Fatty acid composition of diacyl, alkylacyl, and alkenylacyl phospholipids of control and arachidonate-depleted rat polymorphonuclear leukocytes

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Abstract Phospholipid fatty acid composition and phospholipid subclass distribution of control and arachidonate-depleted rat polymorphonuclear leukocytes (PMN) were compared. The 20:4-depleted PMN contained significantly higher amounts of 16:1, 18:1 and 20:3 (Δ5,8,11) and lower amounts of 18:2 and 20:4 than the phospholipids from control cells. Choline-containing glycerophospholipids (CGP) were the major phospholipids of both control and 20:4-depleted cells representing 34% and 37% of the total phospholipids, respectively. Significant amounts of ethanolamine-containing glycerophospholipids (EGP) (29% and 30%) and sphingolipids (20% and 18%) were also present in both cell types. Serine-containing glycerophospholipids (SGP) together with inositol-containing glycerophospholipids (IGP) constituted 16% and 13% of the phospholipids in control and 20:4-depleted cells, respectively. CGP from control cells had significantly higher amounts of 16:0 and 18:2 and lower amounts of 18:0 and 20:4 than EGP, whereas CGP from 20:4-depleted cells has higher amounts of 16:0 and 16:1 and lower amounts of 20:3 than EGP. Analysis of the subclass composition of CGP and EGP revealed that both control and 20:4-depleted cells contained significantly large amounts of alkylacyl-GPC and alkenylacyl-GPE. Small amounts of alkylacyl-GPE and alkenylacyl-GPC were also observed. The predominant fatty acyl residues found in the 1,2-diacyl-GPC, alkylacyl-GPC of control cells were 16:0, 18:0, 18:1, 18:2, and 20:4, while those of 20:4-depleted cells were 16:0, 16:1, 18:0, 18:1, and 20:3. More than 60% of CGP-bound 20:4 of control cells and about 70% of the CGP-bound 20:3 of 20:4-depleted cells were found in their alkylacyl species. The distribution of fatty acids in EGP subclasses was more or less similar to the corresponding CGP subclasses except that the alkenylacyl-GPE from control and 20:4-depleted cells had the higher concentration of 20:4 and 20:3, respectively. Thus, the alkylacyl-GPC and alkenylacyl-GPE from control cells contain 2–3 times more arachidonate than the corresponding diacyl species. The distribution of 20:3 in 20:4-depleted cells follows the pattern of 20:4 in control cells. These results also show that both control and 20:4-depleted cells have ample precursor substrates to support the synthesis of PAF (platelet activating factor). —**Ramesha, C. S., and W. C. Pickett.** Fatty acid composition of diacyl, alkylacyl, and alkenylacyl phospholipids of control and arachidonate-depleted rat polymorphonuclear leukocytes. *J. Lipid Res.* 1987. **28:** 326–331.

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Platelet activating factor (PAF) has long been associated with anaphylaxis and allergic responses (1). More recently, this factor has also been shown to mediate other aspects of acute inflammatory responses including vascular permeability, PMN activation, cell migration, and pain (2). The structure of PAF has now been identified as 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (3, 4) and several cell types including PMN have been shown to synthesize PAF in response to various stimuli (5–8). One of the mechanisms by which the stimulated PMN synthesize PAF is by a deacylation–reacylation pathway in which the 1-O-alkyl-2-acyl-GPC of the stimulated cell is hydrolyzed by phospholipase A_2 releasing long chain fatty acid and 1-O-alkyl-2-lyso-GPC (9, 10); the latter is acetylated by acetyltransferase to form PAF (11, 12).

