Utilization of Polyunsaturated Fatty Acids by Human Diploid Cells Aging in vitro

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ABSTRACT

Cultures of human diploid cell strain IMR-90 were supplemented with γ-linolenic acid, 18:3ω6, by constant infusion over 72 hr. Cell growth was twice that observed when the same amount of fatty acid was supplied as a single dose at the start of a 72-hr incubation. Using the infusion method, growth of cells receiving monoenoic or polyenoic fatty acids was compared. The age of these cells in vitro was measured in terms of the culture mean population doubling level (PDL). Population doubling level refers to the mean number of doublings elapsed since establishment of a primary culture. At PDL from 24-53, the growth of cells from cultures supplemented with oleic acid was similar to that of noninfused cultures. Gamma linolenic acid, 18:3ω6, and to greater extent arachidonic acid, 20:4ω6, however, caused suppression of cell multiplication at PDL $\leqslant$ 32, but not at PDL $\geqslant$ 44. The polyunsaturated fatty acid (PUFA) levels in cell phospholipids were reduced by exogenous oleic acid to half that of nonsupplemented cells at all PDL tested. Conversely, the PUFA levels in phospholipids were elevated by a factor of 1.6 at all PDL when cultures were infused with 18:3ω6. Triglyceride levels at the end of 72 hr were similar, but much higher than the controls, regardless of the fatty acid supplied. Growth inhibition, modification of phospholipid acyl group content and triglyceride levels were not appreciably affected when the amount of monoenoic or polyenoic fatty acid infused into the cultures was doubled. The elongation of 18:3, as well as the distribution of 18:3 and its elongation products, between triglyceride and phospholipid, was dependent on whether the 18:3 was of the ω3 or ω6 family.

INTRODUCTION

Only recently have there been attempts to modify the phospholipid acyl group composition of normal diploid cells having a finite life span in vitro (1,2). The growth of these cells is apparently more sensitive to the addition of exogenous fatty acid salts than is that of transformed cell lines (1, and Lynch, unpublished observations). Although the reason for this difference is unclear, the addition of fatty acid salts as a single dose to transformed or nontransformed cells results in the rapid formation of cytoplasmic triacylglycerol (1-4). This accumulation may be great enough to disrupt cells or, at the very least, cause shape changes (3). Although this may be of little consequence to transformed cells, the shape of nontransformed cells may play an important role in regulating their passage through the cell cycle (4). In earlier studies, to reduce triglyceride accumulation in strain L-fibroblasts, a transformed cell line, the fatty acid salt solution was infused into suspension cultures over a 48-hr period (5). This resulted in modification of the phosphoglyceride fatty acid composition to at least as great an extent as that observed after the administration of the same amount of fatty acid as a single dose at the start of the 48-hr period while maintaining cellular triglyceride at much lower levels.

The purpose of this study was 3-fold: (a) to determine whether the previously reported inhibition of growth of normal human diploid lung fibroblasts (IMR-90) by oleic acid (1) or polyunsaturated fatty acids (PUFA) could be eliminated or reduced by infusing fatty acids into the culture; (b) to define conditions which maximally depress or enhance phosphoglyceride PUFA composition of diploid cells while minimizing triglyceride accumulation; and (c) to determine whether, as has been shown for endogenous biosynthesis of fatty acid (6), the age in vitro of the culture affects the use of exogenous fatty acids by IMR-90 cells.

MATERIALS AND METHODS

Cell Culture

Human female fetal lung fibroblasts, strain IMR-90, at population doubling level 10 (PDL 10) were purchased from the Human Aging Cell Repository of the Institute for Medical Research (Camden, NJ). Population doubling level refers to the mean number of cell doublings elapsed since establishment of a primary culture. This cell strain has been characterized thoroughly and is similar to the strain WI-38 employed in other studies of aging in vitro (7). In this laboratory, these cultures normally cease multiplying after 55 population doublings.

Stock cultures of cells were routinely propagated as monolayers in plastic tissue culture flasks with a 75-cm^2 growth area.

Monolayers were covered with 12 ml of Eagles Minimum Essential Medium (MEM) (Grand Island Biologicals, Inc., Grand Island, NY) supplemented to a level of 10% with nonheat-inactivated fetal calf serum (Grand Island Biologicals, Inc., Grand Island, NY). For buffering, the medium contained 5 mM N-2 hydroxethyl-piperazine-N'-2-ethane-sulfonic acid (HEPES), pH 7.3. All stock cultures were antibiotic-free and were maintained at 36.5 C in a humidified atmosphere of 5% CO_2 (New Brunswick CO-20 Incubator, New Brunswick, NJ). Cultures were periodically monitored for mycoplasma contamination (8).

