J. Biochem. 128, 545–551 (2000)

Metabolism of Palmitic and Docosahexaenoic Acids in Reuber H35 Hepatoma Cells[1]

Marina Martínez-Cayuela, Mª Carmen García-Pelayo, Ana Linares, and Eduardo García-Peregrín[2]

Department of Biochemistry and Molecular Biology, University of Granada, Granada, Spain

Received June 5, 2000; accepted July 7, 2000

In this work, we have modified the fatty acid composition of Reuber H35 hepatoma cells by supplementation of the culture medium with a saturated (palmitic) or a polyunsaturated (docosahexaenoic) acid. These fatty acids were incorporated into total lipids and phospholipids of hepatoma cells. Palmitic acid readily increased the percentage of its monounsaturated derivative (16:1 *n*-7). When both fatty acids were supplemented at the same concentration, the percentage of docosahexaenoic acid in the total lipids and phospholipids of Reuber H35 cells increased more than that of palmitic acid. Although the levels of 16:0 increased, the addition of docosahexaenoic acid to the culture medium decreased the percentages of monoenoic acids. From our results, it can be concluded that palmitic and docosahexaenoic acids modify the fatty acid composition of Reuber H35 hepatoma cells. The profound changes induced by docosahexaenoic acid, especially those in the phospholipid fraction, may be of great interest given the main role of these components in the regulation of chemical and physical properties of biological membranes and/or membrane systems.

Key words: fatty acid composition, hepatoma cells, phospholipids, total lipids.

Cell cultures have been widely used to study different aspects of lipid metabolism, avoiding the complex background of the biological factors present in the intact organism. Mammalian cultured cells are able to utilize the lipid supplied to them, almost exclusively as a serum supplement, in the medium (*1*, *2*). If the lipid supplement is nonlimiting, the fatty acid composition of the cells reflects that of the serum lipids.

The specialized functions of differentiated mammalian cells require the utilization of certain fatty acids (*3*, *4*). The liver is an essential organ for the maintenance of fatty acid homeostasis as a result of its vigorous synthesis of different lipids. Hepatic cell lines derived from human tumors have been used to represent an alternative model for studies on human hepatic lipid metabolism (*5*). Fatty acids are an integral part of the structural and dynamic nature of the cellular membrane. For this purpose, specific fatty acids are selectively incorporated into the different classes of membrane lipids. It is also well known that phospholipids are major constituents of biological membranes. The effects of their fatty acid composition on the fluidity (*6*) and on the properties of specific membrane proteins such as enzymes, transport systems and ion channels have been described (*7*).

Apart from direct interactions of membrane lipids and proteins, the lipid composition influences the cellular metabolism, since fatty acids serve as precursors of second messengers (*8*, *9*). Thus, the eicosanoid derivatives of polyunsaturated fatty acids (PUFA) play a regulatory role in all body cells.

In the present work we have modified the lipid composition of cultured H35 Reuber hepatoma cells by supplementing the culture medium with specific fatty acids (16:0 and 20:6 *n*-3) with the aim of gaining a better understanding of those events that occur in parallel in the normal human liver. Palmitic acid was used as a saturated fatty acid (SFA) and a precursor of other saturated and/or unsaturated fatty acids formed by elongation and/or desaturation reactions. Docosahexaenoic acid (DHA) was tested as a PUFA and a final product of these metabolic pathways. Both fatty acids differ in their effects on cholesterol metabolism, but the underlying mechanisms of this variation are still poorly understood (*10*, *11*). Recently, we have studied the effects of different free fatty acids on chick microsomal and solubilized 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the main regulatory enzyme of cholesterogenesis, suggesting that these effects appear to depend upon the integrity of the microsomal membrane (*12*). Likewise, this enzyme activity was drastically decreased *in vivo* by supplementing the diet with fish oil (*13*). Because of these considerations, we have optimized the conditions to reach a maximum uptake of fatty acids into the total lipid and phospholipid fractions in order to investigate whether certain membrane lipid modifications would alter the activities of some membrane enzymes implied in the lipid metabolism in normal and tumor cells.

[1] Supported in part by grants from DGICYT (PB91-0723) and Junta de Andalucía (Grupo CTS 141), Spain.

[2] To whom correspondence should be addressed. Tel: +34-958-243250, Fax: +34-958-249945

Abbreviations: DHA, docosahexaenoic acid; DMEM, Dulbecco's modified Eagle's minimum essential medium; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MUFA, monounsaturated fatty acid(s); PUFA, polyunsaturated fatty acid(s); SFA, saturated fatty acid(s).

MATERIALS AND METHODS

Reagents were purchased as follows: fetal bovine serum and cell culture medium from Cultek; bovine serum albumin, standard fatty acids for gas chromatography, palmitic acid and DHA from Sigma. All other reagents were of analytical grade.

