Selective depletion of non-esterified fatty acids in fetal bovine serum-supplemented culture medium by human fibroblasts proliferating in low-density culture

W.J. Bettger, E.R. Driscoll, and S. Karmiol

Cellular Nutrition and Metabolism Research Unit, Department of Nutritional Sciences, College of Biological Science, University of Guelph, Guelph, Ontario, Canada

An experiment was designed to investigate the effect of human fibroblasts, proliferating at low density in culture and accumulating 20:3n-9 and 22:3n-9 into cellular phospholipids, on the concentration of polyunsaturated fatty acids (PUFA) in the culture medium. The cells caused a significant reduction in the total mass of non-esterified fatty acids but did not alter the mass or the fatty acid composition of medium cholesterol ester, triglyceride, or phospholipid. The cells caused a partial depletion of individual saturated fatty acids, monounsaturated fatty acids, and 18:2n-6 but effectively removed all the 20- and 22-carbon polyunsaturated, non-esterified fatty acids. The accumulation of n-9 PUFA in cellular phospholipids occurs under culture conditions where there is a significant depletion of bioavailable PUFA in the culture medium.

Keywords: non-esterified fatty acids; fibroblasts; cell culture

Introduction

Human skin fibroblasts accumulate 20:3(n-9) and 22:3(n-9) in cellular phospholipids when cultured in medium supplemented with low levels (≤0.4%) of fetal bovine serum (FBS)[1] or with 5% delipidized bovine serum.[2] Though the accumulation of (n-9) polyunsaturated fatty acids (PUFA) occurs in both quiescent and proliferating cell populations,[3] the effect is greater in multiplying cells and appears to be proportional to the rate of proliferation of the cell population.[3,4] Cultured human fibroblasts, like many other cell types, readily utilize lipids present in FBS-supplemented media to spare the biosynthesis of lipids for biological membranes;[4-6] however, fibroblasts cultured in medium containing very low levels of FBS may synthesize n-9 PUFA and incorporate them into membrane phospholipid because the mass of bioavailable forms of PUFA in the culture medium are depleted by the proliferating cells or the low serum culture environment significantly impairs the ability of the cells to absorb and metabolize exogenous PUFA containing lipid. This experiment was designed to determine whether human fibroblasts, proliferating at low cell density and accumulating n-9 PUFA in cellular phospholipids, cause a depletion of PUFA in non-esterified fatty acids, triglycerides, phospholipids, or cholesterol esters from an FBS-supplemented culture medium.

Dr. Karmiol's current address is the Department of Pathology, University of Michigan, Ann Arbor, MI 48109-0602, USA
Address reprint requests to Dr. Bettger at the Cellular Nutrition and Metabolism Research Unit, Department of Nutritional Sciences, College of Biological Science, University of Guelph, Guelph, Ontario, N1G 2W1, Canada.
Received June 11, 1991; accepted October 2, 1991.

Materials and methods

Cells and culture conditions

Human fibroblasts (HF-2) were obtained from a primary culture of newborn foreskin, passaged in cell culture, and stored frozen in liquid nitrogen as previously described.[1,7] Prior to assay, the cells, which had been cultured in MCDB 110[1,8] plus 10% FBS, were gradually weaned off the serum supplement.[1] The cells were removed from the plate with a

cold temperature trypsinization technique[1] and plated into poly-D-lysine-coated[1] culture plates containing 5 mL of MCDB 110 and 0.4% vol/vol of FBS (Flow Lab Inc., McLean, VA, USA lot #291-050) at 37° C. The cells, population doubling number 15-20, were plated at a density of 200 cells/60 mm dish and were cultured for 10 days at 37° C with >95% humidity.

