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L Fibroblast Phospholipid Acyl Group Composition and Triacylglycerol Levels: Response to Continuous Fatty Acid Infusion (41091)

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Abstract. Two methods of administering fatty acids to cells in culture were compared for their effects on growth, phospholipid acyl group composition, and triacylglycerol formation. Solutions of fatty acid salts were delivered to suspension cultures of L fibroblasts either by infusion at a constant rate over 48 hr, or an amount of fatty acid equal to that infused was added as a single dose complexed to albumin at the start of the 48-hr incubation period. With an initial cell density of 2.5×10^5/ml, 5–18 μmole of Na oleate could be delivered by either method with no adverse effects on the cells. Linoleate (18:2) at the high end of this range, however, decreased culture growth by as much as 50% when the infusion method was employed and 15% when the fatty acid was added as a single dose. At low doses, 5 μmole, 18:2 had little effect on the growth rate. Over a 48-hr period infusion of 5 μmole of 18:2 caused the dienoic acyl group content of phospholipid to increase 22-fold, while the single-dose method increased it by a factor of 18. Time-course studies showed that the triacylglycerol content in the latter case increased to max levels from the 6th to the 24th hr at least 60 times greater than that of control cells not supplemented with fatty acid. The max increase in triacylglycerol above control levels during infusion of 18:2 was only 8-fold and occurred at 24 hr. The infusion method at low levels of 18:2 is at least as effective as the single-dose method in modifying phospholipid acyl group composition, but had the advantage of maintaining lower levels of triacylglycerol.

Mammalian cells cultured *in vitro* are a useful model system for the study of the role of membrane phospholipid acyl group composition in determining the biological (1–3) and physical–chemical (4, 5) properties of cell membranes. Typically, in such experiments, pure fatty acids differing in their chain length and/or degree of unsaturation, are added to the culture medium at zero time and the cells are harvested for testing and analysis after a suitable incubation period.

In many cell culture systems, where the medium contains little or no serum protein, the fatty acid is complexed to bovine serum albumin in order to decrease the toxic effects of these detergent-like molecules (4, 6, 7). The use of commercially available albumin preparations in such studies, however, may introduce unwanted artifacts. It has been shown, for example, that at the same concentration of protein, variations in the quality and quantity of lipid contaminants associated with different commercially available albumins have a marked effect upon the excretion of sterol by mouse L fibroblasts (8). It is also likely, in studies where fatty acids are added to the medium as an albumin complex that the fatty acids derived by hydrolysis of the lipid contaminants present in the albumin may compete with the added fatty acid for incorporation into cell lipids.

Regardless of whether the fatty acid salt is added alone or bound to albumin a rapid accumulation of cytoplasmic triacylglycerol follows if the preparation is added to the cells as a single dose at the start of an incubation (6, 9). Although this may be of little consequence to cells growing in suspension culture, shape changes accompanying the formation of triacylglycerol droplets within anchorage-dependent diploid cells may retard their progress through the cell cycle (10). Moreover large increases in cytoplasmic triacylglycerol may complicate the interpretation of data from experiments in which lipophilic probe molecules are used to obtain information on the physical state of cell membrane lipids (5, 11).

In the present study an alternative method of adding fatty acids to cells in culture was devised. Solutions of fatty acid salts without albumin were infused at a

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constant rate into suspension cultures of L fibroblasts over a 48-hr period, and techniques were applied to determine the effect of such treatment on cell growth, phospholipid acyl group composition, and triacylglycerol content. Results are compared with those from duplicate cultures to which the same amount of fatty acid, complexed to albumin, was added as a single dose at the start of a 48-hr incubation period.

Materials and Methods. *Cell Culture.* Suspension cultures of L fibroblasts were maintained at 37° in 50 ml of Waymouth's medium (MB752/1, Associated Biomedic Systems Inc., Buffalo, N.Y.) in a 125-ml Erlenmeyer flask equipped with a Teflon-lined screw cap. The medium was supplemented with Methocel, 15 Hz, 0.025% (Dow Chemical Co., Indianapolis, Ind.); $CaCl_2$, 19.6 mg/liter; and thymidine, 30 μM. Horse serum (Grand Island Biol. Co., Grand Island, N.Y.) delipidized with a mixture of ethanol:diethyl ether (3:1 v/v) (12) was added to the medium to a final concentration of 2.5%. Suspension cultures were agitated at 120 rpm on a Laboratory rotator Model G-2 (New Brunswick, Scientific Co., New Brunswick, N.J.). Stock cultures were routinely subcultured to maintain cell densities between 6×10^4 and 1×10^6 cells/ml; no antibiotic was present in the medium.