When confluent, cells were detached using a releasing medium of 0.005% trypsin TRL (Worthington Biochemical Corp., Freehold, NJ) in an isotonic solution containing 0.5 mM NaEDTA, buffered with 5 mM HEPES. Sodium bicarbonate was added to the trypsin solution to a concentration of 7 mM just prior to use; the final pH was 7.2-7.3. Briefly, the growth medium was replaced with trypsin solution (4.5 ml) and the culture was incubated for 5 min at 37 C. The action of the trypsin was lessened by addition of fresh medium (8.5 ml) and the cells then detached by gentle tapping. Clumps of cells were disrupted by passing the cell suspension up and down 3 or 4 times using a 10-ml pipet with a narrow orifice. The suspension (3 ml) was transferred to fresh medium (9 ml, 1:4 split ratio) and 0.5 ml diluted with saline to obtain a cell count using a Coulter counter model ZBI (Coulter Electronic, Hialeah, FL). The remaining cell suspension was used to seed flasks (25 cm^2 growth area) for experimental purposes. Stock cultures at PDL < 40 attained confluency within 7 days of a 1:4 split, whereas those at higher passage levels required 10-14 days. Between subculture, the medium was changed every 3 or 4 days. The yield from confluent cultures under these conditions was between 20-30 x 10^6 cells/flask.

Preparation of Fatty Acid Salt Solution

Oleic (18:1ω9), α-linolenic (18:3ω3), γ-linolenic (18:3ω6) and arachidonic (20:4ω6) acids were purchased from Applied Science, Inc. (State College, PA) and used without further purification. Based on gas chromatography (GC) of their methyl esters, the purity of these fatty acids was judged to be at least 99%. (All fatty acids are denoted by number of carbon:number of double bonds; the figure following the ω symbol indicates the first C-atom, starting from the CH_3 end of the chain, at which a double bond is encountered). Sealed ampoules of these fatty acids were opened in a nitrogen atmosphere and the contents quickly diluted with sufficient heptane to yield a final concentration of ca. 50 mg/ml. An accurately measured volume was dried under N_2 in a clean tared vial to determine the fatty acid concentration more precisely. The stock solutions were then stored in liquid N_2. Before use, the required amount of stock solution was washed routinely with 2 vol of a mixture containing methanol/H_2O (3:1, v/v) to remove any trace quantities of lipid hydroperoxides which might be present (9). The washed heptane phase was then dried under N_2 and the fatty acid solution immediately dissolved in 0.12% NaOH; the salt was frozen in liquid N_2.

Preparation of MEM-fatty Acid Salt-albumin Complex

Fatty acid-free bovine serum albumin purchased from Miles Laboratory, Inc. (Elkhart, IN) was dissolved in Eagles MEM. The mixture was warmed rapidly to 56 C, the fatty acid salt added while stirring and the resulting solution chilled quickly to 4 C in an ice bath (6). Amounts of albumin and fatty acid were adjusted so that the molar ratio of fatty acid: albumin was equal to 4:1. These solutions were either stored overnight in liquid N_2 or used immediately. Just prior to use, the solution was passed through a sterile 0.22-μm millipore filter (Millipore Corp., Bedford, MA). Samples of the infusate were tested for malonaldehyde, a product of lipid peroxidation, at the start and finish of the 72-hr incubation period (10). The amounts measured were at the lower limit of detection with the method used. Furthermore, the amount of PUFA recovered by extraction from the infusate was determined by GC after methylation in the presence of heptadecanoic acid and agreed, within experimental error, with the expected value.

Addition of Fatty Acid-albumin Solution to Monolayer Culture

For experiments, cell suspensions from freshly trypsinized cultures were diluted with fresh medium and seeded into plastic flasks (25 μm^2 growth area), at an initial density of ca. 3.2 x $10^4/cm^2$ and left undisturbed for 3 days. At this time, when cells had increased to 4.8 x $10^4/cm^2$ and were in log phase, the medium was removed and fresh medium (2.0 ml) containing penicillin (10,000 units) and streptomycin (.013 g) was added. The MEM solution of fatty acid-albumin complex was then added to the system by one of 2 methods: (a) the total amount of fatty acid-albumin in a vol of 3.0 ml was added as a single dose at the start of a 72-hr incubation; or (b)

the same vol of complex was infused into the culture at a constant rate throughout the 72 hr. This was done using a constant infusion pump (Sage Instruments, Orion Research, Inc., Cambridge, MA) fitted with a 5.0-ml syringe which was connected by No. 22 teflon tubing to the experimental flask. Control cultures received the same vol of additional medium with the appropriate amount of albumin, but without fatty acid. All experimental flasks were placed on a platform rocker (Bellco Glass, Inc., Vineland, NJ) in the CO_2 incubator and gently rocked at 8 cycles/min for the duration of the experiment.