Reuber H35 hepatoma cells were grown in monolayer in Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 100 units/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml amphotericin B, 4 mmol/liter glutamine, and 10% (v/v) fetal bovine serum. They were usually seeded at a density of 3×10^4 cells/cm^2 and maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2. The medium, with or without supplementation with free fatty acids, was replaced by fresh medium every 2 days, even for the prolonged culture. Cells were always used within 24 h of reaching confluence, when the density was about 22–27 $\times 10^4$ cells/cm^2. This was reached 4 days after initial inoculum in control experiments and those carried out with palmitic acid, and 6 days after initial inoculum in experiments carried out with DHA. Cell viability, assessed by trypan blue exclusion, was in excess of 92% in all cases.

Stock solution of DHA was prepared in hexane and stored under N_2 at –80°C to protect from oxidative damage. On the day of the experiment, aliquots of this solution were evaporated to dryness, and the DHA was redissolved in 95% ethanol. Stock solution of palmitic acid was directly prepared in 95% ethanol and kept at 4°C. Both fatty acids were added to the cell cultures coupled to fatty acid–free bovine serum albumin in the ratio of 2 mol of fatty acid to 1 mol of albumin. These complexes were constituted in stoppered flasks by adding the appropriate volume of the ethanolic free fatty acids solution to the albumin previously dissolved in culture medium. These solutions were gently stirred and sterilized by filtration through 0.2 μm filters. Final concentration of ethanol in the culture medium was always less than 0.1%.

On days 0, 2, 4, and 6, culture media were removed and analyzed for fatty acid content. Cells in confluence were also collected for lipid analysis. In that case, cells were washed twice with phosphate-buffered saline (pH 7.4); they were then detached with the aid of 0.05% trypsin/0.02% EDTA solution, pelleted by centrifugation and, finally, suspended in 0.9% saline solution.

Total lipids from the cells and the medium were extracted according to Folch *et al.* (*14*). The organic phase was then evaporated to dryness under nitrogen, and the residue was taken up in 200 μl of hexane. Samples were applied to 0.25 mm silica gel G-60 thin-layer chromatography plates for separating phospholipids by means of a solvent system containing hexane/diethylether/acetic acid (80:20:1) (*15*). Under these conditions, the phospholipid fraction remained at the origin. Fatty acid methyl esters of total lipids and phospholipids were prepared by the method of Lepage and Roy (*16*) and analyzed in a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector. The optimum separation was obtained by using a DB-2330-N J&W Scientific capillary column (30 m × 0.25 mm). The oven temperature was programmed as follows: 60°C, 3 min; 60–150°C, 15°C/min; 150–211°C, 2°C/min; 211°C, 3 min; 211–235°C, 4°C/min; and 235°C, 20 min. Fatty acid methyl esters were identified by comparison of relative retention times with standards.

Results are expressed as mean values ± SE of three experiments. Each experimental value was the mean of duplicate determinations. Data were analyzed by one-factor analysis of variance (ANOVA). When the overall F statistic was significant ($p < 0.05$), analyses of significance were determined by the Bonferroni/Dunn test at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

It is well documented that most cells in culture can synthesize lipids from glucose and amino acids available in the medium; but when lipids are present in the extracellular medium, it has been shown that the *de novo* biosynthesis of fatty acids and cholesterol is inhibited and the cells take up lipids from the medium (*2*). Little is known about cholesterol metabolism in Reuber H35 cells. In previous experiments, we have found that H35 hepatoma cells contained low HMG-CoA reductase activity as well as other cholesterogenic enzyme activities, probably as a result of the presence of cholesterol in the culture medium. Thus, it would be of interest to determine whether sera with different lipid compositions had different effects on the fatty acid composition of cells, and if so, whether these effects were directly related to the activity of some enzymes bound to the membrane.

Firstly, it was necessary to obtain information on the fatty acid levels in the culture medium as well as in the hepatoma cells grown in this medium. As shown in Table I, fatty acid composition in cells was directly related to that of fetal bovine serum. This serum contained 16:0 and 18:1 *n*-9

TABLE I. **Fatty acid composition of total lipids of Reuber H35 hepatoma cells and their culture medium.** Cells were grown at 37°C in DMEM containing 10% fetal bovine serum. Results (% of fatty acids) are expressed as mean values ± SE of triplicate determinations. Statistical significance is indicated by $^*p < 0.05$ with respect to the cells.

Fatty acid	Cells	Culture medium
14:0	1.21 ± 0.02	1.81 ± 0.01 *
16:0	14.97 ± 0.06	24.10 ± 0.07 *
18:0	16.65 ± 0.08	14.73 ± 0.01 *
24:0	1.84 ± 0.02	1.18 ± 0.05 *
16:1 n-9	1.37 ± 0.02	2.26 ± 0.02 *
16:1 n-7	6.08 ± 0.04	2.45 ± 0.02 *
18:1 n-9	35.99 ± 1.34	21.17 ±0.04 *
18:1 n-7	9.25 ± 0.06	5.12 ± 0.01 *
20:1 n-7	1.85 ± 0.04	0.00
22:1 n-9	0.96 ± 0.02	0.02 ± 0.01 *
24:1 n-9	1.17 ± 0.02	2.08 ± 0.08 *
18:2 n-6	1.21 ± 0.03	3.60 ± 0.07 *
20:2 n-6	1.24 ± 0.04	0.00
20:3 n-6	0.39 ± 0.02	1.94 ± 0.02 *
20:4 n-6	2.26 ± 0.03	4.82 ± 0.04 *
18:4 n-3	0.10 ± 0.04	6.73 ± 0.13 *
20:5 n-3	0.50 ± 0.02	1.09 ± 0.02 *
22:5 n-3	1.18 ± 0.03	3.04 ± 0.04 *
22:6 n-3	1.51 ± 0.04	3.86 ± 0.04 *