Lipid analysis

After the 10-day incubation, the tissue culture plates were cooled on ice, the medium collected, then the cells were gently rinsed three times with solution A (30 mmol/L HEPES-NaOH [pH 7.6], 10 mmol/L glucose, 130 mmol/L NaCl, 1.0 mmol/L Na_2HPO_4, and 3 mmol/L KCl) at 4° C. The wash solutions and the tissue culture medium were combined for lipid analysis; cells were harvested for lipid analysis as previously described.[1] Lipids were extracted from the cells and the culture medium by the method of Bligh and Dyer.[9] Total phospholipids and individual neutral lipid classes were isolated by thin-layer chromatography using a system of heptane/isopropyl ether/acetic acid (60:40:3, vol/vol/vol).[10] Fatty acid methyl esters (FAMEs) were produced by incubation in 6% H_2SO_4 in methanol at 78° C for 2 hr.[11] The FAMEs were analyzed by gas-liquid chromatography and identified as previously described.[1,4] Culture medium MCDB 110 does not contain any lipid by formulation; analysis of MCDB 110 prepared in this laboratory using purified chemicals and water[1] had undetectable levels of PUFA in non-esterified fatty acids, phospholipids, cholesterol esters, and triglycerides.

Statistical analysis

All data comparing the fatty acid composition of culture medium without cells to culture medium plus cells were analyzed by the Student *t* test.

Results and discussion

In experimental trials 1 and 2, the fibroblasts cultured in MCDB 110 plus 0.4% FBS accumulated 20:3n-9 and 22:3n-9 in cellular phospholipids, indicative of an inadequate supply of a bioavailable n-6 and n-3 PUFA[1,3,4] *(Table 1)*. In both experimental trials, the cells depleted the medium of detectable levels of n-3 and n-6 non-esterified PUFA, except for small amounts of remaining linoleate *(Tables 2 and 3)*. The depletion of 20 and 22 carbon n-6 and n-3 PUFA from the medium by growing cells strongly suggests that a decrease of bioavailable PUFA over time is a causative factor in the synthesis and accumulation of cellular n-9 PUFA. Whether the low serum culture environment also impairs the rate of uptake of fatty acids or impairs the ability of the cells to incorporate n-6 or n-3 PUFA into cellular phospholipids requires further investigation. The cultured HF-2 cells caused a similar degree of depletion of n-6 and n-3 PUFA from the non-esterified fatty acid pool in experimental trials 1 and 2 but had significantly different 20:3n-9:20:4n-6 and 22:3n-9:22:4n-6 ratios in cellular phospholipids in the two trials.

The cells in culture selectively decreased the non-esterified pool of fatty acids; the concentration *(Table 2)* and the fatty acid composition *(Table 4)* of triglycerides, phospholipids, and cholesterol esters were unaffected *(Table 2)*. It is clear that this effect was not specific for PUFA; the total amount of loss of fatty acids was in the following order: saturates > monounsaturates > polyunsaturates. However, only 20- and 22- carbon PUFA were completely depleted from the medium; these experiments did not ascertain whether these PUFA were more rapidly removed from the culture medium than saturated and monounsaturated fatty acids. The content of non-esterified 14:1 fatty acid was significantly increased in the medium, suggesting a leakage or secretion of some fatty acids from the cultured fibroblasts into the culture medium. The lack of an increase in the concentration of 20:3n-9 in medium phospholipids *(Table 4)* suggests that the extent of exchange of 20:3n-9–phospholipid between cells and medium lipoprotein is negligible under these conditions. The results of our experiment differ from results described by Daniel et al.[12] for cultured rat embryo fibroblasts. Non-transformed rat embryo fibroblasts

Table 1 Phospholipid fatty acid composition of human skin fibroblasts cultured in MCDB 110 plus 0.4% fetal bovine serum

Fatty acids	Fatty acid composition of cells[a,b]
	mol%
Saturates	
14:0	3.5 ± 0.2
16:0	23.3 ± 0.7
18:0	16.2 ± 2.4
Monounsaturates	
14:1	0.2 ± 0.1
16:1n-9[c]	6.5 ± 0.3
16:1n-7	8.3 ± 0.4
18:1n-9	19.7 ± 0.5
18:1n-7[c]	7.7 ± 0.2
20:1n-9	0.3 ± 0.1
Polyunsaturates	
18:2n-9[c]	1.2 ± 0.1
18:2n-7[c]	2.1 ± 0.2
18:2n-6	0.6 ± 0.1
20:3n-9	3.7 ± 0.3
20:3n-6	0.5 ± 0.1
20:4n-6	2.5 ± 0.3
22:3n-9	1.7 ± 0.2
22:4n-6	0.5 ± 0.1
22:5n-3	0.9 ± 0.1
22:6n-3	1.0 ± 0.2
20:3n-9:20:4n-6	1.8 ± 0.3
22:3n-9:22:4n-6	3.9 ± 0.7

