Differential Translational Effects of Myristic Acid and Eicosapentaenoic Acid on 3-Hydroxy-3-Methylglutaryl-CoA Reductase From Reuber H35 Hepatoma Cells

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The mechanisms by which saturated and polyunsaturated fatty acids may exert their effects on levels of blood cholesterol and human atherosclerosis have not been fully established. In this work, we studied the translational effects of myristic (14:0) and eicosapentaenoic (20:5) acids on 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase from Reuber H35 hepatoma cells. This enzyme is an intrinsic membrane, 96-kDa protein whose proteolysis releases an enzymatically active, 52- to 56-kDa, soluble fragment. We optimized an immunoblot procedure for quantifying small amounts of both the native and the soluble forms of HMG-CoA reductase from Reuber H35 hepatoma cells. We demonstrated that the upregulation of HMG-CoA reductase by myristic acid is due to an increase of the HMG-CoA reductase protein; therefore, protein synthesis would be required for the increase of HMG-CoA reductase activity caused by this fatty acid. In contrast, the downregulation of HMG-CoA reductase caused by eicosapentaenoic acid is not due to decreased protein synthesis, since similar levels of protein were found in the presence and absence of this fatty acid. Results obtained with cycloheximide as a protein-synthesis inhibitor confirm these findings. Exp Biol Med 228:781–786, 2004.

Key words: eicosapentaenoic acid; HMG-CoA reductase; myristic acid; Reuber H35 hepatoma cells

INTRODUCTION

Several epidemiological studies have shown a correlation among the consumption of different fatty acids, the levels of blood cholesterol, and the degree of atherosclerosis and coronary heart disease (1–6). Although it has been postulated that saturated fatty acids show a hypercholesterolemic effect opposite that of polyunsaturated fatty acids (PUFA), the effect of the different fatty acids on the endogenous cholesterol biosynthesis is not clear (7–10). The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase catalyzes the conversion of HMG-CoA to mevalonate, the rate-limiting step of the cholesterol biosynthetic pathway. This enzyme is a glycoprotein of the endoplasmic reticulum with a molecular weight of 97 kDa and consists of three regions: a cytosolic catalytic domain, a small linker, and a multiple transmembrane domain. The regulation of HMG-CoA reductase is very complicated and occurs at the transcriptional, translational, and posttranslational levels, as well as by direct modulation of the enzymatic activity by reversible phosphorylation, oxidation events, or membrane fluidity changes (11, 12).

To avoid the difficulty of interpreting results from dietary studies, we used cultures of rat hepatoma cell line Reuber H35 as the experimental model. By using cell cultures, Murthy *et al.* (13) demonstrated that HMG-CoA reductase activity from CaCo-2 cells is modified when membrane fatty acids saturation is altered by growing cells in different fatty acids. More recently, we demonstrated that HMG-CoA reductase activity from Reuber H35 hepatoma cells increases when cells are cultivated in a medium supplemented with saturated fatty acids, whereas reductase activity decreases when n-3 PUFA are the fatty acids supplemented (14). Myristic acid appears to be the principal saturated fatty acid that raises plasma cholesterol levels (15), whereas eicosapentaenoic acid (EPA) is believed to be one of the major active fatty acids that have a hypolipidemic action (16). Although the effects on plasma cholesterol are less consistent, different works have demonstrated that EPA reduces cholesterol levels by inhibiting HMG-CoA reductase activity (8, 10, 13, 14, 17, 18). To determine whether HMG-CoA reductase synthesis from Reuber H35 hepatoma cells is involved in the hypercholesterolemic and hypocho-

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lesterolemic effects of myristic acid and EPA, respectively, we quantified HMG-CoA reductase protein from these cells.

Standard immunoprecipitation procedures for soluble proteins are well established. However, results with membrane proteins are not clear. Thus, weak and irreproducible signals are common problems in immunoblots of hydrophobic integral membrane proteins (19). This is due, in part, to the low abundance of the protein or to an inefficient electrophoretic transfer. It is well known that the HMG-CoA reductase is a very labile microsomal protein that can even be broken in the presence of protease inhibitors (20). We have also demonstrated that HMG-CoA reductase activity from Reuber H35 hepatoma cells grown in whole serum is very low (21). Due to this, it was necessary to assay different electrophoretic and transfer conditions to be able to quantify HMG-CoA reductase protein from Reuber H35 hepatoma cells.