as the main fatty acids (20–25%), followed by 18:0 and lower percentages of 18:4 *n*-3, 18:1 *n*-7, and 20:4 *n*-6 fatty acids. This composition differs from that reported for other sera (*17*). Total lipids from H35 hepatoma cells grown in this medium were enriched in 18:1 *n*-9, as well as in 18:1 *n*-7 and 16:1 *n*-7 acids. However, the percentage of 16:0 was

TABLE II. **Effects of supplementing the culture medium with palmitic acid on fatty acid composition of total lipids and phospholipids of Reuber H35 hepatoma cells.** Results (% of fatty acids) are expressed as mean values ± SE of 3 experiments. Duplicate determinations were made in each experiment. Statistical significance is indicated by $^{a}p < 0.05$ with respect to the control; $^{b}p < 0.05$ with respect to 100 μM.

	Concentration of palmitic acid (μM)					
	0	100	200	0	100	200
Fatty acid	Total lipids			Phospholipids		
14 0	1 21 ± 0 02	1 10 ± 0 05	1 09 ± 0 06	1 34 ± 0 04	1 55 ± 0 10	1 37 ± 0 08
16 0	14 97 ± 0 06	15 29 ± 0 24	21 39 ± 0 75^{ab}	19 02 ± 0 45	21 24 ± 0 19^{a}	28 41 ± 0 19^{ab}
18 0	16 65 ± 0 08	15 74 ± 0.02	15.23 ± 0 56	23 76 ± 0 20	24.23 ± 0 31	22 63 ± 0 23^{ab}
24 0	1 84 ± 0 02	1 07 ± 0 03^{a}	0 78 ± 0 01^{ab}	3 08 ± 0 33	1 91 ± 0 05^{a}	1 31 ± 0 02^{a}
16 1 n-9	1 37 ± 0 02	1 39 ± 0 01	1 14 ± 0 17	1 05 ± 0 13	1 08 ± 0 04	1 06 ± 0 02
16 1 n-7	6 08 ± 0 04	7 94 ± 0 12^{a}	11 64 ± 0 18^{ab}	5 32 ± 0 51	5 24 ± 0 12	7 65 ± 0 44^{ab}
18 1 n-9	35 99 ± 1 34	33 57 ± 0 28	25 79 ± 0 57^{ab}	28 00 ± 0 38	22 91 ± 0 21^{a}	16 73 ± 0 22^{ab}
18 1 n-7	9 25 ± 0 06	10 08 ± 0 12	10 05 ± 0 40	6 33 ± 0 10	7 13 ± 0 90	6.00 ± 0 13
20 1 n-7	1 85 ± 0 04	1 79 ± 0 02	0 97 ± 0 06^{ab}	1 28 ± 0 07	1 03 ± 0 02^{a}	0 53 ± 0 01^{ab}
22 1 n-9	0 96 ± 0 02	1 50 ± 0 02^{a}	1 58 ± 0 03^{a}	2 82 ± 0 07	5 65 ± 0 11^{a}	7 12 ± 0 25^{ab}
24 1 n-9	1 17 ± 0 02	0 92 ± 0 01^{a}	0 69 ± 0 02^{ab}	1 35 ± 0 03	1 22 ± 0 06	0 85 ± 0 03^{ab}
18 2 n-6	1 21 ± 0 03	1 21 ± 0 04	1 15 ± 0 20	1 08 ± 0 12	0 82 ± 0 09	0 75 ± 0 14
20 2 n-6	1 24 ± 0 04	1 25 ± 0 11	0 87 ± 0 13^{ab}	1 04 ± 0 04	0 99 ± 0 03	0 73 ± 0 10^{ab}
20 3 n-6	0 39 ± 0 02	0 47 ± 0 08	0 46 ± 0 07	0 13 ± 0 03	0 08 ± 0 04	0 10 ± 0 04
20 4 n-6	2 26 ± 0 03	3 06 ± 0 05^{a}	3 34 ± 0 08^{ab}	1 91 ± 0 02	2 08 ± 0 03	2 26 ± 0 17
20 5 n-3	0 50 ± 0 02	0 62 ± 0 01	0 74 ± 0 01^{a}	0 15 ± 0 04	0 09 ± 0 03	0 10 ± 0 03
22 5 n-3	1 18 ± 0 03	1 31 ± 0 12	1 35 ± 0 20	0 67 ± 0 03	0 64 ± 0 01	0 67 ± 0 04
22 6 n-3	1 51 ± 0 04	1 67 ± 0 01	1 70 ± 0 03^{a}	0 94 ± 0 01	0 93 ± 0 02	0 91 ± 0 06

TABLE III. **Fatty acid composition of total lipids from culture medium supplemented with palmitic acid and obtained after different times of cell growth.** Results (% of fatty acids) are expressed as mean values ± SE of 3 experiments. Duplicate determinations were made in each experiment. For each palmitic acid concentration, statistical significance is indicated by $^{a}p < 0.05$ with respect to 0 days (control); $^{b}p < 0.05$ with respect to 2 days.