[a]The fatty acid composition of human skin fibroblasts prepared for low-density growth assays in this laboratory have been previously reported[4] to have a 20:3n-9:20:4n-6 ratio of 0.11 ± 0.02 and a 22:3n-9:22:4n-6 ratio of 0.33 ± 0.12 (n = 4) in total cellular phospholipids at time zero. HF-2 of fibroblasts cultured with medium supplemented with 10.0% fetal bovine serum had a 20:3n-9:20:4n-6 ratio of 0.03 ± 0.01 and a 22:3n-9:22:4n-6 ratio of 0.04 ± 0.01 (n = 5).
[b]Mean ± SEM, n = 8. Data from experimental trials 1 and 2 were combined. Each independent trial consists of n = 4 replicates of 15 flasks of cells.
[c]Tentatively identified. FAMEs were separated by the number of double bonds by argentation chromatography prior to gas chromatographic analysis; a modified equivalent chain length calculation was used subsequently to identify peaks without standards.[1,4]

Table 2 Effect of cell growth on the lipid composition of medium MCDB 110 plus 0.4% fetal bovine serum

Form of fatty acids	Concentration of fatty acids*	
	Culture medium	Culture medium + cells
	nmoL/75 mL	
Trial #1†		
Non-esterified fatty acid	55.32 ± 2.18^{A}	21.16 ± 4.20^{B}
Trial #2‡		
Non-esterified fatty acid	41.05 ± 4.22^{A}	15.73 ± 3.42^{B}
Phospholipid	71.05 ± 2.80	64.20 ± 2.71
Cholesterol ester	111.25 ± 1.18	103.80 ± 1.40
Triglyceride	16.30 ± 3.96	18.54 ± 4.10

*Mean ± SEM, n = 4 replicates of 15 flasks of cells in each trial. Values having a different superscript are significantly different ($P < 0.05$) by a Student t test.
†The triene:tetraene ratio of the cells after the 10 day growth period in trial #1 was 20:3n-9:20:4n-6, 2.65 ± 0.06 and 22:3n-9:22:4n-6, 4.98 ± 0.82; n = 4 replicates of 15 flasks of cells.
‡The triene:tetraene ratio of the cells after the 10 day growth period in trial #2 was 20:3n-9:20:4n-6, 0.9 ± 0.1 and 22:3n-9:22:4n-6, 2.34 ± 0.30; n = 4 replicates of 15 flasks of cells.

Table 3 Effect of cell growth on the composition of individual non-esterified fatty acids in medium MCDB 110 plus 0.4% fetal bovine serum

Fatty acids	Concentration of fatty acids*,†	
	Culture medium	Culture medium + cells
	nmoL/75 mL	
Saturates	28.65 ± 1.90^{A}	12.60 ± 1.62^{B}
14:0	1.63 ± 0.19	1.48 ± 0.27
16:0	15.79 ± 0.92^{A}	6.87 ± 0.97^{B}
18:0	11.23 ± 0.82^{A}	4.25 ± 0.46^{B}
Monounsaturates	12.71 ± 0.91^{A}	5.47 ± 1.09^{B}
14:1	0.07 ± 0.01^{A}	0.27 ± 0.08^{B}
16:1n-9	0.83 ± 0.05	0.56 ± 0.18
16:1n-7	1.00 ± 0.11	0.73 ± 0.13
18:1n-9	8.03 ± 0.63^{A}	2.96 ± 0.70^{B}
18:1n-7	2.11 ± 0.11^{A}	0.53 ± 0.07^{B}
20:1n-9	0.68 ± 0.12^{A}	0.34 ± 0.06^{B}
Polyunsaturates	6.16 ± 0.62^{A}	0.29 ± 0.07^{B}
18:2n-9	0.18 ± 0.02	ND
18:2n-6	1.53 ± 0.16^{A}	0.29 ± 0.07^{B}
20:3n-9	0.17 ± 0.01	ND
20:3n-6	0.74 ± 0.06	ND
20:4n-6	2.66 ± 0.25	ND
22:3n-9	ND	ND
22:4n-6	0.17 ± 0.03	ND
22:5n-3	0.42 ± 0.07	ND
22:6n-3	0.29 ± 0.06	ND

