Platelet-Activating Factor Stimulates Prostaglandin Synthesis in Cultured Cells

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SUMMARY The effects of platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) on phospholipase activity were studied in aortic smooth muscle cells and renal epithelial cells. When platelet-activating factor was added to cells prelabeled with [3H]arachidonic acid, it induced rapid hydrolysis of phospholipids. Up to 28% of incorporated [3H]arachidonic acid was released into the medium from both aortic and renal cells. A transient rise of diacylglycerol was also seen after the addition of platelet-activating factor to these cells. The accumulation of diacylglycerol and monoacylglycerol was relatively small when compared with the total amount of released free arachidonic acid. The amount of [3H]arachidonic acid released was comparable to the loss of phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine, which indicates that platelet-activating factor stimulates phospholipase A2 and C activity in aortic smooth muscle cells and renal epithelial cells. Platelet-activating factor also enhanced prostaglandin biosynthesis in these cells.

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KEY WORDS • platelet-activating factor • phospholipase A2 • phospholipase C • aortic smooth muscle cells • renal epithelial cells • prostacyclin

PLATELET-activating factor (PAF), a mediator of anaphylaxis and inflammation, is released from leukocytes after immunological stimulation in various species, including humans, rats, rabbits, and pigs, and has been identified as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (alkylacetetyl-GPC). This bioactive phospholipid is released from a number of mammalian cells on stimulation and appears to be involved in several important cellular events. For example, it causes the aggregation and degranulation of platelets and stimulates arachidonic acid release and subsequent thromboxane synthesis. It also causes aggregation and degranulation of polymorphonuclear neutrophils as well as triggers arachidonic acid release and subsequent production of bioactive mediators, including monohydroxylated and dihydroxylated lipoxygenase products. An independent study demonstrated that the renal medulla contains a hypotensive lipid. This antihypertensive polar renomedullary lipid (APRL) was also identified as alkylacetetyl-GPC and has been shown to possess marked hypotensive activity when given orally or intravenously to one-kidney, one clip (Goldblatt) hypertensive rats. Blank et al. further suggested that APRL is a powerful vasodilator.

Little is known about the effects of APRL on phospholipid hydrolysis in cells other than platelets and leukocytes. If other cells are affected directly by APRL and are stimulated to release prostaglandins, it may have a physiological importance. To study this possibility, we recently examined phospholipase A2 and C activities in Swiss mouse 3T3 fibroblasts stimulated by PAF. We have found that PAF directly stimulated phospholipase A2 and C activities in these cells. The 3T3 cells are not specific for renovascular systems, however, and renovascular-specific cells should be used to clarify the vasodilator effect of PAF on renovascular cells. Therefore, the present study attempted to show that aortic smooth muscle cells and renal epithelial cells hydrolyze phospholipids when stimulated by PAF. Phospholipase A2 and C activities were examined in these cells to determine the mechanism of action of PAF on renovascular systems.

Materials and Methods

[5,6,8,9,11,12,14,15-3H]arachidonic acid (135 Ci/mol) was obtained from Amersham (Arlington Heights, IL, USA). The carrier lipids lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, monoylcglycerol (MG), diacylglycerol (DG), triacylglycerol, and arachidonic acid were obtained from Sigma Chemical (St. Louis, MO, USA), as was 1-O-hexadecyl-2-acetyl-phosphatidylcholine (C16-PAF). The 1-O-octadecyl-2-acetyl phos-
phatidylycerine (C18-PAF) was purchased from Backen Feinchemicales (Bubendorf, Switzerland). All other materials were of reagent purity.

Renal epithelial cells (LLC-RK1) were obtained from Flow Laboratories (Rockville, MD, USA) and maintained in Dulbecco’s modified Eagle’s medium (Flow Laboratories) supplemented with 10% fetal bovine serum. Aortic smooth muscle cells from 3- to 4-week-old Wistar rats were isolated from medial explants essentially by the methods of Ross. Aortas were cut open to expose the intimal surface. The intima and adventitia were stripped using gauze and a razor blade, and the explants then were cut into approximately 4-mm sections. The intimal sides of the explants were attached to Petri dishes. Once the explants had adhered to the dishes, Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum was carefully added. The dishes were then incubated at 37°C in an atmosphere of 95% O₂, 5% CO₂.

