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Production of platelet-activating factor by washed rabbit platelets[1]

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Abstract Production of platelet-activating factor by washed rabbit platelets under stimulation with the ionophore A23187 was investigated utilizing two groups of platelet preparations. The first platelet preparation contained 0.03 ± 0.02% contaminating white cells, while the second preparation contained 0.48 ± 0.27% white cells. The latter preparation produced platelet-activating factor, mainly 1-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, 8.3 ± 6.3 pmol (mean ± standard deviation) with a range of 2.6 to 21.4 pmol (n = 9), followed by small quantities of 1-octadecenyl- and 1-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine. In contrast, there was no production of 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine by the former platelet preparation having 0.03% leukocytes. These quantitative analyses were carried out by the selected ion monitoring technique and it was concluded that it is necessary to consider the presence of contaminating white cells in studies on the production of platelet-activating factor by platelets. —**Oda, M., K. Satouchi, K. Yasunaga, and K. Saito.** Production of platelet-activating factor by washed rabbit platelets. *J. Lipid Res.* 1985. **26:** 1294–1299.

Supplementary key words monocytes • AGEPC • selected ion monitoring

Platelet-activating factor (PAF), which was identified chemically as being 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC) (1), is now recognized as a feasible chemical mediator of inflammation in man as well as the established chemical mediator of anaphylaxis in rabbit (2).

PAF was originally detected in rabbit basophils upon IgE-dependent antigen stimulation (3). Human neutrophils, monocytes (4, 5), and murine macrophages (6) were shown to generate PAF during phagocytosis or by the stimulation of cells with reagents such as formyl-methionyl-leucyl-phenylalanine, C5a, and calcium ionophore. The chemical structure of PAF from human neutrophils has recently been identified as AGEPC by the technique of selected ion monitoring (SIM) (7, 8) and fast atom bombardment mass spectrometry (9, 10).

In addition to the above inflammatory cells, platelets also synthesize PAF in response to the stimulation of thrombin, collagen, and calcium ionophore (11, 12). From these studies a hypothesis that PAF might mediate the so-called third pathway of aggregation, which is independent of ADP and thromboxane A_2, was deduced (13, 14). However, controversy arose concerning the platelet origin of PAF (15, 16). Marcus et al. (16) reported that ionophore was the stimulus to produce PAF by human platelets, but the mechanism was calculated to represent only a minute quantity of AGEPC. Furthermore, no AGEPC was recoverable with thrombin stimulation. Therefore, Marcus et al. (16) concluded that washed platelets are not a significant source of AGEPC as a result of cellular responses. One of the causes for this discrepancy may be attributed to small amounts of white cells in platelet preparations. Bearing the above in mind, we investigated PAF production by platelets using two groups of platelet preparations that differed in their content of white cells.

EXPERIMENTAL PROCEDURES

Chemicals

C-18:0 AGEPC was the product of Bachem Feinchemikalien AG, Bubendorf, Switzerland. C-16:0 AGEPC and C-16:0 d_3 AGEPC were synthesized chemically (7) in the presence of a catalytic amount of perchloric acid (17). A mixture of AGEPC including C-18:1 AGEPC was synthesized by successive mild acid and alkaline hydrolyses of choline glycerophospholipids from human polymorphonuclear leukocytes (PMN), and reacetylation of the resulting 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine as described by Mueller, O'Flaherty, and Wykle (18). Ionophore

Abbreviations: PAF, platelet-activating factor; AGEPC, acetyl glyceryl ether phosphocholine; C-16:0 AGEPC, 1-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; DMSO, dimethylsulfoxide; EGTA, ethylene glycol-bis-(β-amino ether)-N,N'-tetraacetic acid; PRP, platelet-rich plasma; top-2/3 platelets, washed platelets prepared from the top two-thirds of platelet-rich plasma; bottom-1/3 platelets, washed platelets prepared from the bottom one-third of platelet-rich plasma and buffy coat fraction; TLC, thin-layer chromatography; tBDMS, *tert*-butyldimethylsilyl; SIM, selected ion monitoring.