An intriguing consequence of this biosynthetic mechanism is that 1-O-alkyl-20:4-GPC could serve as a common intermediate for both eicosanoids and PAF biosynthesis (13–15). Such an intermediate would be feasible with respect to enzymatic economy as well as providing the spatial and temporal relationship necessary to express the known synergism associated with PAF and arachidonate metabolites. This hypothesis is supported by the fact that *1*) 1-O-alkyl-2-20:4-GPC versus other molecular species of 1-O-alkyl-2-acyl-GPC has been shown to be a very efficient substrate for PAF biosynthesis (13); *2*) PMN hydrolyze PAF to lysoPAF and selectively reacylate the lysoPAF with 20:4 (14); *3*) the akylacyl-GPC of PMN

Abbreviations: PAF, platelet activating factor; PMN, polymorphonuclear leukocytes; CGP, choline-containing glycerophospholipids; EGP, ethanolamine-containing glycerophospholipids; IGP, inositol-containing glycerophospholipids; SGP, serine-containing glycerophospholipids; 20:4, arachidonic acid; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; lysoPAF, 1-O-alkyl-glycerol-3-phosphocholine; alkylacyl-, 1-O-alkyl-2-acyl-; alkenylacyl-, 1-O-alk-1-enyl-2-acyl-; BHT, butylated hydroxy toluene; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; PLA_2, phospholipase A_2.

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from different species is enriched with 20:4 (16–19); *4*) the PAF synthesis is inhibited in PMN depleted of their 20:4 (20).

In our attempt to understand the interrelationship of PAF and 20:4 metabolism, we studied both synthesis and metabolism of PAF in control and 20:4-depleted rat.PMN. Even though the synthesis of PAF is inhibited in 20:4-depleted PMN (20), the rate or the extent of metabolism of PAF to 1-O-alkyl-2-acyl-GPC is unaffected in these cells (21). Analysis of the molecular species of the 1-O-alkyl-2-acyl-GPC formed revealed that, in spite of a large amount of 16:0, 16:1, 18:0, 18:1, and 20:3 in 20:4-depleted PMN and 16:0, 18:0, 18:1, 18:2, and 20:4 in control PMN, these cells metabolized PAF into mainly dienoic and dienoic plus tetraenoic species of the alkyl-acyl-GPC, respectively (21). These observations indicated that the incorporation of dienoic and tetraenoic fatty acids into PAF is highly selective, just as the biosynthesis of PAF is highly selective. However, it is also possible that a certain class of phospholipid is rich in dienoic and tetraenoic fatty acids from which fatty acids are utilized for PAF inactivation, just as only a certain pool of phospholipid may be available for PAF biosynthesis. In order to test this possibility and to see whether the 20:4-depleted PMN have sufficient 1-O-alkyl-2-acyl-GPC to support PAF synthesis, we analyzed the phospholipid subclass and the fatty acid distribution in control and 20:4-depleted PMN.

EXPERIMENTAL PROCEDURES

Materials

Phospholipid standards, phospholipase C (*Bacillus cereus*), benzoyl bromide, 4-dimethyl aminopyridine, and primulin dye were purchased from Sigma Chemical Company, St. Louis, MO. Silicar-CC4 was from Mallincrodt, St. Louis, MO, and silica gel G TLC plates were from Analtech, Inc., Newark, DE. 1-O-Alkyl glycerol standards were from Serdary Research Laboratories, London, Ontario, Canada, and fatty acid methyl esters were from Supelco, Inc., Bellefonte, PA, Fat-free test diet, obtained from ICN Nutritional Biochemicals (Cleveland, OH), contained 20% casein, 55.4% sucrose, 15–16% alphacel, and 6.0 g/kg of a special fortified mixture that contained all needed vitamins and choline chloride. All chemicals were reagent grade or better.

Animals and diet

Male Wistar rats were used throughout the study. Weanling rats were fed either regular rat chow (control) or the fat-free test diet (20:4-depleted) ad libitum for 3–4 months before they were used for the experiments.

Isolation of cells

Peritoneal PMN from individual control rats or rats fed fat-free test diet were collected 14–16 hr after an intraperitoneal injection of 6 ml sodium caseinate (12% in saline). Cells were washed twice with heparinized saline and suspended in Tris buffer (0.02 M, pH 7.4) to a final density of 3×10^7 cells/ml.

