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Modification of Phospholipids Fatty Acid Composition in Reuber H35 Hepatoma Cells: Effect on HMG-CoA Reductase Activity

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Abstract There is controversy about the effect of saturated and polyunsaturated fats on 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the main regulatory enzyme of cholesterogenic pathway. Results from dietary studies are difficult to interpret because diets normally contain a mixture of fatty acids. Therefore, we have used Reuber H35 hepatoma cells whose phospholipids were enriched in different individual fatty acids and have studied their effects on the cellular reductase activity. Lauric, myristic, eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids were supplemented to the culture medium coupled to bovine serum albumin. The four fatty acids were incorporated into phospholipids from cells grown in media containing whole serum or lipoprotein-poor serum (LPPS). Reductase activity of cells cultivated in a medium with LPPS was three to four times higher than those cultivated in medium with whole serum. Saturated fatty acids increased reductase activity of cells grown in medium with whole serum, whereas n-3 polyunsaturated fatty acids (PUFA) decreased it. However, both saturated and polyunsaturated fatty acids increased reductase activity when serum lipoproteins were removed. In conclusion, this is one of the first reports demonstrating that saturated and n-3 PUFA only show differential effects on HMG-CoA reductase activity in the presence of lipoproteins. J. Cell. Biochem. 90: 586–591, 2003.

Key words: HMG-CoA reductase; Reuber H35 hepatoma cells; n-3 polyunsaturated fatty acids; saturated fatty acids; phospholipids

Different risk factors have been implicated in the high incidence of atherosclerosis and coronary heart disease found in many Western societies, an elevated plasma cholesterol concentration [Eisenberg, 1998], being one of the most important risk factor. Dietary fatty acid composition can influence serum cholesterol levels. Thus, the hypercholesterolemic effects of saturated fatty acids are well established [Grundy and Denke, 1990], whereas in polyunsaturated fat enriched diets it has been demonstrated to decrease plasma total and low density lipoprotein (LDL) cholesterol levels [Hayes and Khosla, 1992]. In this sense, we have reported that coconut oil induced a rapid hypercholesterolemia in young chicks [Gil-Villarino et al., 1998] and that supplementation of 10% menhaden oil to the chick diet for 7 days produced a significant hypocholesterolemia and hypotriglyceridemia [Castillo et al., 1999]. More recently, we have demonstrated that replacement of saturated fat in chick diet with menhaden oil produced a significant reversion of the hypercholesterolemia previously induced by coconut oil feeding [Castillo et al., 2000]. Saturated fatty acids can not be regarded as a single entity, because differences in the chain length seem to influence plasma cholesterol concentration differently [Hayes et al., 1991; Khosla and Hayes, 1991]. Myristic acid appears to be the principal saturated fatty acid that

Abbreviations used: ACAT, acyl-CoA cholesterol acyltransferase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LDL, low-density lipoproteins; LPPS, lipoprotein-poor serum; PUFA, polyunsaturated fatty acids.

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raises plasma cholesterol [Hayes and Khosla, 1992].

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase is the enzyme that catalyzes the formation of mevalonate from HMG-CoA, the rate-limiting step in the biosynthesis of cholesterol. This enzyme is regulated by different mechanisms which include transcriptional, translational, and posttranslational control, as well as regulation of its catalytic activity by phosphorylation–dephosphorylation processes, or membrane fluidity changes [Sato and Takano, 1995]. Several authors have reported a decreased HMG-CoA reductase activity resulting from consumption of fish oils, which are rich in n-3 polyunsaturated fatty acids (PUFA) [Murthy et al., 1988; Choi et al., 1989]. In our laboratory, we have demonstrated that menhaden oil drastically decreased reductase activity from chick liver, whereas no changes were observed after diet supplementation with saturated fat rich in myristic and lauric fatty acids [Castillo et al., 1999a]. Likewise, Mizuguchi et al. [1993] reported that a highly purified ethyl ester of all-cis-5, 8, 11, 14, 17-eicosapentaenoate significantly inhibited liver HMG-CoA reductase.