Cells grown to confluence were subcultured at 1.2 × 10³ cells per dish in 2 ml of Dulbecco’s modified Eagle's medium containing 0.3% fetal bovine serum in Eagle’s medium supplemented with 20% fetal bovine serum. Aortic smooth muscle cells and renal epithelial cells were 20 ± 2 and 23 ± 2%, respectively. The plated cells were washed three times with 2 ml of the medium and then incubated at 37°C with the indicated concentration of PAF (C16-PAF or C18-PAF) in 0.6 ml of serum-free medium. The lipids released into the medium were extracted with 2 ml of ethyl acetate and analyzed.

Cellular lipids were obtained for thin-layer chromatography by removing the medium, rapidly washing the cells twice with 2 ml of medium, and then scraping the cells from the dishes using a rubber policeman. The lipids were extracted by the method of Folch et al. Phospholipids were analyzed using a method previously described by Shier. Neutral lipids were separated by thin-layer chromatography using the solvent system described in the next paragraph. Thin-layer chromatography was first performed using isopropyl ether/acetone acid (96:4; vol/vol) and then again with a solvent system containing petroleum ether/diethyl ether/acetone acid (90:10:1; vol/vol/vol). The respective spots (MG, DG, triacylglycerol, and arachidonic acid) were then scraped and counted in a scintillation spectrometer.

Plated cells prelabeled with [³H]arachidonic acid were washed three times with fresh medium and then incubated with the indicated concentration of PAF in Dulbecco’s modified Eagle’s medium up to 60 minutes at 37°C. The prostaglandins released into the medium were extracted with ethyl acetate, and the solvent was evaporated under vacuum. The residues were applied to thin-layer chromatography and developed with ethyl acetate acetic acid (90:1; vol/vol) applied twice. To separate prostaglandin (PG) E₂ and 6-keto-PGF₁α, the plates were first allowed to develop up to 15 cm from the origin and, after drying, were again allowed to develop up to 17 cm from the origin in the solvent system containing the organic phase from ethyl acetate/acetonic acid/2,2,4-trimethylpentane/water (15:20:50:100; vol/vol/vol/vol). The zones corresponding to authentic prostaglandins (PG E₂, PGF₂α, 6-keto-PGF₁α) and arachidonic acid were scraped and counted in a liquid scintillation counter. Further identification of the reaction products was done using a method involving high-performance liquid chromatography. The retention times of 6-keto-PGF₁α thromboxane B₂, PGF₂α, and PGE₂ were 3, 5, 7, and 12 minutes, respectively.

Cells were cultured in the same manner as described for the assay of phospholipid hydrolysis except that no tritiated arachidonic acid was used. Following the treatment of renal cells with PAF for 30 minutes, the medium (600 µl) was removed and centrifuged to remove any nonadherent cells and 250-µl aliquots were tested for ß-galactosidase and lactate dehydrogenase activities.

**Results**

Renal and aortic cells responded to treatment with C16-PAF and C18-PAF at 10⁻³ M by releasing, in 1 hour, up to 28% of the incorporated [³H]arachidonic acid into the culture medium in the form of chromatographically identifiable free arachidonic acid (Figure 1). A detectable release began within 5 minutes and continued for at least 90 minutes (Figure 2). As shown in Figure 3, prostaglandin synthesis was also stimulated in both kinds of cells when PAF was added. Aortic cells released mainly 6-keto-PGF₁α in a concentration proportional to that of PAF. Mainly PGE₂, with trace amounts of other prostaglandins, was produced by adding PAF to renal cells. The addition of PAF stimulated prostaglandin synthesis in both cell types at a concentration of 10⁻³ M, while maximal response occurred at concentrations of 10⁻² or 10⁻¹ M.