Preparation and administration of fatty acid salts. Oleic acid (18:1) and linoleic acid (18:2) were obtained from Applied Science, Inc. (State College, Pa.) and used without further purification.[1] The contents of a freshly opened vial were immediately dissolved in heptane (50 mg/ml) and stored in liquid nitrogen. Just prior to use the fatty acid solution was diluted to a convenient concentration with heptane, and washed free of lipid peroxides with 2 vol of a 3:1 (v/v) mixture of methanol:water (13). The washed heptane phase was dried in a stream of nitrogen gas; the residue was promptly dissolved in 0.12% NaOH, and sterilized by passage through a 0.22 μm Millipore filter (Millipore Corp., Bedford, Mass.). Fatty acid albumin complexes (4:1 M ratio) were prepared by the addition of the fatty acid salt solution to 100 ml of complete cell culture medium, containing albumin, which had been heated to 56° (14). After chilling to 4° in an ice bath, the mixture was sterilized by passage through a 0.22-μm Millipore filter. Appropriate amounts of these solutions were added as a single dose to the culture at the start of an incubation. For those experiments in which a solution of albumin-free fatty acid salt was infused into the culture for various periods of time, the sterile fatty acid preparation in 0.12% NaOH was delivered through sterilized Teflon tubing from a syringe mounted on a syringe pump (SAGE Model 355, Orion Research Inc., Cambridge, Mass.) at a rate of 9.1×10^{-2} ml/hr. The change in volume of culture medium after infusion was less than 10%. There was no perceptible change in the phenol red indicator present in the medium when the alkaline fatty acid salt was added to the culture by either method. Whether the fatty acid was added as a single dose or by infusion, the initial cell population densities were adjusted to approximately 2×10^5/ml at the start of the experiment.

Cell harvesting. Suspension cultures were centrifuged at 600g for 5 min at 4°, resuspended in half the original volume in a harvesting solution containing glucose, 5.5 mM; NaCl, 0.14 M; KCl, 9.4 mM; $NaHCO_3$, 1.8 mM; Na_2 EDTA, 0.5 mM; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), 10 mM; and NaOH, 5 mM; the final pH was 7.2; and the osmolarity was adjusted to 375 mosm/liter by addition of sucrose. An increase in osmolarity insured complete recovery of cells when their numbers were less than 5×10^6 (15, 16). The cells were pelleted a second time and then extracted with chloroform:methanol (2:1 v/v).

Extraction and separation of lipids. Details of these methods are published elsewhere (17, 18) and, therefore, are summarized only briefly below. Twenty-five micrograms of triheptadecanoin (Applied Science, Inc.) was added to the washed cell pellet which was then extracted three times

[1] Fatty acids are abbreviated as chain length:number of double bonds.

with a mixture of chloroform:methanol (2:1 v/v); the combined extracts were concentrated to 7.5 ml, and washed as described by Folch (19). A measured aliquot equivalent to the lipid extract from 5×10^5 cells was removed for a total lipid phosphorous determination (20); the remaining extract was used for separation of lipid classes by thin-layer chromatography.