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A23187 was the product of Calbiochem, San Diego, CA and was stored in dimethylsulfoxide (DMSO) solution at −70°C. *tert*-Butyldimethylchlorosilane/imidazole reagent was obtained from Applied Science, State College, PA. Gelatin powder was purchased from E. Merck, Darmstadt, FRG. Phospholipase C (EC. 3.1.4.3) was prepared from the culture medium of *Bacillus cereus* (19). Thrombin was the product of Mochida Pharmaceutical Co., Tokyo, Japan. Solvents used were all reagent grade.

Buffers

Basic Tyrode's solution was made up as follows: NaCl, 8.00 g/l; KCl, 0.195 g/l; $NaHCO_3$, 1.02 g/l; $MgCl_2$ $6H_2O$, 0.213 g/l D-glucose, 1.00 g/l, and gelatin, 2.5 g/l. Tyrode's EGTA pH 6.5 solution was basic Tyrode's solution containing 0.1 mM EGTA adjusted to pH 6.5 with dilute HCl. Tyrode's Ca^{2+} pH 7.2 solution was basic Tyrode's solution containing 1.3 mM Ca^{2+} adjusted to pH 7.2 with dilute HCl. All Tyrode's solutions were prepared shortly before experimental use.

Preparation of platelets

Unless otherwise mentioned, all experiments were performed more than three times on different platelets and values are shown as mean ± SD.

The entire preparation of washed platelets, essentially following the method of Pinckard, Farr, and Hanahan (20), was performed at room temperature with plastic containers and siliconized pipettes. Briefly, blood (45 ml) was collected from the central ear artery of an adult male white rabbit, weighing from 3.5 to 4.5 kg, into 5 ml of 3.8% trisodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 500 *g* for 15 min. The top two-thirds of PRP (starting material of top-2/3 platelet) and the bottom one-third of PRP plus buffy coat (starting material of bottom-1/3 platelet) were carefully removed and both were layered over a half volume of Ficoll-paque, respectively. They were centrifuged at 750 *g* for 20 min. After the platelet-poor plasma layer was discarded, the banded platelets were removed with a Pasteur pipette and transferred to other tubes. The platelets were resuspended in Tyrode's EGTA pH 6.5 solution, which was then layered over a half volume of Ficoll. After centrifugation at 750 *g* for 15 min, the upper Tyrode's and lower Ficoll layers were removed in the preparation of top-2/3 platelets. In the case of bottom-1/3 platelets, banded platelets were transferred to another tube after removing the upper Tyrode's solution. In this way, the remaining erythrocytes were eliminated. The platelets were resuspended again in Tyrode's solution and were sedimented by centrifugation at 750 *g* for 15 min. They were again resuspended on an appropriate volume of Tyrode's solution and the volume was adjusted to 1.25×10^9 platelets/ml in the same solution. From 100 ml of citrated blood, $1.4 \pm 0.3 \times 10^{10}$ (n = 12) of top-2/3 platelets and $1.6 \pm 0.7 \times 10^{10}$ (n = 13) of bottom-1/3 platelets were obtained, respectively. In another set of experiments, platelets were obtained directly from buffy coat according to the procedure for bottom-1/3 platelets. In this preparation, $0.6 \pm 0.2 \times 10^{10}$ platelets (n = 3) were recovered from 100 ml of citrated blood. By microscopic examination, the numbers of white cells in these platelet preparations were 0.03 ± 0.02% (n = 12) in top-2/3 platelets, 0.51 ± 0.25% (n = 13) in bottom-1/3 platelets, and 0.67 ± 0.24% (n = 3) in platelets from buffy coat fraction, respectively.

Top-2/3 and bottom-1/3 platelet preparations both exhibited similar responsiveness for aggregation to AGEPC and thrombin. The degree of aggregation was assessed in an aggregometer (Nikko Hematracer PAT-4A) as described previously (21).

