Polyunsaturated fatty acid accumulation in the lipids of cultured fibroblasts and smooth muscle cells

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Abstract The lipid content per cell of cells in tissue culture depended on the cell type. Fibroblasts derived from human neonatal foreskin contained less triglyceride and phospholipid and more cholesteryl ester than smooth muscle cells derived from guinea pig aorta. When fibroblasts and smooth muscle cells were challenged with 120 μM polyunsaturated fatty acid, the fibroblasts accumulated much less fatty acid than smooth muscle cells. Fibroblasts and smooth muscle cells challenged with either 8,11,14-eicosatrienoic acid, 5,8,11,14-eicosatetraenoic acid, or 7,10,13,16-docosatetraenoic acid accumulated the polyunsaturated fatty acid, its microsomal chain elongation products, and its extramicrosomal retroconversion products in cellular lipids. A new retroconversion sequence was observed, 8,11,14-eicosatrienoic acid to 6,9,12-octadecatrienoic acid. Microsomal desaturation products did not accumulate. The total fatty acid content of the phospholipid fraction was unchanged in cells challenged with a fatty acid. The polyunsaturated fatty acid and its derivatives exchanged with fatty acyl groups in the cellular phospholipid fraction. These fatty acyl groups were transferred to the triglyceride fraction and the total cellular content of each fatty acid was conserved. The total fatty acid content of the triglyceride fraction was markedly increased in cells challenged with a fatty acid. The polyunsaturated fatty acid and its derivatives accumulated in the triglyceride fraction. The triglyceride fraction contained an unusual triacyl derivative of the polyunsaturated fatty acid. These data support the hypothesis that microsomal fatty acyl-CoA intermediates are shunted into neutral lipid droplets when cells are stimulated to accumulate lipid.—**Gavino, V. C., J. S. Miller, J. M. Dillman, G. E. Milo, and D. G. Cornwell.** Polyunsaturated fatty acid accumulation in the lipids of cultured fibroblasts and smooth muscle cells. *J. Lipid Res.* 1981. **22:** 57–62.

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Triglycerides and cholesteryl esters accumulate as intracellular lipid droplets in several tissues (1–8). The triglyceride droplets in the renal medulla contain large amounts of 8,11,14-eicosatrienoic acid (8,11,14-20:3) and 7,10,13,16-docosatetraenoic acid (7,10,13,16-22:4) (3, 5, 6). The cholesteryl ester droplets in the adrenal (1, 2, 8) and the fatty streak lesion of the aorta (1, 2) also contain large amounts of 8,11,14-20:3 and 7,10,13,16-22:4. These fatty acids are synthesized from 9,12-octadecadienoic acid (9,12-18:2) by a microsomal desaturation-chain elongation pathway (9). The microsome is also the site of the acyl CoA synthetase (10) required in the microsomal biosynthesis of triglycerides and cholesteryl esters from acyl CoA intermediates. We have proposed (1) that this unusual fatty acid composition of triglyceride and cholesteryl ester droplets is characteristic of tissues in which microsomes are stimulated to shunt fatty acyl CoA intermediates into neutral lipid droplets.

In the present investigation, we examine our hypothesis (1) that fatty acyl-CoA intermediates are shunted into neutral lipid droplets during accelerated lipid accumulation. Smooth muscle cells and fibroblasts in culture were challenged to accumulate triglyceride droplets by the addition of specific polyunsaturated fatty acids to the medium (**Fig. 1**). Cellular lipids were then isolated and analyzed. If fatty acyl-CoA intermediates are shunted into lipid droplets, triglycerides should accumulate fatty acids characteristic both of microsomal desaturation-chain elongation (9) (Fig. 1) and of extramicrosomal retroconversion (11) (Fig. 1). Furthermore, these fatty acyl groups should exchange into the phospholipid fraction without a net increase in the total fatty acid content of this fraction (Fig. 1).

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; FBS, fetal bovine serum; unsaturated fatty acids are designated as follows: numerical position of each double bond-number of C atoms: number of double bonds.