The neutral lipid classes were separated from the phospholipids by thin-layer chromatography on 0.45-mm-thick layers of silica gel H impregnated with 0.11% ammonium sulfate (21). A mixture of hexane: diethyl ether:glacial acetic acid, 60:39:1, separated the total lipid in order of increasing R_f into the following classes: (i) phospholipid, (ii) sterol, (iii) diglyceride, (iv) fatty acid, (v) triacylglycerol, and (vi) sterol ester. The developed chromatogram was sprayed with a 0.1% aqueous solution of 8-anilino-1-naphthalenesulfonic acid (22) and the gel area containing triacylglycerol and phospholipid was scraped into screwcap tubes. Methyl esters of the glycerolipid acyl groups were prepared by alkaline methanolysis and then extracted into heptane (23). To separate the different methyl esters, an aliquot of this heptane solution was injected directly onto a 6-ft-long glass column packed with 10% SP-222-PS (Supelco Inc., Bellefonte, Pa.) which was mounted in a Varian Series 2440 gas chromatograph (Varian Assoc., Waltham, Mass.) equipped with a flame ionization detector and a Hewlett–Packard 3350A electronic integrator (Hewlett–Packard Corvallis, Oreg.). Fatty acid methyl esters were identified by comparing their retention times with those of commercial standards (Applied Science, Inc.). To quantitate the amount of triacylglycerol present, the area of methyl-heptadecanoate was compared with the area of all other fatty acid methyl esters.

Results. Starting at an initial population density of 2–2.5 $\times$ 10^5/ml, cells multiplied at control rates when the amount of 18:1 infused into the culture over 48 hr was equal to or less than 18.2 μmole (Table I). At higher levels clumping of cells was evident, and of those not clumped, 20% were unable to exclude trypan blue, suggesting that the increase in generation time was due at least in part to cell death.

At the lowest concentration of 18:1 tested, the 18:1 content of cell phospholipid rose by a factor of 1.16 above that of control cells (Table I). A threefold increase in the amount of 18:1 infused throughout 48 hr further elevated this figure, only slightly, to 1.23. Regardless of the quantity of 18:1 administered, most of the enhancement in 18:1 content was accounted for by a decrease in the 16:0 and 16:1 content of phospholipid. Since the amount of lipid-soluble phosphorus per 10^6 cells at all infusion rates was the same as control cells, the observed increase in phospholipid 18:1 content was not the result of accumulating surplus membrane in each cell.

A nonlinear elevation of cell triacylglycerol content was observed as the amount of fatty acid infused over 48 hr (Fig. 1) was increased. The greater than propor-

TABLE I. EFFECT OF INFUSING DIFFERENT AMOUNTS OF Na OLEATE ON GROWTH RATE AND PHOSPHOLIPID ACYL GROUP COMPOSITION OF L FIBROBLASTS[a]

μmole Na oleate infused per 48 hr	Doubling time (hr)	Fatty acid composition (%) (w/w)				
		16:0	16:1	18:0	18:1	18:2
0	19.1 ± 1.3	17.2 ± .6	3.1 ± .1	10.9 ± .8	67.0 ± 2.7	1.0 ± .2
5.2	18.7 ± 1.6	8.6 ± .9	2.1 ± .2	9.7 ± .5	77.9 ± .8	1.6 ± .3
10.5	18.7 ± 0.9	8.8 ± .4	2.0 ± .3	7.3 ± .6	79.4 ± 1.8	1.5 ± .4
18.2	21.1 ± 2.6	7.4 ± .4	1.5 ± .1	6.6 ± .3	82.9 ± .4	1.2 ± .2
23.0	25.8 ± 1.4	7.2 ± .5	1.5 ± .1	6.1 ± .2	83.7 ± .5	1.1 ± .3

[a] Suspension cultures of strain L fibroblasts (12.5×10^6 cells/flask) were infused with sodium oleate (18:1) at a constant rate for 48 hr. Cell numbers were measured and doubling times calculated. The phospholipid fatty acid methyl esters were separated and quantitated by gas chromatography. All data represent the mean ± SD of three to six determinations. For details see Materials and Methods.

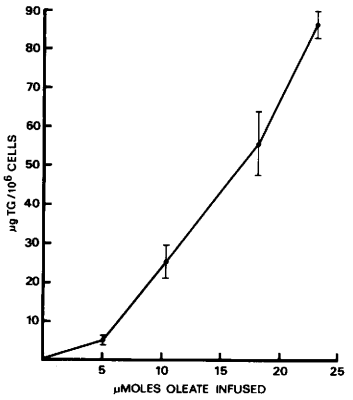

FIG. 1. Triacylglycerol (TG) content of L fibroblasts depends on the amount of Na oleate infused. Cultures of strain L fibroblasts (12.5×10^6 cells per culture) were infused for 48 hr with four different concentrations of 18:1. After addition of 25 μg triheptadecanoin as an internal standard, the triacylglycerol fraction was isolated. Fatty acid methyl esters were prepared, separated, and quantitated by gas chromatography. All data are the mean and SD of three to six determinations.

tional increase noted when 23 μmole of 18:1 was supplied may have resulted from the reutilization by living cells of lipids released from dead cells as well as a decrease in cell multiplication rate which in turn would diminish the rate at which triacylglycerol droplets could be distributed between daughter cells.