Activation of platelets

More than 1×10^{10} washed platelets, adjusted to a cell concentration of 5×10^8 platelets/ml Tyrode's Ca^{2+} pH 7.2 solution, were challenged with the ionophore A23187 at a final concentration of 2.5×10^{-6} M, the optimum condition as described by Chignard et al. (11). At the same time, DMSO, the vehicle for the ionophore, was added to another sample of platelets as a control. The mixture was incubated with constant shaking at 37°C for 15 min. In another set of experiments, thrombin was used as a stimulus at a concentration of 2 units/ml (11).

Extraction and isolation of PAF

Platelets with Tyrode's solution were used directly as a source of PAF determination. Briefly, to the cells in Tyrode's solution, 0.2 vol of cold 0.1 M disodium EDTA and 0.02 vol of formic acid were added. After the mixture was transferred to another vessel, total lipids were extracted by the method of Bligh and Dyer (22) using chloroform with (SIM assay) or without (bioassay) 100 ng of internal standard (C-16:0 d_3 AGEPC). Components of the dried extracts, reconstituted in a minimal volume of chloroform, were applied to silicAR CC-7 column and the total phospholipids were obtained by methanol elution. The total phospholipids were separated into individual components by preparative silica gel H TLC (0.6-mm thick) with chloroform–methanol–water 65:25:4 (v/v) as the solvent system. Areas corresponding to PAF and PC, which were identified from the positions of standard phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine stained by exposure to iodine vapor, were removed by scraping and extracted. However, this method was later found to result in a decrease of C-18:1 AGEPC. Alternatively, the PAF position was judged from standards that were located by their fluorescence under ultraviolet light after being lightly sprayed with rhodamine 6G

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(23). The PAF fraction was further processed by neutral alumina column chromatography and silica gel H TLC (0.3 mm) using a solvent system of methanol–water 2:1 (v/v) (24).

Derivatization of PAF for SIM assay

The purified PAF plus internal standard was subjected to hydrolysis with phospholipase C and the resulting diglyceride was quickly converted to a tBDMS derivative (25). The derivative was further subjected to silica gel H TLC (0.3 mm) in a solvent system of hexane–diethyl ether 9:1 (v/v) in order to remove 1-acyl-2-acetyl-3-tBDMS, which migrates slower than 1-alkyl-2-acetyl-3-tBDMS (23).

The recoveries of the internal standard throughout isolation and derivatization procedures were between 10 and 20% as judged from peak areas of internal standard in SIM traces.

Condition of selected ion monitoring

A JEOL JMS-DX 300 apparatus equipped with a JMA 3100 computer was used. The selected ion was $(M - 57)^+$, m/z 415 for C-16:0 AGEPC, m/z 418 for the internal standard, m/z 441 for C-18:1 AGEPC, and m/z 443 for C-18:0 AGEPC. Each was abundantly produced under electron impact by a scission of the *tert*-butyl radical from the parent ion. Other conditions for gas–liquid chromatography, mass spectrometry, and computer processing were essentially as described before (7).

Gas–liquid chromatography

A Shimadzu GC-8AG apparatus with a flame ionization detector was used. The column was a glass tube (2 m × 3 mm) packed with 1% OV-1 on Chromosorb W (80/100 mesh). Temperatures for column and injection were 230 and 270°C, respectively. Peak area of the tBDMS derivative of AGEPC was calculated automatically with a Shimadzu C-RIA Chromatopac computer.

Calibration curves for AGEPC

The calibration curves for quantification of C-16:0 and C-18:0 AGEPCs were prepared as described previously (7). However, no single C-18:1 AGEPC was available so, alternatively, we analyzed AGEPC, synthesized from human PMN, both by GLC and SIM at $(M-57)^+$. By calculating the ratio of peak areas of both C-16:0 and C-18:1 AGEPC, a tentative calibration factor for C-18:1 AGEPC relative to C-16:0 AGEPC was determined to be 1.1 ± 0.1 (n = 4) (26).

PAF bioassay

The PAF fraction, purified from stimulated platelets, was detected by its ability to aggregate washed rabbit platelets in an aggregometer (21) as an alternative procedure. The results are expressed in pmol of AGEPC, calculated from a calibration curve established with C-16:0 AGEPC.