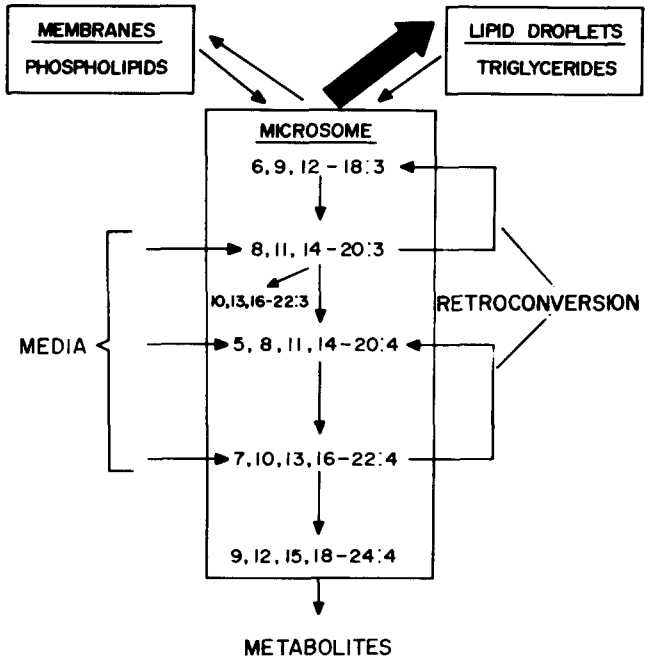

Fig. 1. Outline of polyunsaturated fatty acid metabolism in cultured cells showing fatty acid accumulation (heavy arrow) in neutral lipid and fatty acid equilibration in phospholipid when cells are challenged by the addition of fatty acid to the medium. The microsomal desaturation-chain elongation and extramicrosomal retroconversion products are outlined for each fatty acid. Microsomal lipoxygenase and microsomal cyclooxygenase products of the fatty acids are shown as metabolites in the diagram.

MATERIALS AND METHODS

Tissue culture

Primary cultures of fibroblasts were established from neonatal foreskin and primary cultures of smooth muscle cells were established from guinea pig aorta obtained from prepubertal males. The experimental details for our tissue culture procedures are provided elsewhere (12–15). Cells were used at passage numbers 3 to 5. Subcultures were grown to confluency (16) in 150 cm^2 Falcon Petri dishes containing 25 ml of medium. Medium used for fibroblast experiments was prepared from 1 × Eagle's minimum essential medium containing Hank's salts and 25 mM HEPES buffer (GIBCO, Grand Island, NY) supplemented with 50 μg per ml gentamycin sulfate (Schering, Kenilworth, NJ), 2 mM glutamine (GIBCO), 1 × nonessential amino acids (Microbiological Associates, Walkersville, MD), 1 mM sodium pyruvate (Microbiological Associates), 2.2 mg of sodium bicarbonate per ml, and 10% fetal bovine serum (Reheis, Phoenix, AZ, Lot P. 34112). Medium used for smooth muscle cell experiments contained in addition, 1 × essential amino acids and 1 × essential vitamins (Microbiological Associates), and 20% fetal bovine serum (Reheis, Lot. P. 34112) (12–15).

Fatty acids, purchased from Nu Chek (Elysian, MN), were checked for purity by GLC and were shown to be peroxide-free by TLC (14). Fatty acids were dissolved in 95% ethanol and diluted 1:10 and then 1:50 to a final 1:500 dilution with medium containing either 10% fetal bovine serum (fibroblasts) or 20% fetal bovine serum (smooth muscle cells). The final fatty acid concentration was 120 μM. Medium on confluent cells was replaced by 25 ml of medium containing the fatty acid. Control cells were re-fed with medium that did not contain the fatty acid supplement. Cells in some flasks were rinsed with 0.02% EDTA, detached with 0.1% trypsin, and counted in a hemocytometer. Cells in other flasks were detached mechanically after 24 hr and analyzed for lipids (15). The total cell count and the total DNA content per culture (17) for confluent cells did not vary significantly between control and treatment groups. Cells from two Petri dishes were pooled and their fatty acid content reported either as nmoles per culture or nmoles per 10^6 cells.