When a polyunsaturated fatty acid, 18:2, was similarly tested, a reduction in culture growth was apparent as the amount of fatty acid delivered over 48 hr was increased above 5 μmole. After infusion with 18 μmole of fatty acid, cell numbers were half those in control cultures (Fig. 2A); the infusion of 5 μmole of 18:2 caused only a slight reduction of culture growth (Fig. 2B). It is unlikely that peroxidation of lipid in the syringe and tubing played a significant role in causing this decrease in apparent growth rate for the following three reasons. (i) Based on gas chromatograph measurements, using heptadecanoic acid as an internal standard, the concentration of polyunsaturated fatty acid in the infusate at the start of the experiment and 48 hr later was identical, (ii) The trace amount of thiobarbituric acid-reactive material (24) produced in the infusate during that time was not affected by decreasing the temperature to 4°; and (iii) In one experiment *d*-α-tocopherol was mixed with the fatty acid prior to preparation of the salt (*d*-α-tocopherol:fatty acid = 1:5 w/w). No change in the amount of thiobarbituric acid-reactive material was noted. Using the more conventional method of supplying fatty acids as an albumin complex, little adverse effects on growth were noted even when larger quantities of 18:2 were supplied (Fig. 2A).

In the following experiments, 5 μmole of 18:2 was used to determine the extent to which the modification of phospholipid acyl group composition and triacylglycerol content was dependent upon the method of administering the fatty acid. In contrast to the effects observed with 18:1, much greater changes in phospholipid acyl group composition were induced in cells supplemented with 18:2 by either method (Table II). During a 48-hr incubation the 18:2 content increased by a factor of 13 when the exogenous fatty acid, complexed to albumin, was supplied as a single dose, and 16 when supplied as a continuous infusion. Regardless of the method employed, 25% of the exogenous fatty acid incorporated into phospholipid was elongated to a product tentatively identified as 20:2. This elongation product has been previously identified in a related cell line, the mouse LM cell (25).

The time course for modification of phospholipid acyl groups was influenced by the method of administering exogenous 18:2 (Fig. 3). Six hours after the start of an infusion, when only 12% of the total dose had actually been delivered, the enrichment in dienoic acyl group content was 70% of that observed when the total amount had been added at time zero. Equivalent modification was achieved at 24 hr using either method of administration, but by 48 hr the modification was greater in cells from cultures infused with 18:2 than it was in those receiving the same amount of fatty acid at zero time. The total phospholipid content of the cells was independent of the amount of fatty acid added, the extent of modification, and the method of addition.