Quantitation of phospholipid

Lipid phosphorus was determined by the method of Bartlett (27), after digestion of the lipid sample with 70% perchloric acid. Ether-linked phospholipid was determined as chloroform-soluble phosphorus after mild alkaline hydrolysis of the desired phospholipid fraction with 0.25 N KOH in 95% methanol for 60 min at room temperature (8). The 1-alkyl species was not further separated from the 1-alk-1'-enyl species, because this latter species is very low in choline glycerophospholipids from rabbit platelets (28).

RESULTS

Selected ion monitoring traces of PAF from ionophore-stimulated platelets

Representative SIM traces of PAF fractions from top-2/3 and bottom-1/3 platelets after stimulation with ionophore A23187 are shown in **Fig. 1**. In bottom-1/3 platelets, monitoring at m/z 415, 441, and 443 gave peaks that had the same retention times as standard C-16:0, C-18:1, and C-18:0 AGEPCs. On the other hand, the PAF fraction from top-2/3 platelets did not give significant peaks at m/z 415, 441, and 443 even when the injected amount was twice that of bottom-1/3 platelets, as shown by the peak areas at m/z 418 of internal standard in both preparations. Monitoring at m/z 117, due to acetic acid, also formed a major, sharp peak corresponding to C-16:0 AGEPC, and a small broad peak close to C-18:1 AGEPC in the bottom-1/3 platelet preparation. Whereas in the top-2/3 platelet preparation, monitoring at m/z 117 formed only a minor peak due to C-16:0 AGEPC, even though the injected amount was doubled (data not shown).

The PAF fraction from bottom-1/3 platelets without ionophore A23187 stimulation gave no peak. This was the case when the top-2/3 and bottom-1/3 platelets were stimulated with thrombin at a concentration of 2 units/ml of Tyrode's solution. These results demonstrated that AGEPC did not exist naturally in platelet preparations but was newly generated only in bottom-1/3 platelets by the stimulation of ionophore A23187.

Quantitation of PAF

Quantitation of PAF and the putative precursor of ether-linked choline glycerophospholipids is shown in **Table 1**. In the preparations of top-2/3 platelets, containing 0.03% white cells, 94 nmol of total phospholipid was

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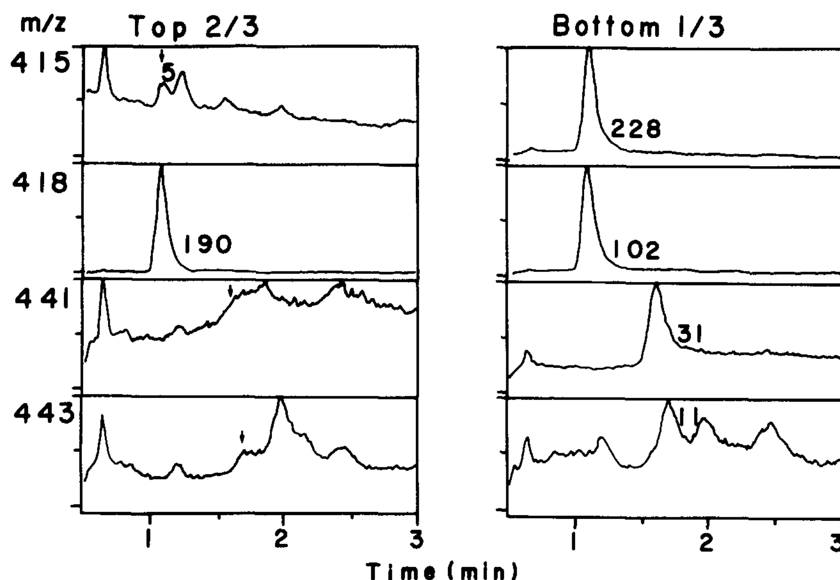