	Concentration of palmitic acid (μM)				
	0	100		200	
Fatty acid	0 days	2 days	4 days	2 days	4 days
14 0	1 81 ± 0 01	1 99 ± 0 07	1 94 ± 0 13	2 15 ± 0 23	2 22 ± 0 18
16 0	24 10 ± 0 07	31 59 ± 0.38^{a}	27 11 ± 0.25^{ab}	46 96 ± 0 70^{a}	38 24 ± 0 75^{ab}
18.0	14.73 ± 0 01	14.57 ± 0 28	16 05 ± 0 62	11.08 ± 0 08^{a}	12 99 ± 0 46^{ab}
24 0	1 18 ± 0 05	0.88 ± 0 03^{a}	0.50 ± 0 05^{ab}	0.55 ± 0 05^{a}	0 56 ± 0 02^{a}
16 1 n-9	2 26 ± 0 02	2 30 ± 0 15	2 36 ± 0 20	1 93 ± 0 14	1 94 ± 0 18
16:1 n-7	2.45 ± 0 02	4 23 ± 0 07^{a}	3.48 ± 0.13^{ab}	5 13 ± 0 32^{a}	6 04 ± 0 16^{ab}
18:1 n-9	21 17 ± 0 14	21 50 ± 0 04	24 48 ± 0 45^{ab}	15.98 ± 0 01^{a}	20 15 ± 0 34^{ab}
18 1 n-7	5 12 ± 0 10	5.67 ± 0.12^{a}	6.17 ± 0 15^{a}	5.14 ±0 07	5 33 ± 0.50
22 1 n-9	0 02 ±0.01	1 18 ± 0 12^{a}	1.41 ± 0 18^{a}	0 57 ± 0 11^{a}	0 79 ± 0 05^{a}
24 1 n-9	2.08 ± 0 08	1.40 ± 0.07^{a}	1 66 ± 0 11^{a}	0.82 ± 0 09^{a}	0.94 ± 0 03^{a}
18:2 n-6	3 60 ± 0 17	2 91 ± 0 15^{a}	3.32 ± 0 12	1 93 ± 0.11^{a}	2.22 ± 0 21^{a}
20 3 n-6	1 94 ± 0.02	1 26 ± 0 02^{a}	1.36 ± 0.05^{a}	0.76 ± 0 04^{a}	0 89 ± 0 09^{a}
20.4 n-6	4.82 ± 0 04	3 31 ± 0.11^{a}	3 94 ± 0 20^{ab}	2.16 ± 0 23^{a}	2 34 ± 0.10^{a}
18:4 n-3	6.73 ± 0 13	1.25 ± 0 15^{a}	0.83 ± 0 09^{a}	1 08 ± 0.17^{a}	0 96 ± 0.11^{a}
20:5 n-3	1.09 ± 0 02	0 40 ± 0 09^{a}	0 42 ± 0 11^{a}	0.65 ± 0 19	0.56 ± 0 09^{a}
22 5 n-3	3 04 ± 0 04	1.86 ± 0 04^{a}	2.21 ± 0 12^{ab}	1 30 ± 0.22^{a}	1 33 ± 0 42^{a}
22 6 n-3	3.86 ± 0.04	2 47 ± 0 07^{a}	2 73 ± 0 13^{a}	1.75 ± 0 28^{a}	1 99 ± 0.25^{a}

clearly lower in the cells than in the culture medium. Other minor differences were observed in the fatty acid composition of both cells and medium. It is clear that the fatty acid composition of Reuber H35 hepatoma cells is similar to

TABLE IV. **Effects of supplementing the culture medium with docosahexaenoic acid on fatty acid composition of total lipids and phospholipids of Reuber H35 hepatoma cells.** Results (% of fatty acids) are expressed as mean values ± SE of 3 experiments. Duplicate determinations were made in each experiment. Statistical significance is indicated by $^{a}p < 0.05$ with respect to the control; $^{b}p < 0.05$ with respect to 100 μM.