The addition of PAF caused a rapid decrease of radioactive phosphatidylincholine, phosphatidylinositol, and phosphatidylethanolamine in both cell types (Table 1). This decrement was measurable within 10 minutes, and progressively decreased for up to 60 minutes (Figure 4). Since the degradation of phospholipids might have been catalyzed by phospholipase A₂ and C, the accumulation of DG was measured in both cell types and the medium to determine phospholipase C activity. The increase in the labeled DG was detectable within 1 minute and peaked at 20 minutes (Figure 5). The decrease of phospholipids was accompanied by an increase in DG (see Table 1). The decline in levels of DG after 30 minutes in both cell types and medium might have been caused by its reincorporation into phospholipids, as previously reported. Although MG accumulation also occurred, the accumulation of DG and MG was relatively small compared with the free arachidonic acid released.
FIGURE 1. Effect of the concentration of platelet-activating factor (PAF) on the release of arachidonic acid in aortic and renal cells. Triplicate cultures of 1.2 x 10^5 aortic and renal cells prelabeled with [3H]arachidonic acid were incubated with the indicated concentration of 1-O-hexadecyl-2-acetylphosphatidylcholine (C16-PAF; o) or 1-O-octadecyl-2-acetylphosphatidylcholine (C18-PAF; •) for 1 hour at 37°C. The total arachidonic acid released is presented as a percentage of total [3H]-arachidonic acid incorporated into cells. Points represent means ± SEM.

FIGURE 2. Time course of the effect of PAF (o) versus control (•) on the release of arachidonic acid from aortic and renal cells. Aortic and renal cells were incubated for the indicated periods at 37°C with 1 μM of C16-PAF as described in Methods. See Figure 1 for key to abbreviations.

FIGURE 3. Effect of the concentration of PAF on the release of prostaglandins from aortic and renal cells. Cells were incubated with the indicated concentration of C16-PAF (o) or C18-PAF (•) for 1 hour at 37°C. The 6-keto-prostaglandin F_1α (6-keto-PGF_1α) released from aortic cells and prostaglandin E_2 (PGE_2) released from renal cells were extracted and analyzed as described in Methods. Total released radioactivity is presented as a percentage of total [3H]arachidonic acid incorporated into the cells. Points represent means ± SEM. See Figure 1 for key to abbreviations.
These findings suggested that PAF promoted the release of arachidonic acid from phospholipids in aortic and renal cells by stimulating phospholipase A,
and C. To examine this possibility, we investigated the decrease of cell phospholipid levels after the addition of PAF. The cells showed a decrease in radioactive phosphatidylcholine, phosphatidylinositol, and
phosphatidylethanolamine (see Figure 4). Phosphatidylcholine and phosphatidylethanolamine accounted for more than 60% of total radioactivity in the cells that had been prelabeled with [3H]arachidonic acid. The incorporation of [3H]arachidonic acid into the cells was about 23% during the 24-hour incubation. The distribution of incorporated arachidonic acid into the second position of phosphoglycerides was determined by treating the cells with naja naja venom phospholipase A2, which is specific for fatty acid in the second position; 96.3% of the incorporated [3H]arachidonic acid was in the second position of cellular phospholipids.

### Figure 4. Effect of PAF on cellular phospholipids. Renal cells were incubated with C16-PAF (O) or C18-PAF (●) for up to 1 hour at 37°C. Cellular phospholipids were extracted and analyzed by thin-layer chromatography as described in Methods. The radioactivity in cells is presented as a percentage of the total [3H]arachidonic acid incorporated into the cells. Points represent means ± SEM. PC = phosphatidylcholine; PI = phosphatidylinositol; PE = phosphatidylethanolamine. See Figure 1 for key to abbreviations.

### Figure 5. Effect of PAF (O) versus control (●) on the release of diacylglycerol from aortic and renal cells. Triplicate cultures of 1.2 × 10⁵ cells prelabeled with [3H]arachidonic acid were incubated with 1 μM of C16-PAF for the indicated periods at 37°C as described in Methods. Points represent means ± SEM. See Figure 1 for key to abbreviations.
The effects of PAF on the release of cellular lactate dehydrogenase and \( \beta \)-galactosidase into the external medium also were determined. The incubation of cell populations with \( 5 \times 10^{-5} \) M PAF for 30 minutes resulted in the release of lactate dehydrogenase into the medium (Figure 6), while no concomitant \( \beta \)-galactosidase activity was observed.