Fig. 1. Selected ion monitoring of PAF from ionophore-stimulated rabbit platelets. Two preparations of rabbit platelets, top-2/3 platelets (1.4 × 10^{10} platelets containing 0.02% leukocytes) and bottom-1/3 platelets (1.4 × 10^{10} platelets containing 0.99% leukocytes) were challenged with ionophore A23187. The fractions corresponding to PAF were detected by the rhodamine method, and purified and derivatized as described in the text. Peak areas at m/z 415 and 418 were 5 and 190, respectively, in top-2/3 platelets and those at m/z 415, 418, 441, and 443 were 228, 102, 31, and 11, respectively, in bottom-1/3 platelets, as shown on the right side of each peak. The diagram of each peak was drawn automatically to make the peak top to be 100% by computer; thus each diagram does not indicate the absolute amount of AGEPC. The arrows indicate retention times of each AGEPC.

recovered from 5 × 10^8 platelets and 34% of these phospholipids were choline glycerophospholipids; 13% of this fraction was an ether-linked choline phospholipid. Thus the putative precursor of PAF was calculated to be 4.2 nmol/5 × 10^8 platelets. From this preparation of platelets, there was no significant production of PAF after stimulation with the ionophore A23187.

In contrast, PAF was detected as AGEPC from the bottom-1/3 platelets which was 15 times higher in white cells than in the top-2/3 platelets. Namely, 119 nmol of total phospholipids was recovered from 5× 10^8 platelets and 5.0 nmol was an ether-linked choline glycerophospholipid. From this preparation, AGEPC molecules could be detected by SIM technique, mainly C-16:0 AGEPC, 8.3 ± 6.3 pmol, with a range of 2.6 to 21.4 pmol (n = 9), followed by small amounts of C-18:1 and C-18:0 species.

Next we examined the relationship between PAF production by platelets and their white cell content, using C-16:0 AGEPC, the major species of PAF. A good correlation was obtained (**Fig. 2**) and this was further confirmed by stimulating platelets prepared from buffy coat fraction, in which more white cells were included. Namely, 18.6 ± 10.4 pmol of C-16:0 AGEPC was generated from 5 × 10^8 platelets containing 0.67 ± 0.24% white cells (n = 3).

Characterization of white cells in platelet preparations

White cells in three different platelet preparations were characterized by microscopic examination. The white cells, counted from a smear of platelet preparation treated with May-Giemsa's stain, were mononuclear leukocytes, composed exclusively of lymphocytes and a small amount of monocytes. The percentages of monocytes in mononuclear leukocytes were 3.6 ± 2.8% in top-2/3 platelets

TABLE 1. Phospholipid and AGEPC contents of rabbit platelets challenged with ionophore A23187

	Top-2/3 (n = 8)[a]	Bottom-1/3 (n = 9)[a]
	nmol[b]	
Total phospholipid	93.8 ± 14.1	119.4 ± 20.4
Choline glycerophospholipids	31.6 ± 6.7	39.9 ± 6.4
Ether-linked choline glycerophospholipids	4.2 ± 2.0	5.0 ± 1.6
	pmol	
AGEPC		
C-16:0 AGEPC[c]	0.03 ± 0.07	8.3 ± 6.3
C-18:0 AGEPC[c]	0.06 ± 0.14	0.8 ± 0.7
C-18:1 AGEPC[d]	0.00 ± 0.00	2.7 ± 2.2

[a]Leukocytes included in platelets were 0.03 ± 0.02% in top-2/3 platelets and 0.48 ± 0.27% in bottom-1/3 platelets, respectively. Number of experiments in parentheses.

[b]Values are expressed as mean ± SD per 5 × 10^8 platelets.

[c]PAF fractions, 5 to 8 in top-2/3 platelets and 6 to 9 in bottom-1/3 platelets, were detected by the iodine method and the remaining were detected by the rhodamine method.

[d]Values were obtained from three platelet preparations using a calibration curve for C-16:0 AGEPC and multiplying by 1.1. PAF fractions were purified by a clean-up procedure with rhodamine 6G. Leukocytes included in platelets were 0.01 ± 0.01% in top-2/3 platelets and 0.73 ± 0.37% in bottom-1/3 platelets.