Harvesting Procedures

At the end of the infusion period, cells were trypsinized and shaken free of the plastic surface. The osmolarity of suspending medium was increased to 375 mOsm/ℓ by the addition of harvesting medium made hyperosmolar with sucrose. A hyperosmolar solution has been shown to increase the efficiency of recovery when small numbers of cells are harvested by centrifuging cell suspensions (11,12). The total vol of the suspension was adjusted to 7.0 ml and a 0.5-ml aliquot taken for cell count. The remainder was centrifuged for 5 min at 600 x g at 4 C; the pellet was washed once with trypsin-free releasing medium with an adjusted osmolarity of 375mOsm/ℓ and the cells were sedimented a second time.

Extraction and Separation of Lipids

A 25-μg aliquot of triheptadecanoin was added to the washed pellet, which was then extracted 3 times with $CHCl_3$/MeOH (2:1, v/v) and washed as described previously (13,14). After removing one-fourth of the lipid extract for total lipid phosphorous determination (15), the remainder was dried in a stream of N_2 and concentrated for thin layer chromatography (TLC).

Lipid samples were applied to a thin layer plate coated with a 0.4-mm-thick layer of silica gel impregnated with 0.11% ammonium sulfate (16). The solvent system, hexane/diethyl ether/acetic acid (60:39:1, v/v) separated the total lipid into the following classes in order of increasing R_f: (a) phospholipid; (b) sterol; (c) diglyceride; (d) fatty acid; (e) triglyceride; and (f) sterol esters. After development, the various lipid classes were visualized under ultraviolet (UV) illumination after spraying the plate with 0.1% aqueous solution of 8-anilino-1-napthalene sulfonic acid (ANS). Gel zones containing phospholipid and triglyceride were scraped into 15-ml screw-capped tubes and methylated in a solution of methanol/benzene (60:40, v/v) 1 N in NaOH (17). The resulting methyl esters were extracted into heptane, concentrated in a vol of 5-7 μl and an aliquot of 0.5-1.0 μl injected onto the gas chromatograph column.

Methyl esters of fatty acids were separated on a 6-ft glass column packed with 10% SP-2330-PS on 100/120 Chromosorb A AW (Supelco, Bellefonte, PA) mounted in a Varian Series 2440 gas chromatograph (Varian Associates, Waltham, MA). Areas under each peak were determined electronically by a Model 3380A electronic integrator (Hewlett Packard, Corvallis, OR). With the exception of 22:3ω6 and 20:4ω3, fatty acid methyl esters were identified by comparing their retention times with those of standards available commercially (Supelco). The 22:3ω6 and 20:4ω3 acids were only tentatively identified by comparing their retention times with those of either 18:3ω6 and 20:3ω6, or 20:3ω3 and 20:5ω3, respectively. Triglyceride levels were estimated by comparing the area under the methyl heptadecanoate peak with the sum of areas from the sample.

RESULTS

Cells which received the entire quantity of fatty acid at the start of an experiment (single-dose method) were covered by 5 ml of medium throughout the 72-hr incubation period. Those from cultures receiving fatty acid by infusion over 72 hr (infusion method), however, were initially covered by only 2.0 ml; the remaining 3.0 ml was added slowly during the 72-hr infusion period. Control cells receiving MEM-albumin under both sets of conditions grew to the same extent and had similar lipid compositions. All control cultures in the following experiments were therefore incubated without added fatty acid in a 5 ml vol of medium for the duration of the experiment. This allowed the limited number of spaces for syringes on the infusion pumps to be used for infusion of experimental cultures with fatty acids.

When added as a single dose at the start of a 72-hr incubation, 0.4μmol of 18:3ω6 reduced growth to 54% of control cultures (Table I). Infusing the same quantity of fatty acid into parallel cultures allowed cell growth to continue at 77% of control values. Using either method, the PUFA content in phospholipid and the amount of triglyceride/cell were elevated to a similar extent. As in the case of transformed cells (18), most of the increase in PUFA was at the expense of the monoenoic fatty acid fraction. The relatively high percentage of PUFA in the phospholipid of control cells most likely resulted from use of serum lipids (19). In

an effort to reduce this high PUFA background, fetal calf serum was delipidized as previously described (13,20) and replaced whole fetal calf serum in the medium. After 72 hr of incubation under these conditions, the percentage of PUFA in the phospholipid was reduced by 26%; however, culture growth was depressed by 50%. In all subsequent experiments, whole fetal calf serum was added to the culture medium.

