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Myristic acid, unlike palmitic acid, is rapidly metabolized in cultured rat hepatocytes

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This study was designed to examine and compare the metabolism of myristic and palmitic acids in cultured rat hepatocytes. [1-^{14}C]-Labeled fatty acids were solubilized with albumin at 0.1 mmol/L in culture medium. Incubation with 24-hr cultured hepatocytes was carried out for 12 hr. Myristic acid was more rapidly ($P < 0.05$) taken up by the cells than was palmitic acid (86.9 ± 0.9% and 68.3 ± 5.7%, respectively, of the initial radioactivity was cleared from the medium after 4 hr incubation). Incorporation into cellular lipids, however, was similar after the same time (33.4 ± 2.8% and 34.9 ± 9.3%, respectively, of initial radioactivity). In the early phase of the incubation (30 min), myristic acid was more rapidly incorporated into cellular triglycerides than was palmitic acid (7.4 ± 0.9% and 3.6 ± 1.9%, respectively, of initial radioactivity). However, after 12 hr incubation, the radioactivity of cellular triglycerides, cellular phospholipids, and secreted triglycerides was significantly higher with palmitic acid as precursor. Myristic acid oxidation was significantly higher than that of palmitic acid (14.9 ± 2.2% and 2.3 ± 0.6%, respectively, of the initial radioactivity was incorporated into the β-oxidation products after 4 hr). Myristic acid was also more strongly elongated to radiolabeled palmitic acid (12.2 ± 0.8% of initial radioactivity after 12 hr) than palmitic acid was to stearic acid (5.1 ± 1.3% of initial radioactivity after 12 hr). The combination of elongation and β-oxidation results in the rapid disappearance of C14:0 in hepatocytes whereas C16:0 is esterified to form glycerolipids. This study provides evidence that myristic acid is more rapidly metabolized in cultured hepatocytes than is palmitic acid. (J. Nutr. Biochem. 11:198–207, 2000) *© Elsevier Science Inc. 2000. All rights reserved.*

Keywords: myristic acid; palmitic acid; fatty acid metabolism; β-oxidation; cultured rat hepatocytes

Introduction

The roles of dietary saturated fatty acids relative to monounsaturated and polyunsaturated fatty acids have been investigated mainly in animals and humans[1,2] based on plasma cholesterol level and low density lipoprotein (LDL) metabolism. Indeed dietary saturated fat containing myristate (C14:0), but also laurate (C12:0) and palmitate (C16:0), is generally considered to induce a large increase in plasma cholesterol, especially in LDL cholesterol level.[3] Although the mechanisms underlying these effects are poorly understood and contradictory effects are reported,[4] myristic acid might be the most potent cholesterolemia-increasing fatty acid.[5] However, myristic acid exhibits other important specific functions in the cell: it is known to modify a number of proteins of both eukaryotic and viral origin by acylation,[6] through myristoyl-CoA:protein N-myristoyltransferase (EC 2.1.3.97). Substrate specificity of this enzyme is apparently very high for myristoyl-CoA,[7] which indicates that C14:0 plays an important role in cell regulation. N-myristoylated proteins do have several biological functions, such as signal transduction pathways, vesicular trafficking, and structural roles, which often depend on the presence of myristic acid in the cells.[8–10] The basis for the opposite metabolic effects of myristic acid in terms of nutritional benefits remains under-investigated.

Myristic acid usually accounts for small amounts (approximately 1 wt%) of total fatty acids in animal tissues,[11,12] but is more abundant in milk fat[13,14] (7–12 wt% of total fatty acids) or in copra and palmist oils (15–23 wt% and 15–17 wt%, respectively). Myristic acid utilization has

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been studied mostly in vivo when added to the diets of animals[15,16] and humans.[17,18] Its metabolism has also been investigated in part in cultured muscle cells[19] and in isolated liver cells,[20] but the main data have been obtained by studying protein N-myristoylation[21,22] or the formation of the glycosylphosphatidylinositol anchor of *Trypanosoma brucei* variant surface glycoprotein.[23] However, myristic acid metabolism in the liver remains unclear. The purpose of the present study was to investigate the utilization of myristic acid compared with palmitic acid in cultured rat hepatocytes.

Methods and materials

Reagents and chemicals

Bovine serum albumin (BSA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Williams' medium E (W 4125), insulin (bovine), dexamethasone, collagenase, and cold fatty acids were purchased from Sigma Chemical Co. (St. Louis, MO USA). Penicillin-streptomycin antibiotic mixture was provided by Gibco BRL (Eragny, France). Fetal bovine serum (FBS) was obtained from J. Boy (Reims, France). [1-^{14}C] Myristic acid and [1-^{14}C] palmitic acid were purchased from DuPont NEN (Le Blanc Mesnil, France). Solvent and other chemicals were obtained from Prolabo (Paris, France) or Merck (Darmstadt, Germany), except high purity reagents for high performance liquid chromatography (HPLC) application, which were from OSI (Elancourt, France). Falcon Primaria Petri culture dishes (AES, Combourg, France) were used.

