 1980; Blank *et al.*, 1988; Lee *et al.*, 1988), and since the neutral lipid synthesis was restricted to the glandular epithelial cells, it may be that this is the pathway of choice in these cells. Therefore, the increasing concentration of uterine PAF observed with increasing gestation in the preimplantation period (Angle *et al.*, 1988) may be a reflection of the proliferation of the glandular epithelial cells. The increase in the amount of radioactivity co-migrating with authentic PAF after treatment of the endometrial cells with *N*-carbamyl-PAF could have been due to a displacement of [3 H]PAF from membrane sites preventing the [3 H]PAF molecule from being transported into the cell for further metabolism. We support that view because the PAF analogue-stimulated intracellular [3 H]PAF resynthesis should have led to an increase in the synthesis of the neutral lipids, which did not take place in the endometrial cells. Pretreatment of human neutrophils with *N*-carbamyl-PAF has not been shown to cause any change in [3 H]PAF metabolism, but did stimulate PAF synthesis (Tessner *et al.*, 1989). This latter observation could not be confirmed by Sisson *et al.* (1987). We have no evidence at this time that *N*-carbamyl-PAF treatment altered the number or occupancy of the endometrial surface membrane PAF receptors. We have reported only that [3 H]-*N*-carbamyl-PAF binds with high affinity to purified endometrial membrane sites (Kudolo and Harper, 1989). It is possible that *N*-carbamyl-PAF treatment alters the number or occupancy of these sites, as seen with myelogenous cells, in which it induced up to a 70% reduction in the number of binding sites without any effect of PAF metabolism (O'Flaherty *et al.*, 1989).

Taken together, metabolism of exogenous PAF in short-term incubations may take place after it enters the endometrial cell. Once inside the cell, PAF is subject to rapid deacetylation. The fate of lyso-PAF is cell dependent. In the stroma, PAF synthesis may be limited and restricted to a remodelling pathway, and lyso-PAF:acetyl CoA acetyltransferase may be a limiting factor. The bulk of endogenous uterine PAF synthesis observed during the preimplantation period may take place in the glandular epithelial cells via a unique *de novo* pathway in which 1-*O*-alkyl-2-acetyl-glycerol may serve as the substrate for CDP-choline:1-alkylacetyl-glycerol-*DTT*-insensitive cholinephosphotransferase (Renooij and Snyder, 1981; Kudolo and Harper, 1995) for PAF synthesis.

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