Extraction and fractionation of lipids

Cellular lipids were extracted by the procedure of Bligh and Dyer (22) immediately after the isolation of cells. An aliquot (50 to 100 μg) of the total lipid was loaded onto a small (0.5×5 cm^2) Silicar-CC4 column; the neutral lipids were eluted with 4 ml of 5% methanol in chloroform, following which the phospholipids were eluted with 8 ml of methanol. The phospholipid fraction was concentrated to dryness under a stream of N_2, redissolved in 100–200 μl of chloroform containing 50 μg of BHT. Following the addition of known amounts of 17:0, fatty acid methyl esters from the phospholipids were prepared using methanolic HCl (21). Aliquots (100–200 μg) of total lipids were also spotted on silica gel G TLC plates and the individual phospholipids were separated using solvent system I (chloroform–methanol–water–acetic acid 65:25:3.5:0.5 (v/v). After drying the TLC plates under a stream of N_2, the phospholipid spots were visualized with primulin spray reagent (23) and the CGP and EGP fractions were extracted from the gel by the method of Bligh and Dyer (22). An aliquot of the PC and PE fraction was taken and mixed with a known amount of 17:0 and their fatty acid methyl esters were prepared (21). The rest of the CGP and EGP samples were taken for phospholipid subclass analysis.

Quantitation of phospholipids

An aliquot (100–200 μg) of the total lipid extracted was analyzed by TLC using solvent system I. After visualizing the phospholipid spots with primulin spray reagent (23), the bands were scraped and the lipid phosphorus was determined by the method of Rouser, Siakotos, and Fleischer (24).

Separation and quantitation of 1,2-diacyl, 1-alkyl-2-acyl, and 1-alkenyl-2-acyl-GPC and -GPE

The CGP and CPE fractions isolated by TLC as described above were digested with phospholipase C (25) and the resulting diglycerides were extracted and converted to benzoate derivatives (26). The benzoate derivatives were separated into diacyl, alkylacyl, and alkenylacyl species by TLC using benzene–hexane–ether 50:45:4 (v/v) as the solvent. Standard benzoate derivatives of diacyl, alkylacyl, and alkenylacyl phospholipids were also prepared and separated on TLC alongside samples. The spots were visualized under ultraviolet light, and scraped

directly into screw-top tubes containing known amounts of 17:0 and methylated with methanolic HCl (21). The fatty acid methyl esters were analyzed by GLC and each mole of fatty acid was taken to represent 0.5 mol of diacyl- or 1 mol of alkylacyl- or 1 mol of alkenylacyl-GPC or -GPE.

Analysis of fatty acid methyl esters by GLC

Analysis of the fatty acid methyl esters was performed on a Hewlett Packard 5890 gas chromatograph equipped with a 7673 A autosampler-injector and a 3393A integrator. The methyl esters were separated on a fused silica capillary column (15 m × 0.25 mm i.d.) with an RSL-950 bonded phase (Alltech, Deerfield, IL). Initial column temperature was 60°C which was increased to 160°C at a rate of 30°C/min and then to 240°C with a rate of 10°C, respectively. Aliquots of 1–2 μl of the samples were injected in splitless mode and the fatty acid methyl esters were identified using appropriate standards.

RESULTS AND DISCUSSION

Table 1 shows the phospholipid composition of PMN from control and 20:4-depleted rats. There is no significant difference in the phospholipid distribution between the two groups of cells; CGP is the predominant phospholipid followed by EGP. There are significant amounts of sphingolipid and SGP plus IGP in both the cell types. Similar distribution of CGP and EGP has been reported for other cell types (16–19). Even though the amounts of sphingolipid and PS + PI of rat PMN reported here are higher than those of circulating human PMN (18), the values are comparable to those of rabbit (16, 17) and guinea pig (19) peritoneal PMN.