Lipid analyses

After the 24-hr incubation period, medium was decanted from the Petri dishes and the cells were rinsed three times with 10-ml portions of Dulbecco's phosphate-buffered saline. Cells were detached from the Petri dish and lipid extracted as previously described (15). Preliminary experiments with radioactive fatty acids showed that over 99% of label taken up by the cells was recovered in the total lipid extract. Triheptadecanoin, 200 μg, and cholesteryl heptadecanoate, 100 μg, were added as internal standards to the combined lipid extract from two Petri dishes. Non-lipid material was removed from this extract by chromatography on a Sephadex G-25 column (18). The purified lipid extract was then separated into neutral lipid and phospholipid fractions by chromatography on a silicic acid column (19). Methyl heptadecanoate, 170 μg, was then added as an internal standard to the phospholipid fraction.

Triglycerides and cholesteryl esters were isolated from the neutral lipid fraction by preparative TLC on PLK 5 precoated silica gel plates (Whatman, Clifton, NJ). Plates were predeveloped in chloroform–methanol 2:1 (v/v), then dried at 70°C for 2 hr before use. Neutral lipids were applied and the plates were developed with hexane–diethyl ether–acetic acid 85:15:1 (v/v). The triglyceride and cholesteryl ester bands, located with 2,7-dichlorofluorescein were scraped onto glass Buchner funnels of medium porosity and eluted with 150 ml of ether containing 0.001% 2,6-di-tert-butyl-4-methylphenol as an antioxidant. 2,7-Dichlorofluorescein was removed from the eluate by washing once with 20 ml of 0.5 N

ammonium hydroxide, then twice with 20-ml portions of water. The ether solution was dried over anhydrous sodium sulfate and evaporated to dryness in a Büchi rotary evaporator. The residue was dissolved in chloroform.

In some experiments, a part of the purified triglyceride fraction was separated into triglyceride molecular species by argentation TLC. A 0.5 mm thick silica gel H plate, impregnated with 8% silver nitrate, was developed with chloroform–methanol–acetic acid–water 93:6:0.5:0.5 (v/v). Bands were visualized with 2,7-dichlorofluorescein and triglycerides were eluted as previously described (20).

Phospholipids, triglycerides, and cholesteryl esters were converted to methyl esters by a published procedure (21). Retention time data were obtained by GLC using a Varian Aerograph model 1740 instrument equipped with a 6-ft, 2 mm i.d. glass column packed with 15% EGSS-X on 100/120 Gas Chrom P (Applied Science Laboratories, State College, PA). The column temperature was 180°C. Helium was the carrier gas and the flow-rate was 20 ml per min. Peaks were identified by an analysis of relative retention times and separation factors as described previously (2). Quantitative data were obtained by GLC using a Packard model 428 instrument equipped with a flame ionization detector and a 6-ft, 2 mm i.d. glass column packed with 10% Alltech CS-10 on 100/120 mesh Chrom WAW (Alltech Associates, Arlington Heights, IL). The column temperature was programmed from 167° to 205°C. Nitrogen was the carrier gas and the flow-rate was 20 ml per min. Peak areas were calculated electronically by a Spectra Physics System I computing integrator.

RESULTS

Fibroblasts

Fibroblasts were treated with 6000 nmoles of 7,10,13,16-22:4 per culture and were examined by dark-field microscopy (15). The cells contained lipid droplets in the perinuclear region of the cytoplasm. These lipid droplets were not as numerous as the lipid droplets found when smooth muscle cells were treated with the same amount of fatty acid (15).

Twenty-five percent (1530 nmoles) of the 7,10,13,16-22:4 added to the medium of fibroblast cultures was taken up in cellular lipids as the fatty acid and its proximate derivatives (chain elongation and retroconversion products in Fig. 1) (**Table 1**). Triglyceride, phospholipid, and cholesteryl ester fractions all contained unusually large amounts of 7,10,13,16-22:4