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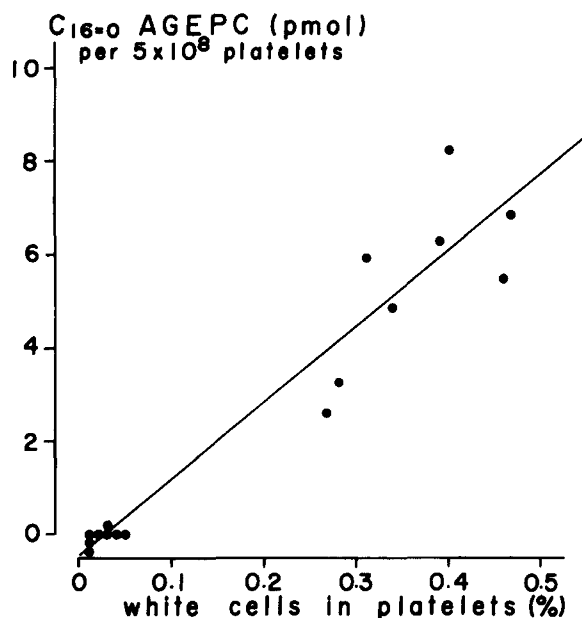

Fig. 2. The correlation on AGEPC production by platelets and number of leukocytes including platelets. The percentages of leukocytes in various platelet preparations are plotted on the x-axis and the amounts of C-16:0 AGEPC per 5 × 10^8 platelets are on the y-axis. The regression equation is Y = −0.38 ± 15.98X, with a correlation coefficient r = 0.95 (n = 16). Platelet preparations containing more than 0.5% white cells produced more C-16:0 AGEPC (0.85 ± 0.17% white cells, 21.6 ± 4.3 pmol, n = 4).

(n = 5), 5.3 ± 2.5% in bottom-1/3 platelets (n = 6), and 4.0 ± 1.0% platelets from the buffy coat (n = 3), respectively.

DISCUSSION

There is a discrepancy in the production of PAF by platelets (11, 16, 29). The most feasible reason for this discrepancy is that it arises from small amounts of white cells in platelet preparations. Indeed, it is impossible to eliminate completely the contaminating white cells from platelet preparations. These white cells are mononuclear leukocytes. Among mononuclear leukocytes, monocytes have been shown to produce substantial amounts of PAF under the stimulation of complement-activated baker's yeast as well as the ionophore A23187 (5, 30). In the discussion of the origin of platelet PAF, especially as a result of stimulation by ionophore A23187, it is essential to consider the possibility that PAF might come from monocytes in these preparations.

In view of this, Alam, Smith, and Silver (31) stimulated platelets, free of white cells, with 2.5 × 10^{-6} M ionophore A23187 at 37°C for 10 min and found, by bioassay, that rabbit platelets (5 × 10^8 cells) synthesized 2.99 ± 3.3 pmol of PAF (range 0.25–8.5 pmol). In contrast, using the SIM technique, we could not detect any significant AGEPC in rabbit platelets containing white cells less than 0.1%. This was also confirmed by an alternative analytical method, the platelet aggregatoin assay. In this latter assay, all PAF fractions, prepared from top-2/3 and bottom-1/3 platelets stimulated with thrombin and ionophore A23187, showed platelet aggregating activity. However, PAF fractions except bottom-1/3 platelets stimulated with ionophore A23187 were far less active. That is, PAF fractions from top-2/3 platelets stimulated with ionophore and with thrombin were 10 and 25 times less active than bottom-1/3 platelets stimulated with ionophore. The SIM technique, being specific for the AGEPC molecule, is 10–100 times less sensitive than the bioassay. Thus we could not detect AGEPC except from bottom-1/3 platelets stimulated with ionophore. The reasons for the differences between this study and that of Alam et al. (31) are not clear at present. There are differences in the methods of platelet preparation and activation which could have effects on the biosynthesis of AGEPC. Accordingly, it is impossible to compare results directly. However, it should be stressed again that AGEPC was generated by platelet preparation in proportion to the increasing amount of contaminating white cells. Moreover, thrombin, a specific stimulus of platelets, had little effect in generating AGEPC. In conclusion, it is suggested that the washed rabbit platelet is not a significant source of AGEPC as a result of cellular responses.

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