The fatty acid composition of total phospholipid and of CGP and EGP is given in **Table 2.** Major fatty acids of the total phospholipids of control cells were 16:0, 18:0, 18:1, 18:2, and 20:4. Small but significant amounts of 16:1 and 22:6 were also present. The 20:4-depleted PMN had similar fatty acid composition except that they had comparatively higher amounts of 16:1 and 18:1 and lower amounts of 18:2 and 20:4 than control PMN. Further, these 20:4-depleted PMN were characterized by the presence of a significant proportion (14.6%) of eicosatrienoic acid (20:3, Δ5,8,11) which is derived from elongation of oleic acid. The total phospholipid fatty acid compositions of the control and 20:4-depleted PMN are comparable to those reported before (27). The fatty acid profile of CGP from control and 20:4-depleted cells closely resembles that of their total phospholipid. On the other hand, PE of control cells contained lower levels of 16:0 and 18:2 and higher levels of 18:0 and 20:4 and PE of 20:4-depleted cells contained lower levels of 16:0 and 16:1 and higher levels of 18:0 and 20:3 than their respective PC. There was 150% more 20:4 in EGP than in CGP of control cells and 200% more 20:3 in PE than in PC of 20:4-depleted cells.

TABLE 1. Phospholipid composition of control and 20:4-depleted PMN

	Wt. % of Total[a] (n = 13)	
Phospholipids	Control	20:4-Depleted
CGP	33.9 ± 1.4	37.4 ± 1.1
EGP	29.1 ± 1.1	30.6 ± 1.1
Sphingolipids	20.2 ± 0.9	18.8 ± 0.8
SGP + IGP	16.7 ± 0.8	13.2 ± 1.1

[a]Each determination is from individual samples of 2–3 × 10^7 PMN. The values are mean ± SEM and are calculated from the lipid phosphorus.

TABLE 2. Subclass composition of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of rat PMN

	CGP (n = 8)		EGP (n = 8)	
	Control	20:4-Depleted	Control	20:4-Depleted
Diacyl	56.7 ± 0.4[a]	60.6 ± 0.4	37.4 ± 1.5	41.0 ± 1.0
Alkylacyl	40.1 ± 0.7	36.6 ± 0.8	10.6 ± 1.0	9.6 ± 0.5
Alkenylacyl	3.2 ± 0.4	2.8 ± 1.1	50.8 ± 0.7	49.3 ± 0.6

[a]Values are mean percentages (weight %) ± SEM. Each determination is from a separate population of cells.

In order to determine whether control and 20:4-depleted PMN have sufficient alkylacyl-GPC to support PAF synthesis and to determine patterns of distribution of 20:4 and 20:3 in the phospholipid subclasses, diacyl-, alkylacyl-, and alkenylacyl-GPC and -GPE were separated and their fatty acid compositions were measured. Table 2 shows the distribution of diacyl-, alkylacyl-, and alkenylacyl-GPC and -GPE in control and 20:4-depleted cells. Both cell types had more or less identical distribution of CGP and EGP subclasses. A considerable portion of CGP from both cell types was the alkylacyl species. The high level (36.6%) of alkylacyl-GPC in 20:4-depleted cells indicate that the inhibition of PAF synthesis observed in these cells (20) was not due to lack of the 1-O-alkyl-2-acyl-GPC precursor substrates of PAF synthesis.

Alkenylacyl-GPC represented only a small fraction (<4%) of CGP from both control and 20:4-depleted cells. Nearly 50% of EGP of both control and 20:4-depleted cells was the alkenylacyl species. A large amount of diacyl and a small amount of alkylacyl species of PE were also present in both cell types. The subclass distributions of CGP and EGP of rat PMN are very similar to those reported for human (18), rabbit (16, 17), and guinea pig (19) PMN.

The fatty acyl distribution of diacyl- and alkylacyl-GPC is shown in **Table 3.** Since alkenylacyl-GPC represented only a small portion (<4%) of CGP and since the fatty acid distribution varied significantly from

TABLE 3. Fatty acid composition of CGP subclasses[a]