*Mean ± SEM, n = 8. Data from trials 1 and 2 are combined; replicates of 15 flasks of cells for each trial. Values having a different superscript are significantly different ($P < 0.05$) by the Student t test.
†ND is not detectable. Detection limit is less than 0.01 nmol of fatty acid.

selectively decreased only the non-esterified 18:2n-6 and 20:4n-6 concentration in the culture medium, did not affect medium phospholipid mass or fatty acid composition, and greatly increased the percentage of PUFA in medium triglycerides;[12] the rat fibroblasts did not accumulate detectable 20:3n-9 in cellular phospholipids under these conditions.[12] However, our data are consistent with the original hypothesis of Howard and Kritchevsky[13] that multiplying populations of human fibroblasts in culture primarily utilize the non-esterified fatty acid fraction of FBS-supplemented culture media as a source of cellular fatty acids. Moreover, we have demonstrated that human fibroblasts synthesize n-9 PUFA and incorporate them into cellular phospholipids under culture conditions where there is a depletion of mass of 20- and 22- carbon n-6 and n-3 PUFA in the non-esterified fatty acid pool(s) but not in triglyceride, cholesterol ester, or phospholipid pools in the culture medium.

The role of non-esterified fatty acids as nutrients for rapidly multiplying populations of cells in vivo and in vitro remains obscure. It is clear that human fibroblasts readily take up non-esterified fatty acids and incorporate these fatty acids into cellular phospholipids and neutral lipids,[14,15] with minimal amounts oxidized for energy.[16] However, the role of non-esterified fatty acids in both promoting and inhibiting growth appears to be independent of the effects of exogenous fatty acids on the composition of cellular phospholipid fatty acids.[4,17] In fetal bovine serum, non-esterified fatty acids are bound to albumin,[17] α-fetoprotein,[18] and fetuin.[19] It is likely that each of these pools of non-

Table 4 Lack of effect of cell growth on the content of 20:3n-9, 20:4n-6, and 22:6n-3 in medium phospholipids, cholesterol esters, and triglycerides

Lipid class	Concentration of fatty acids[1,2]	
	Culture medium	Culture medium + cells
	nmoL/75 mL	
Phospholipid		
20:3n-9	0.51 ± 0.02	0.46 ± 0.03
20:4n-6	3.98 ± 0.12	3.70 ± 0.10
22:6n-3	2.52 ± 0.11	2.28 ± 0.06
Cholesterol ester		
20:3n-9	ND	ND
20:4n-6	9.03 ± 0.43	8.66 ± 0.23
22:6n-3	1.02 ± 0.02	0.98 ± 0.05
Triglyceride		
20:3n-9	ND	ND
20:4n-6	0.43 ± 0.14	0.75 ± 0.34
22:6n-3	ND	ND

[1]Mean ± SEM, n = 4 replicates of 15 flasks of cells in trial 2. There are no significant differences ($P > 0.05$) between culture medium and culture medium plus cells for fatty acid in a lipid class by a Student t test.
[2]ND is not detectable. Detection limit is less than 0.01 nmol of fatty acid.

esterified fatty acids has a distinctive fatty acid composition, is available for uptake at a different rate, or targets fatty acids for specific types of metabolism in the cell.[20-22] The contribution of different exogenous pools of non-esterified fatty acids to the nutritive process of rapidly multiplying cells in vivo and in vitro warrants further investigation.[23,24]

References

1 Karmiol, S. and Bettger, W.J. (1988). Accumulation of (n-9)-eicosatrienoic and docosatrienoic acids in human fibroblast phospholipids. *Lipids* **23,** 891–898

2 Rosenthal, M.D. and Whitehurst, M.C. (1982). Selective utilization of ω6 and ω3 polyunsaturated fatty acids by human skin fibroblasts. *J. Cell. Physiol.* **113,** 298–306

3 Bettger, W.J., Driscoll, E.R., and Karmiol, S. (1990). Effect of cellular growth state on indices of n-6 polyunsaturated fatty acid status in human fibroblasts. *Biochem. Cell Biol.* **68,** 819–822

4 Karmiol, S. and Bettger, W.J. (1991). Lack of an association between cellular phospholipid triene:tetraene ratio and proliferation of human skin fibroblasts in culture. *J. Nutr.* **121,** 595–604