Despite the use of the infusion method to reduce negative effects of fatty acid salts on culture multiplication, the growth of cells receiving PUFA was less than that of control cells or cells receiving oleic acid. Moreover, this residual effect on culture growth was apparent only at low PDL (Table II). At either high or low PDL, cells receiving 18:1 multiplied at the same rate as those from control cultures. The growth of cells from cultures infused with 20:4ω6, though, was markedly decreased when cells were at PDL $\leq$ 32 but was not reduced at PDL $\geq$ 44. The magnitude of the growth effect at low PDL was the same whether cells received 0.4 or 0.8 μmol of fatty acid. Similar, but less striking, effects were noted when 18:3ω6 was supplied.

TABLE I

Growth, Phospholipid Acyl Group Composition and Triglyceride Content of IMR-90 Cells[a] Supplemented with γ-Linolenic Acid by Two Methods[b]

Method of addition[c]	Amount of fatty acid (μmol)	Culture growth[d]	Phospholipid acyl groups (wt %)			Triglyceride (μg/10^6 cells)
			Saturated	Monoenoic	Polyenoic	
Control	---	3.34	30.1 ± 0.6	34.7 ± 1.0	35.2 ± 1.6	1.7 ± 0.5
Single dose	0.4	1.89	26.5 ± 4.2	17.9 ± 1.9	55.8 ± 6.4	10.2 ± 2.4
Infusion	0.4	2.57	28.1 ± 6.0	22.7 ± 3.8	49.2 ± 8.5	14.0 ± 0.2

[a]Cells had completed 32 population doublings and were in log phase at the start of the experiment.

[b]All data represent the mean ± SD of 3 separate experiments.

[c]Cultures received 0.4 μmol of 18:3ω6 either as a single dose at the beginning of a 72-hr incubation period or by infusion throughout that time.

[d]At the start of supplementation, cell numbers/flask = 1.26 x 10^6. The number of cells harvested after 72 hr ÷ the starting number = culture growth.

TABLE II

Growth Inhibition of IMR-90 Cells by Polyunsaturated Fatty Acids As a Function of Population Doubling Level[a]

Population doubling level	Amount of fatty acid (μmol[b])	Initial cell number[c] (cells x 10^{-6})	Increase in cell number of supplemented culture as a fraction of control cultures: Fatty acid supplied		
			18:1ω9	18:3ω6	20:4ω6
24	0.8	0.84	---	0.70[d]	0.55[e]
44	0.8	1.35	---	1.07[d]	0.98[e]
28	0.8	1.36	0.81[e]	---	0.50[e]
52	0.8	1.44	0.95[e]	---	1.09[e]
32	0.4	1.23	---	0.77[f]	0.62[f]
53	0.4	1.13	1.15[e]	1.18[e]	1.32[e]

[a]Population doubling level (PDL) is a measure of the lifespan of diploid cells in culture. In this laboratory, cells cease dividing and cell death is apparent after 52-55 population doublings in vitro.

[b]All fatty acids were supplied over a 72-hr period by infusion as described in Materials and Methods.

[c]This is the cell number 3 days after seeding the flasks, a time when the cells are in log phase.

[d]Results from a single experiment.

[e]Mean from 2 separate experiments.

[f]Mean from 3 separate experiments.

Four different PUFA were infused into log phase cultures of IMR-90 cells at an intermediate PDL and the resulting modification of phospholipid fatty acid compositions was compared with that produced when cells were incubated with 18:1 (Table III). In contrast to the results of a recent study (2) in which no change in phospholipid acyl group composition was reported after 18:1 supplementation, exogenous 18:1 suppressed phospholipid PUFA content of IMR-90 cells to a level half that observed in nonsupplemented control cells, whereas the monoenoic fatty acid content increased by a factor of 1.8. The mass of the triglyceride fraction increased above control values by a factor of 4.5 with its fatty acid composition reflecting closely that of the phospholipid (Table IV). Analysis of the lipid extract from fetal calf serum revealed the presence of 280 μg/ml of fatty acid, nearly all of which was in esterified form. The low level of triglyceride in control cells suggests, however, that this fatty acid is used slowly by the cells and does not affect the results obtained after fatty acid supplementation. The ω3 family of acids observed in the cells are present in the serum. No difference in fatty acid composition or triglyceride content was observed when cells were grown in the presence or absence of albumin.