TABLE 1. Effect of adrenic acid on the lipid composition of fibroblasts

Fatty Acids	Triglyceride		Phospholipid		Cholesteryl Ester	
	Control	22:4[a]	Control	22:4[a]	Control	22:4[a]
			nmol/culture[b]			
Polyunsaturated fatty acids and conversion-retroconversion products						
6,9,12-18:3	n.d.[c]	3.0 ± 1	3,3[f]	3.3 ± 0.6	n.d.	n.d.
8,11,14-20:3	5.3 ± 0.6[d]	20 ± 5.2*↑[e]	27 ± 4.7	19 ± 0.6*↓	3 ± 1	2 ± 1
5,8,11,14-20:4	8.7 ± 3.5	117 ± 28**↑	217 ± 29	188 ± 30	3,10	7.3 ± 3.8
7,10,13,16-22:4	7.0 ± 2.6	1030 ± 215**↑	36 ± 10	352 ± 51**↑	3 ± 2	58 ± 28*↑
9,12,15,18-24:4	n.d.	35 ± 27↑	2	20 ± 17↑	n.d.	5.3 ± 3.5↑
Other fatty acids						
16:0	25 ± 16	92 ± 13**↑	329 ± 34	255 ± 23*↓	15 ± 8.1	13 ± 3.5
9-16:1	7.7 ± 3.5	28 ± 8.9*↑	62 ± 9	30 ± 4**↓	4,9	4,4
18:0	15 ± 6.1	40 ± 6.2**↑	269 ± 59	185 ± 33*↓	5,9	4 ± 1.7
9-18:1	61 ± 16	164 ± 36*↑	518 ± 84	292 ± 29*↓	31 ± 15	16 ± 3.8
9,12-18:2	7.7 ± 3	30 ± 8.7*↑	67 ± 17	35 ± 5.6*↓	3,6	2.7 ± 1.5
9,12,15-18:3	3	4.0 ± 1	13 ± 2.5	8.3 ± 1.1*↓	n.d.	n.d.
7,10,13,16,19-22:5	22 ± 3.6	34 ± 14	76 ± 15	37 ± 6.1*↓	5,9	3.3 ± 2.3
4,7,10,13,16,19-22:6	5.7 ± 4.2	9 ± 3	73 ± 7.6	30 ± 2.5**↓	3,4	2,1
Total fatty acid	166 ± 29	1600 ± 361**↑	1690 ± 271	1460 ± 190	75 ± 47	115 ± 42

[a] Cultures in confluency were incubated with fatty acid (6000 nmol/culture) in media containing 10% FBS for 24 h (see Methods).
[b] Cultures (cells from two Petri dishes) contained 119 ± 20.7 μg DNA (13.8 pg DNA/cell).
[c] Fatty acid not detectable (less than 1 nmol/culture).
[d] Data reported as mean ± S.D.
[e] Fatty acid treatment group differed from control group at $P < 0.05$* and $P < 0.005$** levels. Arrows indicate increase (↑) or decrease (↓) from control.
[f] Fatty acid not detectable in other members of the experimental group.

but only the triglyceride fraction increased significantly in total fatty acid content (Table 1). The polyunsaturated fatty acid and its proximate derivatives exchanged with other phospholipid fatty acids which then showed statistically significant decreases in concentration (Table 1). The fatty acids which decreased in the phospholipid fraction accumulated in significant amounts in the triglyceride fraction (Table 1).

Microsomal chain elongation was demonstrated by the appearance of 9,12,15,18-24:4 in triglyceride, phospholipid and cholesteryl ester fractions (Table 1). Fibroblast microsomes contained very little Δ4-desaturase since 4,7,10,13,16-22:5 was not found when cells were treated with 7,10,13,16-22:4 (Table 1). Extramicrosomal retroconversion was shown by the statistically significant accumulation of 5,8,11,14-20:4 in the triglyceride fraction (Table 1).

Smooth muscle cells

Smooth muscle cells were treated with 6000 nmoles per culture of either 8,11,14-20:3, 5,8,11,14-20:4 or 7,10,13,16-22:4. The mean fatty acid uptake for the three 9,12-18:2 metabolites and their proximate fatty acid derivatives, 83 ± 10%, was the same as the uptake of labeled 5,8,11,14-20:4, 81 ± 9%, obtained with smooth muscle cells in a previous study (15). Fatty acid uptake in smooth muscle cells correlated with the increased number of lipid droplets in these cells compared to fibroblasts (15).