Fatty Acid	Diacyl (Position 1 + 2)		Alkylacyl (Position 2)	
	Control	20:4-Depleted	Control	20:4-Depleted
14:0	tr[b]	0.7 ± 0.3	1.7 ± 0.3	tr
15:0	0.6 ± 0.02	tr	1.5 ± 0.1	0.5 ± 0.05
16:0	28.2 ± 1.4	26.3 ± 1.4	30.3 ± 0.8	24.7 ± 0.4
16:1	2.1 ± 0.1	9.4 ± 0.5	3.8 ± 0.1	11.4 ± 0.5
18:0	21.3 ± 0.7	15.3 ± 0.4	5.4 ± 1.7	4.8 ± 0.4
18:1	18.1 ± 0.6	33.3 ± 0.8	9.6 ± 0.5	25.2 ± 0.4
18:2 Δ6,9		1.7 ± 0.3		1.2 ± 0.1
18:2 Δ9,12	13.7 ± 0.4	tr	14.1 ± 0.7	1.3 ± 0.4
18:3	tr		tr	
20:2		0.6 ± 0.04		tr
20:2	1.1 ± 0.5		tr	
20:3 Δ5,8,11		8.4 ± 0.6		19.9 ± 1.3
20:3 Δ6,9,12	0.8 ± 0.1		0.9 ± 0.1	
20:4	10.8 ± 0.5	1.5 ± 0.2	28.3 ± 1.4	5.4 ± 0.3
20:5	1.1 ± 0.5	0.9 ± 0.1	1.1 ± 0.1	1.0 ± 0.3
22:5	0.3 ± 0.05	tr	0.9 ± 0.1	1.0 ± 0.3
22:6	1.1 ± 0.1	tr	1.2 ± 0.1	0.8 ± 0.1

[a]Values are mean (weight %) ± SEM. Each determination is from a separate population of cells (n = 8–13).
[b]tr, Represents values < 0.5% of total.

one preparation to another, the fatty acyl composition of alkenylacyl-GPC is not given. The alkylacyl-GPC of control cells contained lower proportions of 18:0 and 18:1 and higher proportions of 20:4 than their diacyl-GPC. Similarly, the alkylacyl-GPC of 20:4-depleted cells had lower amounts of 18:0 and 18:1 and higher amounts of 20:3 than their diacyl-GPC. The amount of 20:4 and 20:3 in the alkylacyl-GPC of respective control and 20:4-depleted PMN is about 2.5 times higher than those in their respective diacyl-GPC. Even though the proportions of 20:4 in the alkylacyl-GPC of rat PMN reported here are higher than that in the alkylacyl-GPC of human PMN (18) and guinea pig PMN (19) and comparable to that in rabbit PMN (17), in all the cell types studied including the rat PMN, there is a general enrichment of the alkylacyl-GPC with arachidonate. However, following depletion of PMN with 20:4, the alkylacyl-GPC becomes enriched with 20:3. **Table 4** shows the fatty acyl distribution of PE subclasses. There is enrichment of 20:4 in the alkenylacyl-GPE control cells. Significant amounts of 20:4 are also present in diacyl- and alkylacyl-GPE. These fatty acid profiles of PE subclasses are similar to those reported for other cell types (16–19). Like the peritoneal PMN of guinea pig (19), alkylacyl- and alkenylacyl-GPE of rat PMN contain a significant amount of 18:0. The level of 18:0 has shown to be much lower in the alkylacyl- and alkenylacyl-GPE of circulating human PMN (18) and rabbit alveolar macrophages (17). In cells that have high proportions of 18:0, it is probably associated with unsaturated alkenyl and alkyl side chains of phospholipids. Further, like CGP, enrichment of alkenylacyl-GPE with 20:3 is also observed following 20:4-depletion.

Thus, it appears that enzyme(s) that selectively acylate PC and PE subclasses with 20:4 can also selectively utilize 20:3 in the absence of 20:4. This is in contrast to the observation that 20:4-containing rat PMN inactivate PAF and lysoPAF to alkylacyl-GPC having mainly dienoic and tetraenoic fatty acids in the *sn*-2 position, whereas in the absence of 20:4 and in the presence of 20:3 they metabolize PAF and lysoPAF to alkylacyl-GPC containing mainly dienoic fatty acids (21). One explanation for this is that the enzymes that metabolize PAF (and lysoPAF) to alkylacyl-GPC are different and have a stricter specificity for dienoic and tetraenoic fatty acids than those that are