Both the α and γ isomers of 18:3 were incorporated into cell glycerolipids (Tables III and IV); their fate within the cell, however, differed in 2 respects: (a) large increases in 20:3ω6 were observed in phospholipid triglyceride fractions of cells receiving 18:3ω6. In contrast, most of the 18:3ω3 was incorporated into cell lipids unchanged; (b) the distribution of 20:4 between phospholipid and triglyceride was dependent on whether it was of the ω3 or ω6 family, the ω6 being preferentially incorporated into phospholipid whereas the ω3 was sequestered primarily in the triglyceride fraction. No increases in the more highly polyunsaturated fatty acid 22:4 were observed in either the phospholipid or triglyceride frac-

TABLE III

Concentration and Acyl Group Composition of Phospholipids from IMR-90 Cells[a] Supplemented with Monenoic or Polyenoic Fatty Acids[b]

Phospholipid fatty acids	Phospholipid fatty acid composition (wt %)				
	Fatty acid infused[c]				
	0[d]	18:1	18:3ω6	18:3ω3	20:4ω6
Classes					
Saturated	28.8	24.2 ± 0.1	31.5 ± 0.3	32.5 ± 2.0	35.8 ± 0.8
Monoenoic	33.0	57.0 ± 1.3	19.0 ± 0.6	22.3 ± 2.0	23.4 ± 0.2
Polyenoic	38.2	18.9 ± 1.3	49.6 ± 0.9	45.3 ± 3.8	40.8 ± 0.6
Individual acids					
16:0	9.2	11.6 ± 0.4	15.2 ± 0.4	13.1 ± 2.9	18.4 ± 0.8
16:1	1.7	2.2 ± 0.2	2.0 ± 0.0	3.6 ± 2.4	3.2 ± 0.2
18:0	19.6	12.3 ± 0.1	17.1 ± 0.4	19.4 ± 1.0	17.4 ± 0.1
18:1	31.3	54.8 ± 1.1	17.0 ± 0.6	19.0 ± 0.3	20.2 ± 0.4
18:2	3.4	2.4 ± 0.3	2.9 ± 0.6	3.7 ± 0.4	3.6 ± 0.2
18:3ω6	0.6	tr[e]	8.6 ± 0.1	---	tr
20:3ω6	3.1	1.0 ± 0.2	17.2 ± 0.2	1.7 ± 0.1	2.0 ± 0.3
20:4ω6[f]	15.6	7.4 ± 0.2	10.0 ± 1.2	11.4 ± 1.2	21.3 ± 0.4
20:5ω3	---	---	---	3.9 ± 0.5	---
22:3ω6	---	---	2.2 ± 0.1	tr	---
22:4ω6	2.5	2.0 ± 0.2	2.9 ± 0.0	1.6 ± 0.2	10.2 ± 0.2
18:3ω3	---	---	---	13.3 ± 0.2	---
20:4ω3	---	---	---	2.3 ± 0.4	---
22:5ω3	5.2	2.8 ± 0.3	3.0 ± 0.1	4.3 ± 0.9	2.2 ± 0.1
22:6ω3	7.2	3.6 ± 0.5	3.0 ± 0.2	3.1 ± 0.7	1.1 ± 0.3
μg Lipid P per 10^6 cells	0.7	1.1 ± 0.0	1.1 ± 0.2	1.0 ± 0.1	1.4 ± 0.0

[a]At the start of infusion, cells/flask = 1.2 X 10^6; PDL = 32.

[b]Unless noted otherwise, all values are mean ± SD of 3 separate experiments.

[c]A total of 0.4 μmol of each fatty acid was infused into cultures over 72 hr.

[d]Values are mean of 2 experiments.

[e]tr = trace.

[f]20:4ω6 and 20:3ω3 have identical retention times on the packing material used.

tions. Exogenous arachidonic acid also increased the PUFA content of phospholipid, but to a smaller extent than that observed when either of the 18:3 isomers was used. Most of the increase in PUFA was in the 22:4 fraction; the 20:4 levels in phospholipid did not exceed 21% despite the presence of a triglyceride pool 8 times that of control cells with a 20:4ω6 content of 37%. Regardless of whether cells had completed 45% (PDL 24) or 95% (PDL 53) of their lifespan in vitro, the effects of fatty acid supplementation on the acyl group class distribution was similar in both the phospholipid and triglyceride fraction (Table V). Although not statistically significant, there was a trend toward a small increase in lipid phosphorous per 10^6 cells during supplementation with fatty acid. This may, in part, be related to a decrease in cell multiplication and an increase in cell size.