Microsomal chain elongation was demonstrated by the net accumulation (difference between treatment and control for the sum of fatty acid in triglyceride and phospholipid fractions) of 10,13,16-22:3 (98 nmol) after treatment with 8,11,14-20:3, the net accumulation of 7,10,13,16-22:4 (772 nmol) after treatment with 5,8,11,14-20:4, and the net accumulation of 9,12,15,18-24:4 (141 nmol) after treatment with 7,10,13,16-22:4 (**Table 2**). Smooth muscle cell microsomes had little desaturase activity because 5,8,11,14-20:4 did not accumulate after treatment with 8,11,14-20:3 and 4,7,10,13,16-22:5 was not found after treatment with 7,10,13,16-22:4 (Table 2). Extramicrosomal retroconversion was confirmed by the net accumulation of 6,9,12-18:3 (114 nmol) after treatment with 8,11,14-20:3 and the net accumulation of 5,8,11,14-20:4 (392 nmol) after treatment with 7,10,13,16-22:4 (Table 2).

Smooth muscle cells, like fibroblasts, showed a significant increase in the total fatty acid content of

TABLE 2. Effect of polyunsaturated fatty acids on the lipid composition of smooth muscle cells

Fatty Acids	Triglyceride				Phospholipid			
	Control	8,11,14-20:3[a]	5,8,11,14-20:4[a]	7,10,13,16-22:4[a]	Control	8,11,14-20:3[a]	5,8,11,14-20:4[a]	7,10,13,16-22:4[a]
	nmol/culture[b]							
Polyunsaturated fatty acids and conversion-retroconversion products[c]								
6,9,12-18:3	n.d.[d]	98	31	18	n.d.	16	n.d.	n.d.
8,11,14-20:3	8 ± 2[e]	4560	65	60	42 ± 13	695	25	32
5,8,11,14-20:4	20 ± 4	256	3330	468	515 ± 125	341	773	459
10,13,16-22:3	n.d.	72	n.d.	n.d.	n.d.	26	n.d.	n.d.
7,10,13,16-22:4	20 ± 4	78	642	3960	42 ± 5	46	192	423
9,12,15,18-24:4	n.d.	n.d.	19	109	n.d.	n.d.	12	32
Other fatty acids[f]								
16:0	143 ± 12	325 ± 56**↑[g]			691 ± 143	608 ± 70		
9-16:1	29 ± 8	80 ± 18**↑			87 ± 9	46 ± 8**↓		
18:0	59 ± 13	104 ± 6**↑			503 ± 177	344 ± 29		
9-18:1	230 ± 19	524 ± 59**↑			1060 ± 331	540 ± 61*↓		
9,12-18:2	25 ± 2	104 ± 10**↑			171 ± 43	75 ± 11*↓		
7,10,13,16,19-22:5	44 ± 7	86 ± 2**↑			157 ± 31	79 ± 12**↓		
4,7,10,13,16,19-22:6	28 ± 4	70 ± 9**↑			142 ± 32	47 ± 13**↓		
Total fatty acid	607 ± 42	5880 ± 634**↑			3410 ± 881	2760 ± 257		

[a] Cultures in confluency were incubated with fatty acid (6000 nmol/culture) in media containing 20% FBS for 24 hours (see Methods).
[b] Cultures (cells from two Petri dishes) contained 143 ± 31 μg DNA (10.8 pg DNA/cell).
[c] Single values for each treatment with a polyunsaturated fatty acid.
[d] Fatty acid not detectable (less than 1 nmol/culture).
[e] Data reported as mean ± S.D.
[f] Mean values from three polyunsaturated fatty acid treatments.
[g] Fatty acid treatment group differed from control group at $P < 0.025$* and $P < 0.01$** levels. Arrows indicate increase (↑) or decrease (↓) from control.

the neutral lipid or triglyceride fraction (Table 2). The triglycerides were unusually rich in the polyunsaturated fatty acid and its proximate derivatives (Table 2). Argentation TLC separated a major triglyceride molecular species from cells treated with 5,8,11,14-20:4 which was almost pure triarachidonoyl glycerol since it contained 96.2% 5,8,11,14-20:4 and 3.8% 7,10,13,16-22:4.