The aim of this work was to study the effects of myristic acid and EPA incorporated into cellular membranes on the level of HMG-CoA reductase protein from Reuber H35 hepatoma cells. Although several authors have found only a single 97-kDa band for the microsomal HMG-CoA reductase when cell extracts were immunoblotted (22, 23), we observed substantial amounts of the soluble 52- to 56-kDa HMG-CoA reductase in addition to the native 97-kDa form. Our optimized method permitted evaluation, for the first time to our knowledge, of the possible translational effect of myristic acid and EPA on the HMG-CoA reductase from Reuber H35 hepatoma cells. Levels of HMG-CoA reductase activity found in the presence of the different fatty acids and with cycloheximide as a protein-synthesis inhibitor confirm results obtained by immunoblot and demonstrate the involvement of a translational control of the HMG-CoA reductase mediated by myristic acid. No translational effect was observed with EPA.

MATERIALS AND METHODS

Materials. Cell culture medium and fetal bovine serum were obtained from Cultek, Madrid, Spain. Electrophoretic supplies were obtained from Bio-Rad, Madrid, Spain. Polyvinylidene difluoride membranes and ECL-Plus Western blotting detection system were from Amersham, Little Chalfont, England. Polyclonal antisera to rat HMG-CoA reductase were generously provided by Dr. P. A. Edwards, Departments of Biological Chemistry and Medicine, UCLA School of Medicine, Los Angeles, CA. Recombinant HMG-CoA reductase from *Leishmania* was kindly donated by Dr. D. Gónzalez-Pacanowska, CSIC, Granada, Spain. Total and free cholesterol tests were from DIPAL-Inquebor, Granada, Spain. All other reagents were from Sigma, Madrid, Spain.

Cell Cultures. Reuber H35 hepatoma cells were grown in monolayer in Dulbecco's modified Eagle's medium, as previously described (24). Myristic acid and EPA were assayed at the150 μ*M* concentration. Both fatty acids were added to the cell cultures coupled to fatty acid–free bovine serum albumin in the ratio of 2 moles of fatty acid to 1 mole of albumin. Cycloheximide was assayed at 100 μ*M*. The appropriate volume of an ethanolic solution of cycloheximide was added to the culture medium after 64 hrs of growing cells with the different fatty acids; these cells were incubated for 8 hrs with the protein-synthesis inhibitor. Final concentration of ethanol in the culture medium was always less than 0.1%.

HMG-CoA Reductase Assay. The HMG-CoA reductase activity was measured essentially as described by Shapiro *et al.* (25). This method measures the formation of radioactive mevalonate from [^{14}C]-HMG-CoA, using [^{3}H]-mevalonate as an internal standard. Specific activity of the substrate was 2584 dpm/nmole. Mevalonate from the internal standard, as well as that formed during the reaction, was lactonized and separated from the HMG-CoA by thin-layer chromatography (benzene-acetone; 1:1, v/v). Mevalonolactone was counted for both ^{14}C and ^{3}H. Details of the enzyme preparation and optimal assay conditions in Reuber H35 hepatoma cells have been reported elsewhere (21). Reductase activity was expressed as pmol of mevalonate synthesized per minute per milligram of protein.

Cholesterol and Protein Assays. Cellular cholesterol content was assayed by enzymatic colorimetric methods using total and free cholesterol tests from DIPAL. The homogenate used was obtained as previously described (21). Proteins were determined according to the method of Lowry *et al.* (26).

Electrophoresis and Immunoblotting. Cellular extract, obtained as for enzyme determination, was centrifuged at 12,000 *g* for 10 mins at 4°C. Portions of the supernatant, with 5 μg of protein, in a final volume of 50 μl, were loaded onto slab gels that contained 7.5% polyacrylamide in the separating gel and 4% in the stacking gel (27). Electrophoresis was conducted with a constant voltage of 150 V. Proteins were incubated in the sample buffer at room temperature for 60 mins before their application. The sample buffer consisted of 125 m*M* Tris-HCl, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 0.04% (w/v) bromophenol blue. The separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes in 25 m*M* Tris, 192 m*M* glycine, and 20% methanol for 1 hr at 2°–8°C and with a constant voltage of 100 V. The membranes were blocked with 5% nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline (TBS-T) at room temperature for 1 hr while shaking. After two washes, each for 5 mins in TBS-T, membranes were incubated for 1 hr with a 1/20,000 dilution of rabbit anti-rat HMG-CoA reductase polyclonal antibody. The membranes were then washed four times for 10 mins with TBS-T and incubated with a 1/4,000 dilution of peroxidase-conjugated anti-rabbit IgG, followed by another four washes for 10 mins with TBS-T. Proteins were detected using the ECL kit from Amersham. The relative levels of

immunoreactive HMG-CoA reductase were measured by densitometric scanning.