**Discussion**

Heffner et al.\(^34\) reported that thromboxane \( A_2 \), released from platelets stimulated by alkylacetyl-GPC, caused pulmonary hypertension and lung edema in an isolated rabbit lung perfused with platelets and alkylacetyl-GPC. This finding suggests that prostaglandins released from blood cells play an important role when tissues are stimulated by PAF. Furthermore, several investigators have reported that the renal medulla has antihypertensive polar lipids\(^1,14,17\) identified as alkylacetyl-GPC. The short-term effect of APRL appears to cause generalized relaxation of vascular smooth muscle, mainly of the arterial side. Relaxation of the venous side was observed when a higher dose was used. Smith et al.\(^18\) concluded that APRL appeared to be a potent vasoactive glycerophosphate whose main short-term and long-term effect was vasodilation. They suggested that the short-term vascular response may be mediated by adrenergic antagonism.\(^18\)

Other mechanisms also might induce vascular smooth muscle relaxation, such as prostaglandins released from platelets and leukocytes stimulated by PAF. Alternatively, PAF may stimulate smooth muscle cells directly, and these cells would then release prostaglandins. The present study investigated the latter possibility. Since prostaglandins are quickly removed and inactivated from circulation,\(^36\) the physiological role of vasoactive substances may depend on local synthesis and release. The intracellular endogenous levels of the precursor of prostaglandin are extremely low since most of the arachidonic acid is esterified into the membrane phospholipids.\(^37\)

Phospholipase \( A_2 \) and \( C \) deacylate arachidonic acid from phospholipids. Several studies have suggested an important role for these enzymes in normal and pathological renovascular function.\(^38,39\)

In our experiments, the addition of PAF caused rapid hydrolysis of phospholipids in aortic smooth muscle and renal epithelial cells and also stimulated prostaglandin synthesis. Vogel et al.\(^40\) have postulated that cellular phospholipids are hydrolyzed by phospholipase \( A_2 \) or phospholipase \( C \) (especially phosphatidylinositol-specific phospholipase \( C \)) and the released DG is subsequently converted, first to phosphatidic acid and then to cytidinediphosphodiacylglycerol and phosphatidylinositol. Whether this mechanism is applicable to all systems remains to be proved. In the present study, the addition of PAF to aortic and renal cells clearly led to phospholipase \( A_2 \) and \( C \) stimulation. A higher concentration of PAF was necessary to hydrolyze phospholipids in these cells compared with that needed in platelets and leukocytes.\(^13,14\)

The effects of PAF on the release of cellular lactate dehydrogenase and \( \beta \)-galactosidase into the external medium also were studied. Lactate dehydrogenase and \( \beta \)-galactosidase activities are highly compartmentalized in the cytoplasm and lysosomes, respectively, and have proved to be useful markers for damage to cell surface and lysosomal membranes, respectively. The incubation of cell populations with \( 5 \times 10^{-5} \) M PAF resulted in the release of lactate dehydrogenase into the medium, while concomitant \( \beta \)-galactosidase activity was not observed. These results suggest that the effect of PAF appears at less toxic concentrations in these cells.

Our experiments with these cells prelabeled with arachidonic acid demonstrated that the degradation of phospholipids was accompanied by the accumulation of free arachidonic acid in the medium. After 10 to 20 minutes, early changes in phospholipids were sufficient to account for the changes in DG and MG released from the cells, but the total amount of DG and MG after a 60-minute incubation was relatively small when compared with the amount of released \( [3H] \)arachidonic acid. The amount of free \( [3H] \)arachidonic acid in the medium and the cells was comparable to the loss from phosphatidylethanolamine, phosphatidylinositol, and phosphatidylethanolamine. These results suggest that PAF stimulated not only phospholipase \( A_2 \) but also phospholipase \( C \) in aortic and renal cells. Some of the arachidonic acid released was converted to 6-keto-PGF\(_{1\alpha}\), a stable metabolite of prostacyclin, in aortic smooth muscle cells and prostaglandin \( E_2 \) in renal epithelial cells. These results are physiologically important because prostacyclin acts as a vasodilator and PGE\(_2\) participates in blood pressure regulation in hypertensive states.\(^41,42\)

Although PAF is a potent lipid mediator strongly implicated in the pathogenesis of acute allergic and inflammation reaction and causes vasodilation, little information is available regarding the effect of PAF on cells other than platelets and leukocytes that release arachidonic acid and subsequent metabolites. Our re-
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