The aim of this work was to contribute to clarify the effects of fatty acids on the main regulatory enzyme of cholesterogenesis. To avoid the difficulty of interpreting results from dietary studies, we have used as experimental model cultures of rat hepatoma cells line Reuber H35. These cells have been used for studying stress response or pathways for removal of many xenobiotics [Donato et al., 1994; Wiegant et al., 1997]. Concerning cholesterol metabolism, the possible relationship between the level of sterol carrier protein 2 and cholesterol biosynthesis and intracellular esterification has been investigated in Reuber H35 hepatoma cells [van Amerongen et al., 1985; van Heusden et al., 1985]. We have studied whether membrane lipid composition of these cells could be modified by supplementing the culture medium with specific fatty acids and, if so, the effect of these fatty acids on HMG-CoA reductase. With this purpose, we have assayed eicosapentaenoic (EPA) acid and docosahexaenoic (DHA) acid, two n-3 PUFA very abundant in fish oils, and lauric and myristic acids, considered as two of the most hypercholesterolemic saturated fatty acids. This is the first study that demonstrates the regulation of HMG-CoA reductase of Reuber H35 hepatoma cells by modifications of their membrane fatty acid composition.

MATERIALS AND METHODS

Materials

[3-^{14}C]-HMG-CoA and [2-^{3}H]-mevalonic acid were from Amersham, Buckinbhamshire, UK. Ready Safe liquid was from Beckman, Fullerton, CA. Fetal bovine serum (FBS) and cell culture medium were from Cultek, Madrid, Spain. Whatman silica gel thin-layer chromatography plates were from Merck, Darmstadt, Germany. Bovine serum albumin, Brij 97 detergent, leupeptine and fatty acids were from Sigma, St. Louis, MO. All other reagents used were of analytical grade.

Cell Cultures

Reuber H35 hepatoma cells were a generous gift of Dr. A.M. Vargas, University of Granada, Spain. Cells were grown in monolayer in Dulbecco's modified Eagle's Minimum Essential Medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml amphotericin B, 4 mM glutamine, and 10% (v/v) whole or lipoprotein-poor FBS. They were usually seeded at a density of 3×10^4 cells/cm^2. Cultures were maintained at 37°C in a humidified atmosphere of 95% air–5% CO_2. Cells were fed fresh medium every 2 days and used in the log phase of growth. Lipoprotein-poor serum (LPPS) was obtained from FBS according to the method of Poumay and Ronveaux-Dupol [1985] lightly modified. Briefly, anhydrous KBr was added to the serum to reach a density of 1.25 g/ml; this solution was centrifuged for 16 h at 104,000*g* at 10°C. The yellow top layer was removed and the 12–14 ml of the intermediate layer was defined as LPPS. KBr was eliminated by dialysis for 72 h at 4°C against five changes of 6 L of 150 mM NaCl. LPPS was stored at −20°C and sterilized by filtration before using it.

Measurement of Fatty Acid Incorporation Into Phospholipids

Fatty acids were added to the cell cultures coupled to albumin in the ratio of 2 mol of fatty acid to 1 mol of albumin. These complexes were constituted by adding the appropriate volume of an 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%. Lipid preparations for lipid extraction were obtained as for enzyme determination. Lipid analysis was carried out as previously described [Martínez-Cayuela et al., 2000]. Briefly, total lipids of 60 μl aliquots of cellular homogenate were extracted according to Folch et al. [1957]; 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 [Skipsky and Barclay, 1969]. Fatty acid methyl esters of phospholipids were prepared by the method of Lepage and Roy [1986] and analyzed in a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector.

Measurement of HMG-CoA Reductase Activity

HMG-CoA reductase activity was measured essentially as described by Shapiro et al. [1974]. This method measures the formation of radioactive mevalonate from [^{14}C]-HMG-CoA, using [^{3}H]-mevalonate as an internal standard. Other details of optimal assay conditions in Reuber H35 hepatoma cells have been reported elsewhere [García-Pelayo et al., 2000]. Reductase activity was expressed as pmol of mevalonate synthesized per min per mg protein.