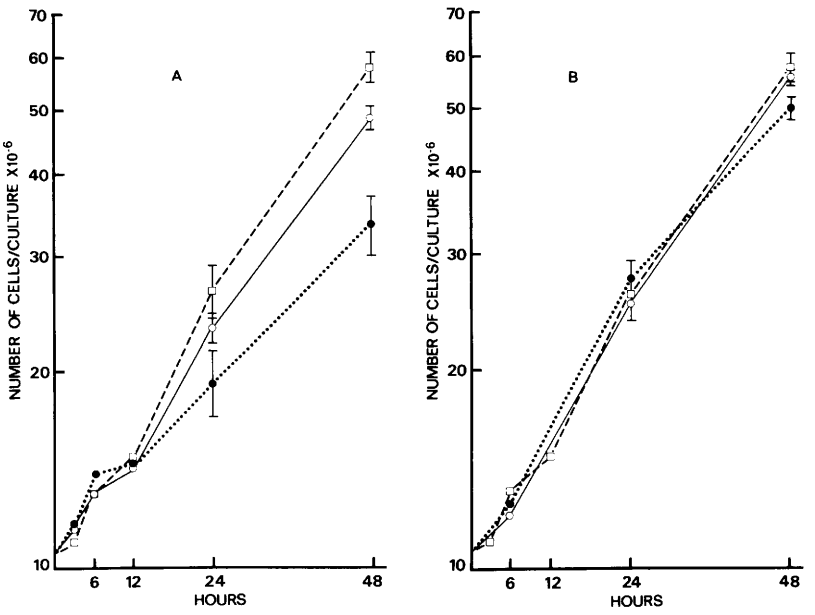

FIG. 2. Growth of L fibroblasts to which fatty acids were administered by two different methods. Cells (12.5×10^6 per culture) were grown in the presence of Na linoleate which was added either as an albumin complex at zero time, or by infusion at a constant rate throughout the incubation. At times indicated aliquots of culture were used to measure population density. All points are the mean ± SD of three to five determinations. (A) 18.2 μmole, (B) 5.2 μmole. Control, □--□; albumin-linoleate, ○——○; linoleate by infusion, ● • • ●.

Although by either method of addition the increase in cell triacylglycerol was similar after 48 hr, the time course for its accumulation was markedly dependent upon the method of adding 18:2 (Fig. 4). Within 6 hr after the addition of the fatty acid albumin complex as a single dose the triacylglycerol was elevated to values 60 times greater than those of control cells, while that of cells from cultures infused with albumin free 18:2 was elevated by a factor of 2–3. After 24 hr the triacylglycerol levels of cells incubated with fatty acid administered as a single dose was similar to that observed at 6 hr. The triacylglycerol content of cells from cultures infused with 18:2 had increased to 10 times that of control cultures but was still only one-third of that formed in cells receiving the single dose of fatty acid–albumin complex. By 48

TABLE II. CHANGES IN PHOSPHOLIPID ACYL GROUP COMPOSITION IN L CELLS AFTER 48-hr EXPOSURE TO THE SAME AMOUNT OF LINOLEATE ADMINISTERED BY TWO DIFFERENT METHODS[a]

Method of administration	Fatty acid composition (%) (w/w)					
	16:0	16:1	18:0	18:1	18:2	20:2
Single dose[b]	13.7 ± 1.2	—	17.5 ± 0.1	22.5 ± 1.3	32.8 ± 1.0	11.1 ± 0.5
Infusion[c]	13.5 ± 1.9	—	15.7 ± 0.8	17.4 ± 1.0	40.2 ± 0.6	12.2 ± 0.9
Control	16.6 ± 1.3	3.8 ± 0.2	11.9 ± 0.6	65.3 ± 3.5	2.4 ± 0.2	—

[a] Suspension cultures of L fibroblasts (12.5×10^6 cells/culture) received 5.2 μmole 18:2 either as a single dose at the start of a 48-hr incubation, or by infusion at a constant rate throughout the same time period. The phospholipid fatty acid methyl esters were separated and quantitated by gas chromatography. All values are the mean ± SD of three to six determinations.

[b] Sodium linoleate bound to albumin (4:1 *M* ratio).

[c] Sodium linoleate solution (no albumin).

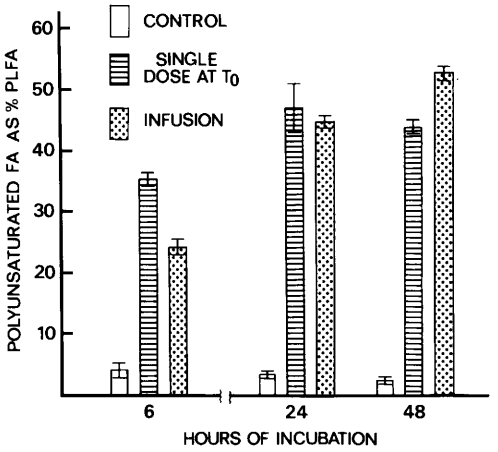

FIG. 3. Differences in time course for modification of L-cell phospholipid polyunsaturated fatty acid (FA) composition related to the method of adding 18:2 to cultures of L cells. Linoleic acid (5.2 μmole) was administered to L cells using the protocol described in legend to Fig. 2. The phospholipid fatty acid (PLFA) composition was determined by gas chromatography after transesterifying with alkaline methanol. The percentages 18:2 and 20:2 were combined and represented the polyunsaturated fatty acids. All data are the mean ± SD of three to six determinations.