	Concentration of docosahexaenoic acid (μM)					
	0	100	200	0	100	200
Fatty acid	Total lipids			Phospholipids		
14:0	1.21 ± 0.02	1.73 ± 0 02^{a}	2.23 ± 0 02^{ab}	1 34 ± 0 04	2.57 ± 0.05^{a}	2 84 ± 0.05^{ac}
16 0	14 97 ± 0 06	21.80 ± 0.18^{a}	26.02 ± 0 82^{ab}	19 02 ± 0.45	29 84 ± 0 17^{a}	34 02 ± 0 17^{ab}
18:0	16 65 ± 0.08	23.30 ± 0 05^{a}	15.50 ± 0 43^{b}	23 76 ± 0 21	33.00 ± 0 09^{a}	21 45 ± 0 16^{ab}
24·0	1 84 ± 0.02	1.75 ± 0 03	1 01 ± 0.02^{ab}	3 08 ± 0 33	2.51 ± 0 06	1.40 ± 0 03^{ab}
16 1 n-9	1 37 ± 0.02	0 09 ± 0 03^{a}	0 00	1 05 ± 0 13	0 84 ± 0.03	0 53 ± 0 03^{abc}
16.1 n-7	6 08 ± 0.04	2.96 ± 0 09^{a}	1 33 ± 0.08^{ab}	5 32 ± 0 51	3.39 ± 0.11^{a}	1 65 ± 0 14^{ab}
18 1 n-9	35.99 ± 1 34	10 45 ± 0 18^{a}	6.73 ± 0 37^{ab}	28 00 ± 0 38	12.23 ± 0.23^{a}	9.81 ± 0.32^{ab}
18 1 n-7	9 25 ± 0.06	3 36 ± 0.12^{a}	3 02 ± 0 20^{a}	6 33 ± 0.11	4 47 ±0 47	3.62 ± 0 05^{a}
20 1 n-7	1.85 ± 0 04	0.10 ± 0 04^{a}	0 00	1 28 ± 0 07	0 00	0 08 ± 0 03^{a}
22·1 n-9	0.96 ± 0.02	0 76 ± 0 06^{a}	0 00	2 82 ± 0 07	0 99 ± 0.10^{a}	0 75 ± 0 28^{a}
24 1 n-9	1 17 ± 0.02	1 01 ± 0 03^{a}	1 09 ± 0 03	1 35 ± 0 03	1 37 ± 0 04	1 37 ± 0 05
18 2 n-6	1.21 ± 0 03	1.22 ± 0 05	0 00	1 08 ± 0.12	0 85 ± 0.07	0 97 ± 0.06
20 2 n-6	1 24 ± 0 04	0 10 ± 0 04^{a}	0 00	1 04 ± 0 04	0 00	0 10 ± 0 04^{a}
20 3 n-6	0 39 ± 0 02	0 92 ± 0 02^{a}	1 12 ± 0 02^{a}	0 13 ± 0 03	0 54 ± 0 05^{a}	0 76 ± 0 05^{ab}
20:4 n-6	2.26 ± 0 03	3.31 ± 0 06^{a}	3 46 ± 0 06^{a}	1 91 ± 0 02	1.95 ± 0.05	2 05 ± 0 09
20 5 n-3	0 50 ± 0 02	6 58 ± 0 11^{a}	5 47 ± 0 33^{ab}	0 15 ± 0 04	1 41 ± 0 03^{a}	2 82 ± 0 05^{ab}
22 5 n-3	1 18 ± 0 03	1.66 ± 0 03	1 57 ± 0 06	0 67 ± 0 03	0 64 ± 0 06	0 75 ± 0 04
22 6 n-3	1 51 ± 0 04	18 89 ± 0 05^{a}	31 46 ± 0 08^{ab}	0 94 ± 0 01	3.46 ± 0 03^{a}	14 33 ± 0 07^{ab}

TABLE V. **Fatty acid composition of total lipids from culture medium supplemented with docosahexaenoic acid and obtained after different times of cell growth.** Results (% of fatty acids) are expressed as mean values ± SE of 3 experiments. Duplicate determinations were made in each experiment. For each docosahexaenoic acid concentration, statistical significance is indicated by $^{a}p < 0.05$ with respect to 0 days (control); $^{b,c}p < 0.05$ with respect to 2 days; $^{d}p < 0.05$ with respect to 4 days.