Statistical Analysis

Data from two independent experiments were pooled together without normalization ($N = 6$) and 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 Scheffe's test.

RESULTS AND DISCUSSION

Cell cultures have been used to study different aspects of lipid metabolism. Thus, cholesterogenic activity of cultured cells can be affected by quality and quantity of lipids in the culture medium. Because of that, we have investigated the effects of different fatty acids on HMG-CoA reductase activity of Reuber H35 hepatoma cells cultivated in medium containing FBS or LPPS. Total cholesterol content of LPPS was about 9.3 times lower than that of FBS (1.86 vs. 11.39 mg/dl of free cholesterol and 22.74 vs. 217.91 mg/dl of cholesteryl ester), while changes in protein content were minimum (34.7 vs. 38.8 mg/dl). We have demonstrated that Reuber H35 hepatoma cells normally grow in absence of lipoproteins, however, many other cells are not able to grow in media without serum [Bailey et al., 1972].

It has been reported that saturated fatty acids and PUFA at concentrations higher than 100 μM inhibited the growth of several cellular lines [Spector and Yorek, 1985]. However, Reuber H35 cells grew in the presence of 50–150 μM lauric, myristic, EPA, and DHA, without a significant modification of cell viability.

The four fatty acids individually supplemented to the culture medium were incorporated into phospholipids from Reuber H35 hepatoma cells grown in FBS (Fig. 1A). Percentage of each fatty acid significantly augmented compared to controls, the increase being proportional to the concentration of fatty acids assayed. Myristic acid was incorporated more clearly than lauric acid. With respect to PUFA, percentage of DHA incorporation was higher than that of EPA. These results demonstrated that, under our experimental conditions, Reuber H35 hepatoma cells actively assimilate saturated fatty acids and PUFA from the culture medium. Similarly, cells grown in LPPS incorporated the fatty acids assayed into their membrane (Fig. 1B). In this case, percentages of incorporation were higher than those found in cells grown in FBS, suggesting that removal of serum lipoproteins from the culture medium turns cells greedy for uptaking the supplemented fatty acid.

Cholesterol is an essential component of mammalian cell membrane. Because of this, cholesterol synthesis must be correctly regulated for the normal cellular growth [Bennis et al., 1993]. HMG-CoA reductase activity from Reuber H35 hepatoma cells cultivated in a medium with LPPS was three to four times higher than those cultivated in a medium with whole serum (Fig. 2), the ratio of specific activities being remarkably constant in all the experiments. In the presence of serum lipoproteins, a feed-back control of the enzyme could regulate cholesterol biosynthesis in Reuber H35 hepatoma cells. Cholesterol requirements of cells growing in a medium with serum are partially satisfied, so that HMG-CoA reductase activity should be low. On the contrary, cells growing in a medium without lipids have to synthesize cholesterol; therefore, HMG-CoA