Statistical Analysis. HMG-CoA reductase activity and ratio of cholesterol to protein data are expressed as mean ± SE values of three experiments. Each experimental value was the mean of triplicate determinations. Data were analyzed by one-factor analysis of variance. When the overall F statistic was statistically significant ($P < 0.05$), analyses of significance were determined by the Scheffé test.

RESULTS AND DISCUSSION

Immunoblot of HMG-CoA Reductase. Standard immunoblotting procedures give a weak signal with integral membrane proteins, especially when proteins are not abundant, because they are poorly transferred (19, 28). Besides being a microsomal protein, HMG-CoA reductase from Reuber H35 hepatoma cells shows very low activity when cells are grown with whole fetal bovine serum (14). Hence, we tested different conditions to improve the detection of HMG-CoA reductase. Urea (6–8 *M*) has been used for the transfer of cell membrane proteins from SDS gels, giving good results (29). In our case, 7 *M* urea did not improve the protein electroelution from gels, not even with a transblotting time of 15 hrs. It has also been demonstrated that omission of methanol increases the amount of the peroxisomal membrane proteins recovered on the transblot membrane (19). However, our data showed that HMG-CoA reductase from Reuber H35 hepatoma cells was more efficiently transferred with the presence of methanol in the electrophoretic transfer buffer.

Ness *et al.* (30) showed that the addition of leupeptin, an inhibitor of cysteine proteinases, prevents HMG-CoA reductase solubilization. Chin *et al.* (31) found that, when HMG-CoA reductase from a line of CHO cells is solubilized in the presence of ethylene glycol-bis-(β-amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA) and leupeptin and examined by immunoblotting, a single band with a molecular weight of 90 kDa is observed. We obtained substantial amounts of the soluble HMG-CoA reductase with a molecular weight of 52–56 kDa, in addition to the 97-kDa native microsomal form, when extracts were immunoblotted; in this case, neither EGTA nor leupeptin completely prevented proteolysis of HMG-CoA reductase from Reuber H35 hepatoma cells. These findings agree with those obtained in previous works where partial solubilization of the enzyme was observed (21).

To achieve a successful separation of the native 97-kDa from the soluble 52- to 56-kDa HMG-CoA reductase, we assessed the use of different acrylamide percentages and volumes of stacking gel. Seven-centimeter gels with 7.5% acrylamide in the separating gel and 1.5 ml of 4% acrylamide in the stacking gel provided the suitable resolution. The rabbit anti-rat HMG-CoA reductase polyclonal antibody was tested in different concentrations by dot blotting against a recombinant HMG-CoA reductase from *Leishmania*. The 1/20,000 dilution gave the best results. Secondary antibody was used at 1/4,000 as the optimal dilution.

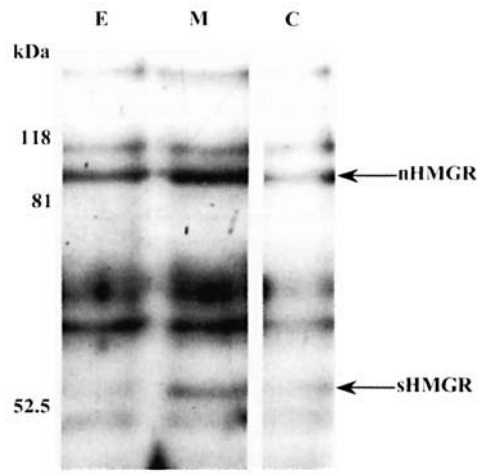

Figure 1. Immunoblot of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase from Reuber H35 hepatoma cells grown in a medium with fetal bovine serum and supplemented with 150 μ*M* myristic or eicosapentaenoic acid. C, control cells. M, cells grown with myristic acid. E, cells grown with eicosapentaenoic acid.

Figure 1 shows the clear separation of the bands corresponding to the native 97-kDa and soluble 52- to 56-kDa HMG-CoA reductase from Reuber H35 hepatoma cells grown under different conditions. Previous densitometric analysis of the area corresponding to the 97-kDa and 52- to 56-kDa bands from the immunoblots revealed that the intensities of these bands were proportional to the quantity of protein loaded on gels, at least between 5 and 20 μg of protein assayed (data not shown). In addition to the HMG-CoA reductase bands, other bands were observed due to nonspecific immunoreactions of the polyclonal antisera.