TABLE 4. Fatty acid composition of EGP subclasses[a]

Fatty Acid	Diacyl (Position 1 + 2)		Alkylacyl (Position 2)		Alkenylacyl (Position 2)	
	Control	20:4-Depleted	Control	20:4-Depleted	Control	20:4-Depleted
14:0	tr[b]	tr	1.2 ± 0.6	tr	tr	
15:0	tr	tr	1.3 ± 0.5	tr	0.6 ± 0.1	tr
16:0	11.6 ± 0.7	26.7 ± 2.5	28.0 ± 1.9	12.2 ± 1.8	11.3 ± 1.3	11.9 ± 0.3
16:1	1.7 ± 0.1	4.1 ± 0.1	2.4 ± 0.2	3.0 ± 0.4	3.2 ± 1.2	3.1 ± 0.3
18:0	41.8 ± 2.3	32.8 ± 1.8	33.1 ± 3.0	32.8 ± 1.6	15.4 ± 2.1	17.7 ± 1.4
18:1	15.7 ± 0.4	17.4 ± 1.8	11.1 ± 0.1	26.2 ± 0.7	13.0 ± 0.5	27.2 ± 0.8
18:2		tr		0.7 ± 0.1	tr	1.0 ± 0.1
18:2	6.2 ± 0.3		4.6 ± 0.5		8.7 ± 0.4	
18:3					0.8 ± 0.1	
20:2				0.5 ± 0.1		tr
20:2	tr				tr	
20:3		13.2 ± 1.9		16.8 ± 1.1		25.2 ± 1.9
20:3	0.9 ± 0.1		1.2 ± 0.3		1.1 ± 0.1	
20:4	20.0 ± 0.6	3.5 ± 0.5	16.5 ± 1.5	3.5 ± 0.2	41.1 ± 3.5	7.8 ± 0.6
20:5	tr	1.9 ± 0.4	1.5 ± 0.8	1.8 ± 0.2	1.2 ± 0.1	2.8 ± 0.1
22:5	0.8 ± 0.1		1.5 ± 0.3		1.2 ± 0.1	tr
22:6	1.9 ± 0.1	tr	1.6 ± 0.2	1.0 ± 0.1	1.6 ± 0.1	0.7 ± 0.1

[a]Values are mean (weight %) ± SEM. Each determination is from an individual population of cells.
[b]tr, Represents values < 0.5% of total.

involved in the acylation of fatty acids to CGP and EGP and their subsequent remodeling. It is also likely that PAF inactivation and phospholipid acylation are compartmentalized.

While the present work does not provide direct evidence for either the inhibition of PAF biosynthesis in 20:4-depleted PMN (20) or for the failure to utilize trienoic fatty acid during PAF and lysoPAF metabolism by these cells (21), we have shown here that the inhibition of PAF synthesis in 20:4-depleted cells is not due to the lack of the needed alkylacyl-GPC precursor substrates and that both control and 20:4-depleted cells have sufficient 1-O-alkyl-2-acyl-GPC to support PAF synthesis by deacylation–reacylation pathway. It is also shown here that, as in other types of cells studied (16–19), the alkylacyl-GPC of rat PMN is enriched with 20:4 and, relevant to mechanisms of PAF synthesis, we find that 20:4 is replaced with an equal distribution among phospholipid subclasses by 20:3 in 20:4-depleted cells. If 1-O-alkyl-2-20:4-GPC serves as the preferred precursor substrate for both PAF and eicosanoids by the action of phospholipase A_2, it is likely that the inhibition of PAF synthesis in 20:4-depleted cells is due to lack of 1-O-alkyl-2-20:4-GPC for which 1-O-alkyl-2-20:3-GPC substrates are not a substitute. In view of our previous (20, 21) and present observations, such a hypothesis would suggest that PAF synthesis and 20:4 mobilization are tightly coupled and that phospholipase A_2 plays a strict and specific role in regulating both PAF and eicosanoid biosynthesis.

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