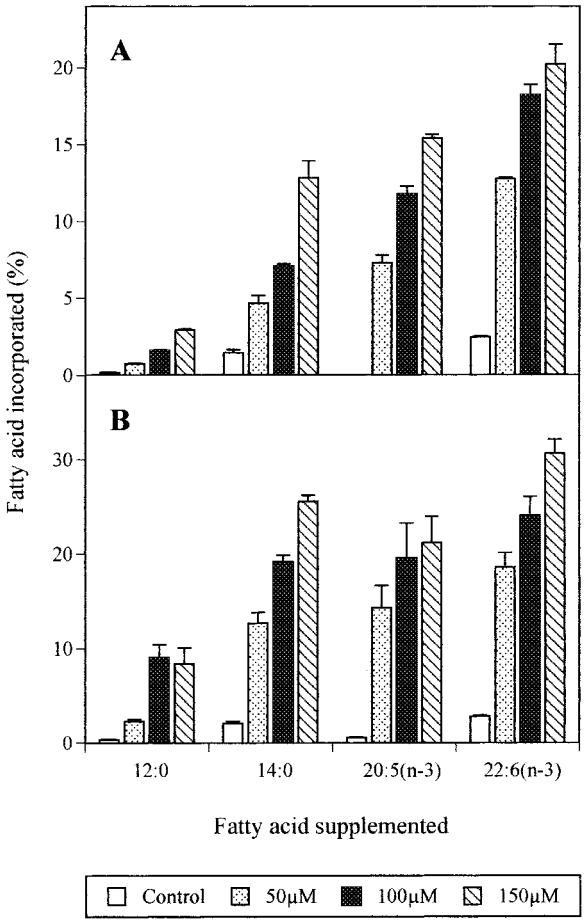

Fig. 1. Percentage of lauric, myristic, eicosapentaenoic (EPA), or docosahexaenoic (DHA) acids in phospholipids of Reuber H35 hepatoma cells grown in a medium with fetal bovine serum (FBS) (**A**) or lipoprotein-poor serum (LPPS) (**B**) and supplemented with each one of the named fatty acids. Results are expressed as mean values ± SEM. Data from two independent experiments were pooled together without normalization (N = 6). All mean values were significantly different when compared 50, 100, 150 μM versus control ($P < 0.05$).

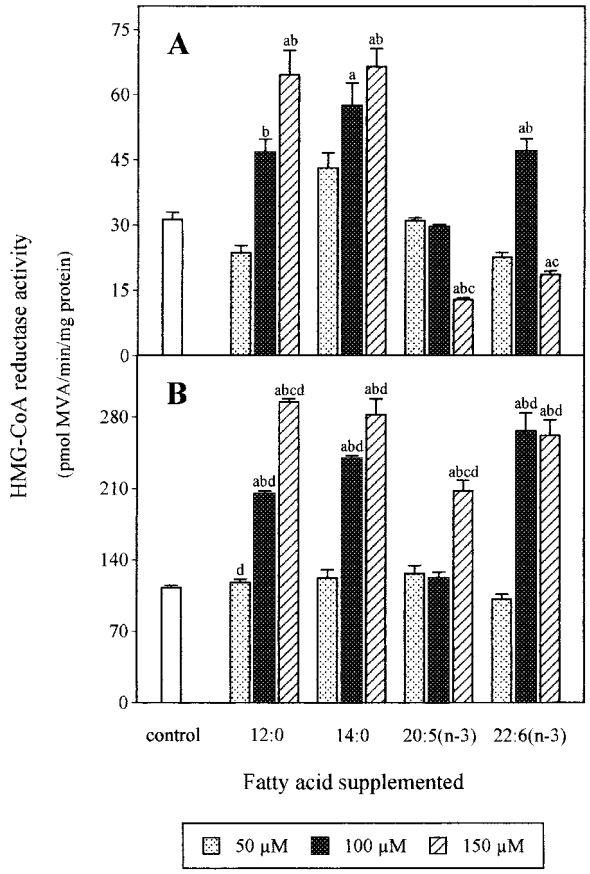

Fig. 2. 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity of Reuber H35 hepatoma cells grown in a medium with FBS (**A**) or LPPS (**B**) and supplemented with lauric, myristic, EPA, and DHA acids. Results are expressed as mean values ± SEM. Data from two independent experiments were pooled together without normalization (N = 6). Statistical significance ($P < 0.05$) is indicated by (**a**) 50, 100, 150 μM versus control; (**b**) 100, 150 μM versus 50 μM; (**c**) 150 versus 100 μM; (**d**) LPPS versus FBS.

reductase activity should augment. It has been suggested that malignancy is associated with lipid metabolism disturbances caused by the absence of different enzyme activities or defective regulator systems [Beirne and Watson, 1976]. Thus, it has been reported that some hepatoma cellular lines do not show the feedback control of reductase activity [Barnard et al., 1984]. Our results demonstrated that malignancy has not to be necessarily coupled to a loss of cholesterogenesis regulation.