Effect of Myristic Acid and EPA on HMG-CoA Reductase Protein. Different epidemiological and biochemical studies suggest that fish oil consumption confers benefits to the cardiovascular function in humans (3, 5, 6). The protective effects of fish oil intake could be caused by the n-3 PUFA. It has been demonstrated that one of the most important factors implicated in atherosclerosis is an elevated plasma cholesterol concentration (3, 32). Diets rich in saturated fatty acids are associated with increased levels of plasma cholesterol; however, the effect of n-3 PUFA is less clear (33, 34). In previous works, we have demonstrated that myristic acid, the main saturated fatty acid that raises plasma cholesterol levels (15), supplemented to the culture medium at a 150 μ*M* concentration, increases HMG-CoA reductase activity of Reuber H35 hepatoma cells, whereas EPA, at the same concentration, decreases it (14). Later results showed that 150 μ*M* myristic acid significantly increased ratios of total cholesterol to protein and free cholesterol to protein in these cells (Table 1). In contrast, 150 μ*M* EPA decreased both ratios under the same experimental conditions. Control

Table 1. Effects of Supplementing the Culture Medium with Myristic and Eicosapentaenoic Acids on Ratios of Free Cholesterol to Protein and Total Cholesterol to Protein of Reuber H35 Hepatoma Cells[a]

	Ratio of free cholesterol to protein (mg/mL)	Ratio of total cholesterol to protein (mg/mL)
Control	0.136 ± 0.003	0.147 ± 0.003
Myristic acid	0.148 ± 0.002*	0.159 ± 0.002*
Eicosapentaenoic acid	0.124 ± 0.001*	0.124 ± 0.002**

[a] Results are expressed as mean ± SE of three experiments. Triplicate determinations were made in each experiment.
*$P < 0.05$ and **$P < 0.01$ with respect to the corresponding control.

values of cholesterol found at the start of incubation in the absence of myristic acid and EPA were similar to those observed at the end of incubation in control cells. Thus, data obtained in our experiments are representative of newly synthesized cholesterol in response to media. These results agree with changes induced in the HMG-CoA reductase activity by both fatty acids.

The molecular mechanisms by which fatty acids exert their effects on the HMG-CoA reductase are unknown. HMG-CoA reductase is regulated at several levels of gene expression (11, 12). Translational regulation of HMG-CoA reductase by sterol and nonsterol products has been demonstrated (35–37). However, translational regulation of this enzyme by fatty acids has not been previously reported. Consequently, we have evaluated the possible translational effects of myristic acid and EPA on HMG-CoA reductase from Reuber H35 hepatoma cells.

The amount of HMG-CoA reductase from Reuber H35 hepatoma cells grown in the presence of 150 μ*M* myristic acid was higher than that from cells grown without fatty acid supplementation (Fig. 2) both in 97-kDa and 52- to 56-kDa bands. Changes in the amount of reductase protein, as determined by immunoblotting, were parallel to changes in the enzymatic activity found in previous works (14). The data presented suggest that the activating effect of myristic acid on the HMG-CoA reductase may take place at the translational level.

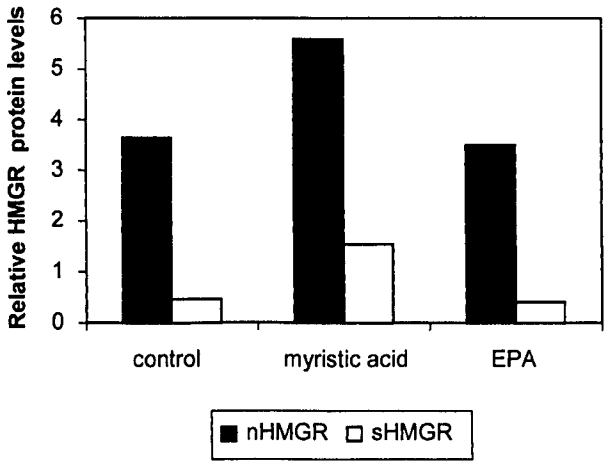

Figure 2. Relative immunoblot density of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase from Reuber H35 hepatoma cells grown in a medium with fetal bovine serum and supplemented with 150 μ*M* myristic or eicosapentaenoic acid. The immunoblot illustrated in Figure 1 was scanned and quantified using Scion Image. Data of relative HMG-CoA reductase levels were calculated by comparing the area of the peaks of the bands corresponding to the native and soluble HMG-CoA reductase with a control band. nHMGR, native HMG-CoA reductase. sHMGR, soluble HMG-CoA reductase.