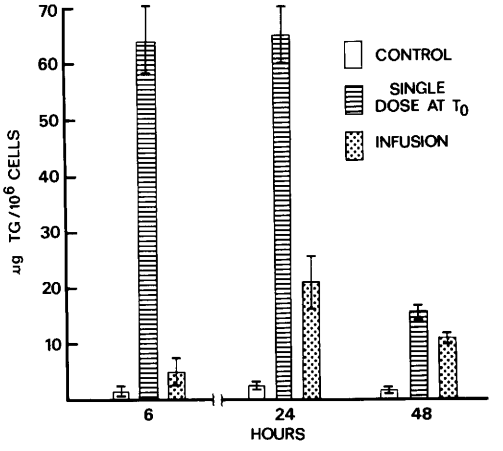

FIG. 4. Differences in triacylglycerol (TG) content of L cells receiving 5.2 μmole 18:2 by two methods. Protocol was similar to that described in Fig. 2 except that 25 μg triheptadecanoin was present during lipid extraction, and the total triacylglycerol fatty acid content was measured by gas chromatography of fatty acid methyl esters formed during transesterification.

hr method-related differences in triacylglycerol content were further decreased. Regardless of the method of administration, 18:2 constituted from 85 to 90% of the fatty acid in the triacylglycerol fraction, when the latter was elevated to its maximum value.

Discussion. Under the conditions of culture described in this study, the addition of 5–25 μmole of albumin-free fatty acid salt as a single dose to L fibroblasts causes inhibition of growth and cell death (unpublished observation). This problem can be greatly reduced if the fatty acid salt is complexed to albumin prior to use. However, when it is necessary to minimize the effects on cells of exogenous albumin or its lipid contaminants (8), infusing solutions of pure fatty acid salts is at least as good as alternative if not superior to the single addition of fatty acid–albumin complex. Although triglyceride levels were elevated to as much as 50 μg/10^6 cells (Fig. 1), solutions containing as much as 18 μmole of albumin-free sodium oleate could be delivered by constant infusion without affecting cell growth. For reasons which are at present unclear, the same quantity of sodium linoleate delivered under identical conditions caused a marked reduction in culture growth; 5 μmole of the latter could be infused into the cultures, however, without affecting cell multiplication. By infusing 5 μmole of 18:2 into cultures of L fibroblasts over 48 hr, the dienoic phospholipid acyl group content was increased from control values of 2 to 52% of total acyl groups, a value 20% greater than that achieved when the same amount of fatty acid was supplied as an albumin complex at the start of a 48-hr incubation. In addition to causing a greater change in phospholipid acyl group composition, the infusion method greatly reduces triacylglycerol accumulation throughout the incubation period. The maximum levels of triacylglycerol were 3–13 times less than those observed when the fatty acid albumin complex was added at zero time.

Although the amount of 18:2 which can be infused into the cultures without marked growth inhibition is 3.5 times less than that which can be added as a fatty acid albumin complex, the resulting difference in phospholipid acyl group composition is insignificant. In fact, increasing the quantity of

18:2 administered as the albumin complex at zero time to 18 μmole results in the same increase in phospholipid dienoic acyl group content as was obtained by infusion of 5 μmole of 18:2. The peak levels of triacylglycerol, however, increased by a factor of 3 (unpublished data).

With the high degree of phospholipid acyl group modification achieved and the relatively small amounts of triacylglycerol accumulated during the infusion of exogenous fatty acid into cells in culture, this method should be especially useful for studies on the utilization of exogenous fatty acids by diploid cells growing in monolayer culture. In fact, recent data have been published (26) which show that infusion of the fatty acid–albumin complex, rather than the pure salt, reduces the adverse effects of exogenous polyunsaturated fatty acids on the growth of human fibroblast finite strain IMR-90 *in vitro* noted previously (27).

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