Further increases in the amount of PUFA delivered to cultures had little effect on glycerolipid acyl group composition. Doubling the amount of exogenous 18:1 increased the monoenoic acid content only slightly and caused no further suppression of the PUFA content in phospholipid (Table V). At the lower dose, 0.4 μmol of 18:1, the increase in 18:1 was compensated for by a decrease in PUFA with no change in saturated fatty acid content of the phospholipid, whereas at higher levels (0.8 μmol), both the saturated and PUFA fraction decreased in the phospholipid. Although there was no large increase in the amount of triglyceride per 10^6 cells at the higher level, the 18:1 content of that lipid class was elevated. There was little dose-dependent increase in phospholipid PUFA noted when the amount of exogenous PUFA was increased from 0.4 to 0.8 μmol, and as was reported in an earlier study with skin fibroblasts (2), arachidonic acid was least effective in eliciting a change in glycerolipid PUFA content. Doubling the quantity of 20:4 infused into the cultures caused the smallest change in the PUFA content of phospholipid despite the presence of

TABLE IV

Concentration and Acyl Group Composition of Triglyceride from IMR-90 Cells[a] Supplemented with Monoenoic or Polyenoic Fatty Acids[b]

Triglyceride fatty acids	Triglyceride fatty acid composition (wt %)				
	Fatty acid infused[c]				
	0[d]	18:1	18:3ω6	18:3ω3	20:4ω6
Classes					
Saturated	67.4	25.2 ± 3.8	17.0 ± 0.5	23.3 ± 5.6	21.8 ± 1.1
Monoenoic	7.9	55.0 ± 2.8	5.2 ± 0.7	9.9 ± 1.0	8.6 ± 2.8
Polyenoic	24.6	19.8 ± 6.6	80.6 ± 2.8	66.8 ± 4.9	69.6 ± 3.9
Individual acids					
16:0	35.2	13.6 ± 0.4	8.8 ± 1.5	14.6 ± 5.6	12.2 ± 2.0
16:1	tr[e]	1.4 ± 0.6	tr	tr	1.7 ± 1.3
18:0	32.2	11.6 ± 3.4	8.2 ± 1.0	8.5 ± 0.3	9.8 ± 1.8
18:1	7.9	54.2 ± 4.0	5.2 ± 0.7	9.9 ± 1.0	7.4 ± 0.9
18:2	2.4	2.2 ± 0.3	1.8 ± 0.5	4.2 ± 0.6	2.6 ± 0.3
18:3ω6	14.4	2.0 ± 1.5	16.3 ± 2.8	tr	3.9 ± 1.0
20:3ω6	5.9	6.4 ± 3.8	38.8 ± 1.6	2.0 ± 0.3	5.2 ± 0.5
20:4ω6[f]	tr	2.8 ± 0.2	6.7 ± 0.1	5.6 ± 0.1	37.4 ± 3.4
20:5ω3	3.2	3.2 ± 1.7	1.6 ± 1.1	7.5 ± 4.6	5.3 ± 4.5
22:3ω6	---	---	9.4 ± 1.3	---	tr
22:4ω6	---	2.0 ± 0.6	1.6 ± 0.3	---	13.2 ± 0.7
18:3ω3	---	---	---	32.5 ± 5.1	---
20:4ω3	---	---	---	10.7 ± 0.7	---
22:5ω3	---	1.4 ± 0.4	1.1 ± 0.4	4.5 ± 0.7	1.2 ± 0.0
22:6ω3	---	---	0.8 ± 0.1	---	---
μg Triglyceride per 10^6 cells	1.12	5.94 ± 1.0	7.6 ± 0.8	8.3 ± 0.4	8.6 ± 1.1

[a]At the start of infusion, cells/flask = 1.2 X 10^6; PDL = 32.

[b]Unless noted otherwise, all values are mean ± SD of 3 separate experiments.

[c]A total of 0.4 μmol of each fatty acid was infused into cultures over 72 hr.

[d]Values are mean of 2 experiments.

[e]tr = trace.

[f]20:4ω6 and 20:3ω3 have identical retention times on the packing material used.