The total fatty acid content of the phospholipid fraction did not increase even though there were very large increases in the polyunsaturated fatty acid and its proximate derivatives (Table 2). These fatty acids accumulated in the phospholipid fraction at the expense of other unsaturated fatty acids. Fatty acid uptake and distribution were examined by treating each related polyunsaturated fatty acid (a 9,12-18:2 metabolite) individually and by calculating mean values for other unrelated fatty acids (Table 2). This analysis showed that phospholipid 9-16:1, 9-18:1, 9,12-18:2, 7,10,13,16,19-22:5, and 4,7,10,13,16,19-22:6 all decreased significantly when cells were treated with polyunsaturated fatty acids (Table 2). These fatty acids all increased significantly in the triglyceride fraction (Table 2). The total amount of each fatty acid was conserved. Thus the amount of each fatty acid in a combined triglyceride-phospholipid fraction did not change when control and treatment groups were compared (**Table 3**). Finally, the cholesteryl ester fraction in smooth muscle cells was too small for fatty acid analysis.

TABLE 3. General conservation of unsaturated fatty acid[a] in smooth muscle cells treated with specific polyunsaturated fatty acids

Fatty Acid	Control	Treatment[b]
	nmol/culture	
9-16:1	120 ± 16[c]	120 ± 20
9-18:1	1300 ± 330	1100 ± 87
9,12-18:2	190 ± 42	180 ± 19
7,10,13,16,19-22:5	200 ± 32	170 ± 13
4,7,10,13,16,19-22:6	170 ± 30	120 ± 22

[a] Sum of fatty acid in triglyceride and phospholipid fractions (see Table 2).
[b] Cells treated with 6000 nmol polyunsaturated fatty acid per culture.
[c] Mean ± S.D. Control and treatment groups did not differ significantly.

TABLE 4. The amount of lipid per cell for fibroblasts and smooth muscle cells in control and treatment cell populations

Cell Population	Triglyceride	Phospholipid	Cholesteryl Esters
	nmol fatty acid/10^6 cells		
Fibroblasts[a]			
Control	19	193	8
Treatment	179	154	14
Smooth muscle cells[b]			
Control	45	259	
Treatment	443	205	

[a] Calculated from data in Table 1.
[b] Calculated from data in Table 2.

DISCUSSION

The lipid composition of cells in tissue culture depends both on the composition of the growth medium and the cell line. Human fibroblasts cultured in 20% fetal bovine serum (22) contained twice as much triglyceride and cholesteryl ester as fibroblasts cultured in 10% fetal bovine serum (Table 1). The increase in neutral lipid undoubtedly reflects the additional free fatty acid, calculated as 700 nmoles from free fatty acid data (14), supplied when 20% fetal bovine serum is used in place of 10% fetal bovine serum.

Human fibroblasts contain much less triglyceride and phospholipid than guinea pig smooth muscle cells (Tables 1 and 2). Total lipid values per cell for the two cell lines are compared in **Table 4.** Fibroblasts that are challenged with a polyunsaturated fatty acid take up a much smaller amount of this fatty acid as triglyceride than smooth muscle cells (Table 4). Nevertheless, fibroblasts and smooth muscle cells both show a marked increase in lipid droplets and the accumulation of neutral lipid when they are challenged with polyunsaturated fatty acids. These results confirm a number of studies which have shown that triglycerides accumulate when cells are challenged with excess free fatty acid (15, 23–25).

In the present investigation, we show that a polyunsaturated fatty acid added to the medium, its microsomal chain elongation products, and its extramicrosomal retroconversion products all accumulate in cellular phospholipids and cellular triglycerides. These fatty acids accumulate in phospholipids by exchanging with fatty acyl groups already present in this lipid fraction. These fatty acids accumulate in triglycerides through a net increase in the concentration of this lipid fraction. The data confirm classic lipid studies (26) which characterized cellular phospholipids as the *élément constant* and cellular triglycerides as the *élément variable*. The data support our hypothesis (1) that microsomal fatty acyl-CoA intermediates are shunted into neutral lipids that are unusually rich in chain elongation and retroconversion products such as 8,11,14-20:3 and 7,10,13,16-22:4 when excess lipid stimulates neutral lipid accumulation (Fig. 1). These studies show that precursor shunting into neutral lipid droplets may con-

trol fatty acid availability for the microsomal biosynthesis of hydroperoxides (lipoxygenase pathway) and endoperoxides (cyclooxygenase pathway).

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