	Concentration of docosahexaenoic acid (μM)						
	0	100			200		
Fatty acid	0 days	2 days	4 days	6 days	2 days	4 days	6 days
14:0	1.81 ± 0 01	1.27 ± 0 05^{a}	1 84 ± 0.12^{b}	2 24 ± 0.13^{ac}	0 77 ± 0 13^{a}	0.79 ± 0 13^{a}	1 33 ± 0 16^{cd}
16 0	24 10 ± 0 07	18.93 ± 0 18^{a}	22 58 ± 0 27^{b}	26 42 ± 1.15^{cd}	5 53 ± 0 74^{a}	10 23 ± 1 28^{ab}	17 46 ± 0 89^{acd}
18 0	14 73 ± 0.01	11.84 ± 0 35^{a}	16 06 ± 0.69^{b}	16 30 ± 1.05^{c}	9 72 ± 0 88^{a}	6 64 ± 1 52^{a}	11 18 ± 0.94^{d}
24:0	1.18 ± 0 05	0.84 ± 0 06	0 57 ± 0 10^{a}	1 05 ± 0.12^{d}	0.88 ± 0 25	0 88 ± 0 30	0 67 ± 0 19
16.1 n-9	2 26 ± 0 02	1.82 ± 0 17	2 00 ± 0 21	2.31 ± 0.32	0.45 ± 0 08^{a}	0 39 ± 0.08^{a}	1.04 ± 0 34^{a}
16:1 n-7	2.45 ± 0.02	1.78 ± 0 07^{a}	1 70 ± 0.12^{a}	2 38 ± 0 15^{cd}	0.55 ± 0 14^{a}	1 08 ± 0 32^{a}	1 35 ± 0.26^{a}
18.1 n-9	21.17 ± 0.14	15.11 ± 0 13^{a}	14 19 ± 0.95^{a}	16.84 ± 1.05^{a}	1.78 ± 0 03^{a}	7 44 ± 0.84^{ab}	10.77 ± 0.57^{acd}
18:1 n-7	5 12 ± 0 10	3.76 ± 0 15	3 27 ± 0 10	4.27 ± 0.17	4 03 ± 0.57	1 97 ± 0 36^{ab}	2.72 ± 0 18^{a}
22·1 n-9	0.02 ± 0.01	0.94 ± 0 13^{a}	1.01 ± 0.17^{a}	1.11 ± 0.21^{a}	0.69 ± 0.13^{a}	0.59 ± 0.06^{a}	0.69 ± 0.15^{a}
24 1 n-9	2.08 ± 0.08	1 21 ± 0 09^{a}	0 66 ± 0 15^{a}	1.41 ± 0.23^{ad}	1 05 ± 0 08^{a}	0 84 ± 0 06^{a}	1.01 ± 0.05^{a}
18:2 n-6	3.60 ± 0.17	2.64 ± 0 15^{a}	2.37 ± 0 24^{a}	2.97 ± 0 04	0.69 ± 0.11^{a}	1 39 ± 0.23^{a}	1.80 ± 0.12^{ac}
20.3 n-6	1.94 ± 0 02	1.25 ± 0 02^{a}	1.16 ± 0.07^{a}	1.43 ± 0.18^{a}	0.88 ± 0.08^{a}	0.73 ± 0.07^{a}	0.69 ± 0.11^{a}
20:4 n-6	4.82 ± 0.04	1 40 ± 0 13^{a}	3.10 ± 0.21^{ab}	3 84 ± 0 04^{acd}	1 79 ± 0 13^{a}	2 21 ± 0 35^{a}	3 19 ± 0 05^{acd}
18·4 n-3	6 73 ± 0.13	1 32 ± 0.20^{a}	1.97 ± 0.19^{a}	1.89 ± 0.23^{a}	1.12 ±0 15^{a}	1.70 ± 0 33^{a}	2.81 ± 0 15^{acd}
20 5 n-3	1 09 ± 0.02	2 83 ± 0 14^{a}	2.62 ± 0 12^{a}	2 34 ± 0.34^{a}	4.07 ± 0.39^{a}	3 21 ± 0.26^{a}	4.19 ± 0 25^{a}
·22·5 n-3	3 04 ± 0.04	2.62 ± 0 07	2 63 ± 0.13	2 36 ± 0 14^{a}	2 53 ± 0.15	1.87 ± 0.27^{ab}	2.28 ± 0 16^{a}
22.6 n-3	3 86 ± 0.04	30.04 ± 2.37^{a}	22.25 ± 2.43^{a}	10 24 ± 1.87^{cd}	63.49 ± 4 18^{a}	57.62 ± 5.00^{a}	36.38 ± 2.06^{acd}

that usually observed in tumor tissues: a high content of monounsaturated fatty acids (MUFA), in particular oleic acid, and lower levels of arachidonic, linoleic, and other PUFA (*3*, *18–20*).

As the free fatty acid fraction is believed to be the main class of lipid that supplies fatty acids to the cells in culture, we studied the possible influence of supplementation with different fatty acids in the culture medium on fatty acid composition of total lipids and phospholipids from cells grown in these modified media. Table II shows that the percentage of palmitic acid in both lipid fractions from hepatoma cells clearly increased when the culture medium was supplemented with this acid. These results showed that Reuber H35 hepatoma cells exhibit a great capacity to assimilate a saturated fatty acid such as palmitic acid from the culture medium under our standard experimental conditions. The palmitic acid incorporated into the tumor cells was actively metabolized. Thus, significant increases in the percentage of 16:1 *n*-7 and 22:1 *n*-9 acids were observed. From the analysis of the pattern of endogenous fatty acid composition of the cells, it is difficult to draw conclusions on individual fatty acid desaturase activities. Probably as a consequence of the increase in the percentages of 16:0 and 16:1 *n*-7 acids, due to 16:0 supplementation, the percentage of the main fatty acid (18:1 *n*-9) drastically decreased. Minor but significant decreases were also found in the levels of 24:0, 20:1 *n*-7, 24:1 *n*-9, and 20:2 *n*-6 acids. The pattern of changes in fatty acid composition was similar in total lipids and phospholipids. Alterations in these lipid species would be of special interest because functional and pathological consequences may be correlated (*21*).