5 Spector, A.S., Mathur, S.N., Kaduce, T.L., and Hyman, B.T. (1981). Lipid nutrition and metabolism of cultured mammalian cells. *Prog. Lipid Res.* **19,** 155–186

6 Rosenthal, M.D. (1987). Fatty acid metabolism of isolated mammalian cells. *Prog. Lipid Res.* **26,** 87–124

7 Kirkland, J.B., Bray, T.M., and Bettger, W.J. (1987). The effect of 3-methylindole on the rates of phospholipid and neutral lipid synthesis in cultured fibroblasts. *Can. J. Physiol. Pharmacol.* **63,** 1788–1792

8 Bettger, W.J., Boyce, S.T., Walthall, B.J., and Ham, R.G. (1981). Rapid clonal growth and serial passage of human diploid fibroblasts in a lipid-enriched synthetic medium supplemented with epidermal growth factor. *Proc. Natl. Acad. Sci. USA* **78,** 5588–5592

9 Bligh, E.G. and Dyer, W.J. (1965). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37,** 911–917

10 Mercer, N.J.H. and Holub, B.J. (1979). Response of free and esterified plasma cholesterol levels in the Mongolian gerbil to the fatty acid composition of dietary lipid. *Lipids* **14,** 1009–1014

11 Mahadevappa, V.G. and Holub, B.J. (1982). The molecular species composition of individual diacyl phospholipids in human platelets. *Biochim. Biophys. Acta* **713,** 73–79

12 Daniel, L.W., Kucera, L.S., and Waite, M. (1980). Metabolism of fatty acids by cultured tumor cells and their diploid precursor fibroblasts. *J. Biol. Chem.* **255,** 5697–5702

13 Howard, B.V. and Kritchevsky, D. (1969). The source of cellular lipid in the human diploid cell strain WI-38. *Biochim. Biophys. Acta* **187,** 293–301

14 Rosenthal, M.D. (1981). Accumulation of neutral lipids by human skin fibroblasts: differential effects of saturated and unsaturated fatty acids. *Lipids* **16,** 173–182

15 Spector, A.S., Kiser, R.E., Denning, G.M., Koh, S.-W.M., and DeBault, L.E. (1979). Modification of the fatty acid composition of cultured human fibroblasts. *J. Lipid Res.* **20,** 536–547

16 Sumbilla, C.M., Zielke, C.L., Reed, W.D., Ozand, P.T., and Zielke, H.R. (1981). Comparison of the oxidation of glutamine, glucose, ketone bodies and fatty acids by human diploid fibroblasts. *Biochim. Biophys. Acta* **673,** 301–304

17 Spector, A.A. (1975). Fatty acid binding to plasma albumin. *J. Lipid Res.* **16,** 165–179

18 Carlsson, R.N.K., Estes, T., DeGroot, J., Holden, J.T., and Ruoslahti, E. (1980). High affinity of α-fetoprotein for arachidonate and other fatty acids. *Biochem. J.* **190,** 301–305

19 Kumbla, L., Cayatte, A.J., and Subbiah, M.T.R. (1989). Association of a lipoprotein-like particle with bovine fetuin. *FASEB J.* **3,** 2075–2080

20 Calvo, M., Naval, J., Lampreave, F., Uriel, J., and Piñeiro, A. (1988). Fatty acids bound to α-fetoprotein and albumin during rat development. *Biochim. Biophys. Acta* **959,** 238–246

21 Uriel, J., Naval, J., and Laborda, J. (1987). αFetoprotein-mediated transfer of arachidonic acid into cultured cloned cells derived from a rat rhabdomyosarcoma. *J. Biol. Chem.* **262,** 3579–3585

22 Cayette, A.J., Kumbla, L., and Subbiah, M.T.R. (1990). Marked acceleration of exogenous fatty acid incorporation into cellular triglycerides by fetuin. *J. Biol. Chem.* **265,** 5883–5888

23 Veerkamp, J.H., Peters, R.A., and Maatman, R.G.H.J. (1991). Structural and functional features of different types of cytoplasmic fatty acid-binding proteins. *Biochim. Biophys. Acta* **1081,** 1–24

24 Potter, B.J., Sorrentino, D., and Berk, P.D. (1989). Mechanisms of cellular uptake of free fatty acids. *Ann. Rev. Nutr.* **9,** 253–270