Several authors have demonstrated that EPA decreases HMG-CoA reductase (8, 10, 13, 38). When EPA was added to the culture medium of cells, the immunoblot analysis revealed that the amount of the 97-kDa and 52- to 56-kDa HMG-CoA reductase protein was similar to that of the control (Fig. 2). These results demonstrated that suppression of HMG-CoA reductase activity previously observed in Reuber H35 hepatoma cells incubated with EPA (14) was not parallel to the decrease in the amount of reductase protein. Thus, the downregulation of HMG-CoA reductase by EPA may be mediated by a mechanism different to the modification of the protein synthesis or degradation.

Effect of Cycloheximide on HMG-CoA Reductase Activity. To confirm these findings and to determine whether protein synthesis was required for the upregulation or downregulation of HMG-CoA reductase from Reuber H35 hepatoma cells, we studied the effect of adding cycloheximide to the culture medium of cells that were growing in the presence of the different fatty acids assayed. We incubated cells with 100 μ*M* cycloheximide for 8 hrs, a period sufficient to produce significant changes in enzyme activity. To compare HMG-CoA reductase activity from cells grown in the presence or absence of the protein-synthesis inhibitor, activity was expressed as the percentage of specific activity of the corresponding controls. Values of reductase activity of cells grown in the absence of fatty acids were 24.94 ± 1.46 pmol of mevalonic acid (MVA)/min/mg of protein and 46.39 ± 5.98 pmol of MVA/min/mg of protein for cells grown with or without cycloheximide, respectively. Results shown in Figure 3 indicate that HMG-CoA reductase activity of cells grown with 150 μ*M* myristic acid in the presence of cycloheximide was significantly lower than that reached in the absence of the inhibitor. These results agree with those obtained by immunoblot, since a greater amount of reductase protein was found in extracts from cells incubated with myristic acid (Fig. 2). Data for cycloheximide confirm that protein synthesis was required for the enhanced HMG-CoA reductase activity observed with myristic acid. Because the percentage of specific activity of control cells grown with cycloheximide was similar to that of cells grown in the presence of myristic

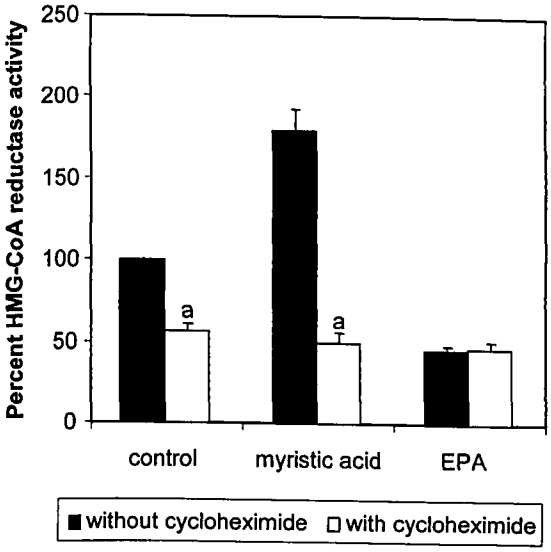

Figure 3. 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity of Reuber H35 hepatoma cells grown with 100 μM cycloheximide in a medium with fetal bovine serum and supplemented with 150 μM myristic or eicosapentaenoic acid. Results are expressed as percentages of specific activity with respect to the control without cycloheximide. In all cases, statistical significance is indicated by $^{a}P < 0.05$ of every condition with respect to the corresponding values obtained without cycloheximide.

acid with cycloheximide, the upregulation of HMG-CoA reductase from Reuber H35 hepatoma cells may be due to an increase in protein synthesis.

With respect to EPA, when the synthesis of proteins was inhibited by the addition of cycloheximide to the culture medium, HMG-CoA reductase activity of cells grown with 150 μM EPA was not significantly different from that of cells grown with this fatty acid but without the inhibitor (Fig. 3). These results also support those obtained by immunoblot, since similar amounts of reductase protein were found in control cells and cells grown in the presence of EPA. In this way, it is possible to rule out an inhibitor effect of EPA on the HMG-CoA reductase activity from Reuber H35 hepatoma cells mediated by a modification of the enzymatic protein synthesis.

Our results show, for the first time to our knowledge, that protein synthesis is involved in the increase of HMG-CoA reductase activity caused by myristic acid in Reuber H35 hepatoma cells, whereas the inhibition of this activity in the presence of EPA is not related to changes in protein synthesis.

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