TABLE V

Effects of Culture Age[a] and Amount of Fatty Acid Infused[b] on Glycerolipid PUFA Content and Triglyceride Levels of IMR-90 Cells[c]

Culture age	Fatty acid	Amount of fatty acid (μmol)	Fatty acid class composition (wt %)						
			Phospholipid			Triglyceride			Triglyceride (μg per 10^6 cells)
			Saturated	Monoene	Polyene	Saturated	Monoene	Polyene	
32[d]	—	—	28.8	33.0	38.2	67.4	7.9	24.6	1.1
32	18:1	0.4	24.2 ± 0.1	57.0 ± 1.3	18.9 ± 1.3	25.2 ± 3.8	55.0 ±2.8	19.8 ± 6.6	5.9 ± 1.4
32	18:3ω6	0.4	31.5 ± 0.3	19.0 ± 0.6	49.6 ± 0.9	17.5 ± 0.5	5.2 ± 0.7	80.6 ± 2.8	7.6 ± 0.6
32	20:4ω6	0.4	35.8 ± 0.8	23.4 ± 0.2	40.8 ± 0.6	21.8 ± 1.1	8.6 ± 2.8	69.6 ± 3.9	8.6 ± 1.1
53[d]	—	—	29.5	27.4	43.1	59.3	29.6	11.1	3.6
53	18:1	0.4	26.3 ± 3.4	49.6 ± 0.8	24.1 ± 4.2	16.4 ± 0.7	66.8 ± 1.3	17.1 ± 0.3	9.9 ± 0.4
53	18:3ω6	0.4	31.8 ± 5.0	20.2 ± 2.8	48.0 ± 7.8	12.4 ± 1.4	10.2 ± 0.1	77.7 ± 1.6	9.4 ± 1.2
53	20:4ω6	0.4	37.3 ± 4.1	25.0 ± 4.1	37.7 ± 3.0	13.0 ± 4.4	14.9 ± 6.5	72.0 ± 11.0	14.6 ± 0.5
24[e]	—	—	30.5	36.8	32.7	73.2	19.8	9.0	0.3
24[e]	18:3ω6	0.8	31.3	16.4	52.3	7.7	5.0	87.3	10.9
24	20:4ω6	0.8	38.2 ± 1	22.0 ± 0.9	39.8 ± 0.1	11.0 ± 1.9	7.2 ± 0.5	81.7 ± 2.4	11.4 ± 0.3
44[d]	—	—	31.9	38.1	30.0	72.0	17.2	10.8	1.7
44[e]	18:3ω6	0.8	35.0	17.4	47.6	18.1	3.9	78.0	7.9
44	20:4ω6	0.8	37.6 ± 2.0	23.4 ± 0.2	39.0 ± 2.2	13.2 ± 0.5	6.8 ± 0.4	80.8 ± 0.8	13.6 ± 0.5
28[d]	—	—	32.3	38.2	29.5	65.4	19.6	14.9	0.6
28	18:1	0.8	22.3 ± 0.1	61.5 ± 1.0	16.2 ± 1.4	12.2 ± 2.5	80.6 ± 1.8	7.3 ± 0.7	11.8 ± 0.2
52[e]	—	—	31.8	31.0	37.2	67.9	32.3	—	0.8
52	18:1	0.8	21.0 ± 0.1	57.8 ± 0.3	21.2 ± 0.4	11.5 ± 0.6	79.4	9.2 ± 0.6	7.9 ± 1.0

[a]Culture age is given as PDL, the mean number of population doublings since the culture was initiated.

[b]Either 0.4 or 0.8 μmol of fatty acid were infused into cultures. Initial cell number/flask = 0.8-1.2 x 10^6.

[c]Unless noted otherwise, all values are the mean ± SD of 3 separate experiments.

[d]Values are mean of 2 experiments.

[e]Values are from a single experiment.

sufficient exogenous fatty acid to elevate cell triglyceride levels by a factor of 10, while increasing the PUFA composition of that lipid class to twice that of the phospholipid. The fatty acid sequestered in triglyceride during infusion was available for phospholipid synthesis as shown by changing the medium at the end of the infusion period and allowing the cells to continue in culture for an additional 3 days. At the end of the infusion period and 3 days later, the percentage of PUFA in phospholipid was virtually identical despite an increase in cell number by a factor of 2 (Table VI). The amount of triglyceride/cell during this same 3-day interval decreased by a factor of 5 as its PUFA composition decreased from 75 to 35%.