Supplementing the culture medium with palmitic acid (100–200 μM) produced drastic changes in its fatty acid composition. Table III shows that the percentage of 16:0 in total lipids from the culture medium increased when the medium was supplemented with this acid. However, the levels of 16:0 in the medium fell considerably after 4 days at all concentrations assayed. The percentage of 18:4 *n*-3 in the medium also decreased drastically, suggesting that both fatty acids had been taken up by the cells. The uptake of the last fatty acid may explain the small but significant increase in the percentage of 20:5 *n*-3 and 22:6 *n*-3 acids observed in the cells at the highest palmitic acid concentration (Table II).

On the other hand, the percentage of 18:2 *n*-6 in the medium also decreased when the medium was supplemented with palmitic acid (Table III), especially at a concentration of 200 μM, suggesting that it had been incorporated into the cells. This may be related to the significant increase observed in the percentage of arachidonic acid (20:4 *n*-6) in total lipids from these cells (Table II).

Table IV shows the effects of DHA supplementation on fatty acid composition of total lipids and phospholipids from Reuber H35 hepatoma cells. It is clear that DHA was assimilated by the cells. At the same concentrations, DHA was incorporated more extensively than palmitic acid. In agreement with the known requirement of PUFA within the phospholipids of cellular membranes for the maintenance of normal fluidity within their lipid phases, the relative enrichment in DHA of the phospholipid fraction was higher with this polyunsaturated precursor than the enrichment in palmitic acid in the same lipid fraction when this SFA was incorporated (see Table II). A significant increase in the percentage of 20:5 *n*-3 was also observed in both lipid fractions, suggesting that retroconversion of 22:6 *n*-3 to 20:5 *n*-3 acids occurs, as described in rats (*22*, *23*) and in humans (*24*). When the medium was supplemented with DHA at a high concentration (200 μM), the relative increase of 20:5 *n*-3 acid was higher in phospholipids (nearly 20 times) than in total lipids (about 10 times). Similar results were found in *in vivo* experiments (*22*). Levels of 14:0 and 16:0 acids also increased, whereas those of MUFA drastically decreased, especially in oleic acid. We have obtained analogous results in previous *in vivo* experiments. The addition of fish oil to the diet for young chicks resulted in an increase in the percentage of palmitic acid as well as a great decrease in those of 16:1 *n*-7 and 18:1 *n*-9 acids (*25*). These data are also in agreement with those found in growing crossbred barrows (*26*) and in rats (*27*) consuming a diet enriched with *n*-3 PUFA. Likewise, the addition of *n*-3 PUFA to the medium of HTC cells decreased the mono-

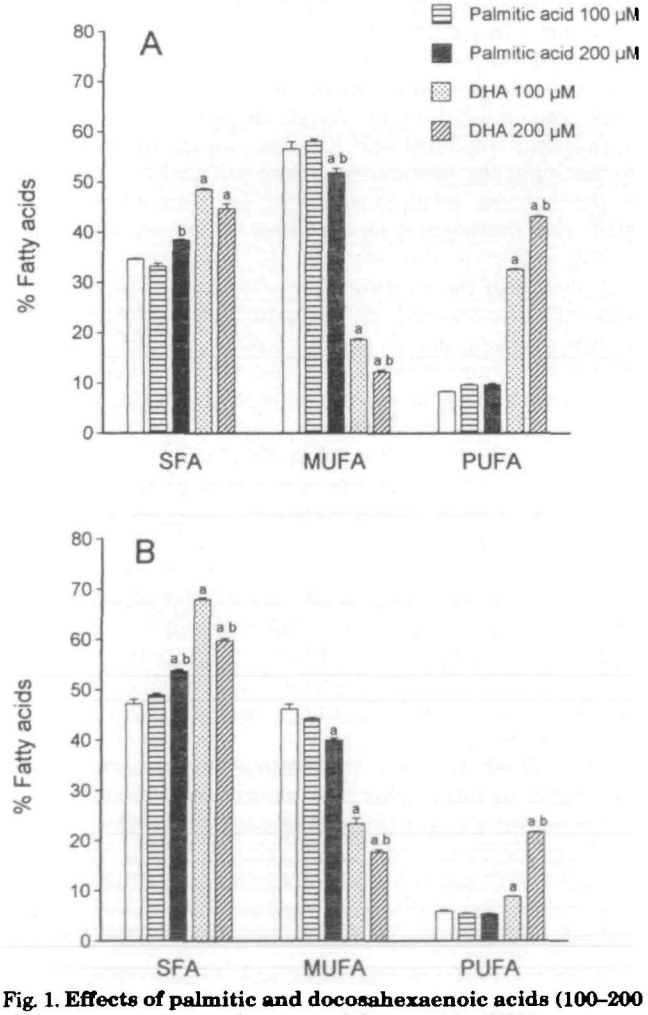

Fig. 1. Effects of palmitic and docosahexaenoic acids (100–200 μM) on percentages of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in total lipids (A) and phospholipids (B) of Reuber H35 hepatoma cells. Results are expressed as mean values ± SE of 3 experiments. Duplicate determination were made in each experiment. For each fatty acid, statistical significance is indicated by $^{a}p < 0.05$ with respect to the control; $^{b}p < 0.05$ with respect to 100 μM.