Cholesterol metabolism in cell cultures can be regulated by changes in the fatty acid composition of the culture medium [Murthy et al., 1988]. HMG-CoA reductase activity from Reuber H35 hepatoma cells was modified when lauric, myristic, EPA, or DHA acids were incorporated into their membranes. When Reuber H35 hepatoma cells were cultivated in a medium with whole serum and supplemented with lauric or myristic acids (100–150 μM), the HMG-CoA reductase activity increased, the increase being proportional to the fatty acids concentration assayed (Fig. 2A). According to Daumerie et al. [1992], the activity of LDL receptor would be determined by the quantity of cholesterol in a cellular putative regulatory pool. Cholesterol concentration in this pool would be in equilibrium with cholesterol sequestered in the ester pool. Saturated fatty acids are not preferential substrate for acyl-CoA cholesterol acyltransferase (ACAT). Because

of this, when cholesterol ester concentrations decreased, the regulatory pool would increase inducing a decrease of LDL receptor activity. Therefore, the decrease of lipoproteins uptake could account for the increase of HMG-CoA reductase activity.

EPA and DHA, supplemented at 150 μM concentration, decreased reductase activity of Reuber H35 cells cultivated in medium with FBS. Lower concentrations did not significantly modify this enzymatic activity, except 100 μM DHA (Fig. 2A). Again EPA and DHA down-regulation could be explained by an indirect effect mediated by LDL receptor. Thus, Rumsey et al. [1995] have demonstrated that HepG2 hepatoma cells grown with EPA showed an increased activity of LDL receptors, originating a greater intake of cholesterol to the cells, so that HMG-CoA reductase activity would decrease. If we consider the existence of a putative cholesterol pool, EPA and DHA, as the best substrates for ACAT, would provoke an increase of the cholesterol ester pool, inducing a decrease of the cholesterol concentration in the regulatory pool and, so, the LDL receptors activity would increase. Thus, the increase in lipoproteins uptake could decrease the HMG-CoA reductase activity. Surprisingly, DHA supplemented to the culture medium at 100 μM concentration increased reductase activity of Reuber H35 cells growing in presence of lipoproteins in all experiments carried out. This contradictory result is comparable to that obtained by other authors [Lindsey et al., 1992]. Anyway, changes in HMG-CoA reductase activity correlated to those found in the amount of cellular cholesterol (0.130, 0.135, and 0.126 mg cholesterol/mg proteína for 50, 100, and 150 μM DHA concentrations, respectively). It seems likely that very high concentrations of certain n-3 PUFA might provoke a different response to that with low ones.

In the absence of lipoproteins, saturated fatty acids increased HMG-CoA reductase activity proportionally to the concentrations of fatty acids assayed, these increases being significant at the higher concentrations (Fig. 2B). EPA also increased enzymatic activity but it was only significant at 150 μM. DHA significantly increased reductase activity at 100 and 150 μM. The fact that the four fatty acids increased enzymatic activity appears to indicate an unspecific effect of them. As we reported above, fatty acids are more easily incorporated into membranes of cells grown in LPPS than into those grown in FBS. The more fatty acids are available to be uptaken, the more actively the cells multiply; therefore, in order to synthesize new membranes, cellular cholesterol requirements increase. That would probably bring about an increase in HMG-CoA reductase activity.

As the activity of membrane-bound enzymes can be modulated by particular phospholipids and by changes in their fatty acids composition, it is impossible to discard a regulatory mechanism of the HMG-CoA reductase by fatty acids mediated in changes of the membrane fluidity. In fact, hydrocarbonated chain length and saturation grade of the fatty acids of membrane phospholipids affect the enzyme activity. Previous experiments [Gil-Villarino, 1998] had shown that different fatty acids incorporated into microsomal membranes from chick liver modified the HMG-CoA reductase activity. These changes correlated to the variations observed in the cholesterol/phospholipids molar ratio, usually considered as an index of membrane fluidity. Our present results showed that the effects of saturated and n-3 PUFA on HMG-CoA reductase activity from Reuber H35 hepatoma cells were clearly dependent on the presence or absence of lipoproteins in the medium. Thus, we have demonstrated that differential effects of the fatty acid assayed on HMG-CoA reductase were only patent in the presence of lipoproteins, experimental conditions closer to those found in vivo.

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