DISCUSSION

The addition of oleic acid to fibroblasts from human skin (GM-10) adversely affected their growth in vitro (1). More recently, using diploid cells derived from primary cultures of human foreskin, the addition of PUFA, especially 20:4ω6, caused suppression of cell growth which was dependent on the concentration of exogenous fatty acid (2). In the same study, 18:1ω9 was without effect, and 18:3ω6 had an effect intermediate between that of 18:1 and 20:4. In this investigation, using amounts of fatty acid similar to those in the study just mentioned (2), the decrease in growth accompanying the addition of PUFA as a single dose at the start of a 72-hr incubation period was greatly reduced if, instead, the PUFA were administered at a constant rate throughout the 72 hr. The difference in triglyceride accumulation at the end of the incubation was negligible; observations by phase microscopy, however, showed that by using the infusion technique, the early accumulation of triglyceride droplets in the cytoplasm could be greatly reduced. The triglyceride, which was accumulated, served as a source of acyl groups, some of which were used for phospholipid synthesis when the cells were returned to the control medium after infusion. These results are in agreement with those obtained with a transformed line of fibroblasts (5).

Providing 20:4ω6 to IMR-90 cells by infusion resulted in inhibition of growth. Unlike the previous study (2), however, doubling the amount of fatty acid supplied did not further depress cell growth. These data suggest that the growth inhibition observed during infusion of PUFA into cultures of low PDL was independent of the nonspecific effects of the exogenous PUFA or of products arising from their autoxidation in the medium. Instead, the exogenous 20:4ω6 or its peroxidation products in the cell may effect a depression of cell growth through their role either as precursors (20) or activators (21), respectively, of prostaglandin biosynthesis. No greater growth inhibition was observed when arachidonic acid levels were doubled, which suggests that the pathways of prostaglandin biosynthesis were saturated or maximally active at the lower level of 20:4. That 18:3ω6 is less effective in suppressing growth may be explained if the rate of converting 18:3ω6 or 20:4ω6 by these cells is slow enough to limit the production of the relevant prostaglandin. In fact, the ratio of 18:3/20:4 increases as the amount of 18:3 supplied is increased, suggesting restricted conversion of 18:3 to 20:4. Regardless of the mechanism, the factors responsible for inhibiting growth of IMR-90 cells become markedly less significant at high PDL. The

TABLE VI

Utilization of Triglyceride Acyl Groups for Phospholipid Synthesis after Transfer of Cells to Fresh Medium Following Infusion with γ Linolenic Acid[a]

Fatty acid[b] infused	Time of harvest[c]		Fraction of acyl groups as polyenoic acids (wt %)		Triglyceride
	End of infusion	3 days post-infusion	Phospholipid	Triglyceride	μg per 10^6 cells
None	+	–	34.0 ± 0.3	–	3.1 ± 0.2
γ18:3	+	–	44.0 ± 0.3	75.9 ± 0.8	35.1 ± 3.5
None	–	+	34.2 ± 0.7	–	2.9 ± 0.3
γ18:3	–	+	44.4 ± 3.0	35.3 ± 4.4	7.2 ± 0.9

[a]All values are mean ± SD of 3 separate experiments.

[b]Cells at an initial density of 1.2 x 10^6/flask were infused for 72 hr with γ18:3 or no fatty acid.

[c]At the end of infusion, half of the cultures were harvested for analysis. Medium was changed in the remaining half, and the cultures incubated an additional 3 days prior to harvest.

reasons for this age-dependent difference are unknown.

Results from these studies suggest the operation of mechanisms which act to prevent PUFA levels from decreasing below 15% or increasing above 50%. Similar limits have been reported for human skin fibroblasts (2) and transformed mouse fibroblasts (18). Limitations on PUFA content may be dictated by the specificities of transacylating enzymes which determine the fatty acid composition in positions 1 and 2 of phospholipid (22). Even with large amounts of either 18:1 or PUFA available within the cell, rapid exchange reactions may prevent a net change greater than that observed in this study. It is unknown whether adaptive changes in the activity of transacylating enzymes are necessary for resistance to extreme changes in the PUFA composition of cell membrane phospholipids. The difference between $\omega 3$ and $\omega 6$ isomers of 18:3 with respect to their elongation and incorporation into phospholipids, as opposed to triglyceride, was not reported in an earlier study (2) in which the $\omega 3$ isomer was employed. Features of shape or packing density may be important in dictating the use of these fatty acids and their elongation products for membrane phospholipid synthesis.

For purposes of comparing the effects of phospholipid PUFA content on the structure and/or function of human diploid cells in culture, the best contrast is produced when cells from cultures infused with 18:3$\omega 6$ are compared with those from cultures infused with 18:1. The PUFA content of membrane lipids in these 2 groups of cells differs by a factor of 3-4. Both fatty acids are incorporated to the same extent into the glycerolipid of cells at high and low PDL and are therefore suitable for long-term studies on the effect of membrane unsaturation on diploid cell function throughout their lifespans in vitro.

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