TABLE VI. **Effects of supplementing the culture medium with palmitic and docosahexaenoic acids on saturated/unsaturated and *n*-3/*n*-6 ratios of total lipids and phospholipids of Reuber H35 hepatoma cells.** Results are expressed as means ± SE of 3 experiments. Duplicate determinations were made in each experiment. For each fatty acid, statistical significance is indicated by $^{a}p < 0.05$ with respect to the control; $^{b}p < 0.05$ with respect to 100 μM.

		Concentration of fatty acid supplemented (μM)				
			Palmitic acid		Docosahexaenoic acid	
		0	100	200	100	200
Sat /unsat	Total lipids	0 53 ± 0 02	0 49 ± 0.01	0 63 ± 0.01^{ab}	0 95 ± 0 01^{a}	0 81 ± 0 02^{ab}
	Phospholipids	0.91 ± 0 03	1 01 ± 0.01	1 19 ± 0 02^{a}	2 11 ± 0 08^{a}	1 51 ± 0.03^{ab}
n-3/n-6	Total lipids	0 62 ± 0 01	0 60 ± 0 03	0 65 ± 0.05	4 98 ± 0 08^{a}	8 41 ± 0 12^{ab}
	Phospholipids	0 42 ± 0.05	0 38 ± 0.02	0 42 ± 0 03	1 65 ± 0 09^{a}	4 73 ± 0 16^{ab}

enoic acid production, suggesting that these fatty acids of *n*-3 series depress the Δ9 desaturase activity (*28*). A similar decrease in Δ9 desaturation activity was reported in different studies as a result of supplementation with *n*-6 PUFA (*29, 30*).

As observed in the case of palmitic acid, the addition of DHA (100–200 μM) to the culture medium produced drastic changes in its fatty acid composition. Data in Table V show that the percentage of 22:6 *n*-3 acid in total lipids from the culture medium drastically increased after 2 days of its supplementation but decreased after 4–6 days, corroborating that this fatty acid had been taken up by the cells. Surprisingly, the percentage of 20:5 *n*-3 acid also increased in the culture medium after its supplementation with DHA. This finding may be explained by the retroconversion of 22:6 to 20:5 in the cells and the later secretion of this fatty acid into the medium. The changes produced in the levels of saturated and monounsaturated fatty acids in the culture medium, mainly those of 16:0 and 18:1 *n*-9 acids, were more pronounced when the medium was supplemented with DHA at a high concentration (200 μM). The percentages of 18:4 *n*-3 and 20:4 *n*-6 in the medium also decreased with the addition of 22:6 *n*-3 acid.

Figure 1 shows the effects of supplementing the culture medium with palmitic and docosahexaenoic acids (100–200 μM) on SFA, MUFA, and PUFA of total lipids and phospholipids from Reuber H35 hepatoma cells. In all classes of fatty acids, palmitic acid produced smaller changes than DHA in both lipid fractions. DHA addition to the culture medium drastically increased the percentage of PUFA and decreased that of MUFA in total lipids. However, these changes tended to be lower in the phospholipid than in the total lipid fraction.

Table VI shows that the saturated/unsaturated fatty acids ratio in total lipids and phospholipids from Reuber H35 hepatoma cells clearly increased when the medium was supplemented with DHA. This increase was more marked than that found after palmitic acid supplementation, mainly due to the great increase in the levels of SFA induced by DHA. The increase originated by DHA in the levels of *n*-3 acids was neutralized by the decrease in those of MUFA, so that total even unsaturated fatty acids decreased as a consequence of supplementing the medium with DHA. The *n*-3/*n*-6 fatty acids ratio drastically increased with the addition of DHA.

In conclusion, all the above mentioned findings indicate that the preincubation of Reuber H35 hepatoma cells with different fatty acids (16:0 or 22:6 *n*-3) modifies the fatty acid composition of these cells. The profound changes in the percentages of MUFA and PUFA induced when the culture medium was supplemented with DHA, especially those changes in the phospholipid fraction, may be of considerable significance, given that these components determine to a great extent the chemical and physical properties of biological membranes and/or membrane enzyme proteins. We also conclude that Reuber H35 hepatoma cells represent an appropriate experimental model for investigating the metabolic reactions of both saturated and unsaturated fatty acids as well as the lipid metabolism associated with them. Moreover, our results emphasize the importance of a precise knowledge of lipid composition of cells and culture media in the *in vitro* studies of cancer cells.

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