Animals and diet

Sprague-Dawley male rats (250 g body weight) obtained from the breeding center R. Janvier (Le Genest-St Isle, France) were freely fed rat chow (nutriment A 04, purchased from Usine Alimentaire Rationnelle, Epinay-sur-Orge, France) and were used to obtain the hepatocytes. They were food-deprived 12 hr prior to hepatocyte preparation. The experimental protocol was in compliance with applicable guidelines from Ministère de l'Agriculture of France.

Cell culture

Hepatocytes were obtained by collagenase perfusion in situ, according to the procedure of Seglen,[24] adapted in this laboratory.[25] Viability, estimated by using the trypan blue exclusion test, was greater than 90%. The hepatocytes were brought to a concentration of 0.4 10^6 cells/mL and plated on Falcon Primaria Petri culture dishes (60 mm diameter) with 4 mL of suspended cells (1.6 10^6 cells) per dish. The culture medium (Williams' medium E) was supplemented with 26 mmol/L $NaHCO_3$, 12.5 mmol/L HEPES, 15 μmol/L BSA, antibiotic mixture (50,000 IU/L penicillin, 50 mg/L streptomycin), 1 μmol/L insulin, and 1 μmol/L dexamethasone. For plating only, the culture medium was supplemented with 7% FBS. After plating, the cells were maintained in a humidified incubator at 37°C under 5% carbon dioxide (CO_2) in air. After 4 hr, the plating medium was changed to a serum-free culture medium.

Preparation of the fatty acid albumin complex containing media

Each radiolabeled fatty acid, diluted with the corresponding nonradiolabeled fatty acid to the final specific activity (183 MBq/mmol), was incubated for 30 min at 70°C with 300 μL potassium hydroxide (KOH) 2 mol/L in ethanol. The fatty acid salt obtained was dissolved at pH 10 in Williams' medium E containing 0.15 mmol/L BSA. The fatty acid:albumin molar ratio was 0.67:1. After 24 hr of gentle shaking, the pH was adjusted to 7.35. The obtained solutions were used as incubation media. The final fatty acid concentration was 0.1 mmol/L unless indicated otherwise in the figure legends. This concentration can be considered physiologic in terms of fatty acid supply and is related to the relatively low concentration of myristic acid (approximately 27 μmol/L) in rat serum.[11]

To see to what extent each labeled fatty acid was complexed with albumin in the incubation medium, 500 μL of each medium was precipitated with 500 μL of trichloroacetic acid 2.5 mol/L. After 15 min at 0°C followed by centrifugation (2,500 × g, 2°C, 10 min), the radioactivity contained in the supernatant was subjected to liquid scintillation counting (Packard Tri-carb 1600 TR, Meriden, CT USA) and expressed as nmol unbound fatty acid per liter. Verification was previously performed to ensure that trichloroacetic acid did not precipitate unbound fatty acids.

Incubation procedure

After 24 hr of culture, incubation was initiated by replacing the culture medium with 2 mL of the appropriate fatty acid containing medium per dish. Incubation was carried out at 37°C in a 5% CO_2 atmosphere.

Lipid extraction

At the end of the incubation, the medium was taken off, centrifuged (2,500 × g, 4°C, 10 min) to remove any cell debris, and kept for lipid extraction. The cells were washed twice with ice-cold phosphate-buffered saline solution (PBS; 150 mmol/L NaCl, 5 mmol/L Na phosphate, pH 7.4) and harvested with a rubber policeman in PBS. Cell suspensions were then centrifuged at 800 × g for 4 min. The supernatant was discarded and the cell pellet kept for lipid extraction. Lipids from the medium (after acidification with 1 mL HCl 3 mol/L) and from the cells were extracted twice separately with 4 mL and 2 mL hexane:isopropanol (3:2 v/v).[26]

Determination of total cellular fatty acid radioactivity

Lipid extracts from the cells were saponified for 30 min at 70°C by 1 mL of 2 mol/L KOH in ethanol. The fatty acids were liberated by acidification and extracted twice with diethylether. Total cellular fatty acids were then subjected to liquid scintillation counting.

Separation of lipid species

Lipid extracts from the cells and from the medium were submitted to thin layer chromatography using silicagel H plates and a mixture of hexane:diethylether:acetic acid (80:20:0.5 v/v/v) for development. The spots corresponding to cellular phospholipids (PL), mono- and diglycerides (MDG), cholesterol, nonesterified fatty acids (NEFA), triglycerides (TG), and cholesterol esters (CE) were then visualized by spraying with dichlorofluorescein, separately scrapped off the plates and then eluted with 2 mL diethylether, except for the phospholipid spot, which was eluted with 2 mL methanol. For medium lipids, however, only NEFA remaining from the initially added fatty acid and secreted TG were collected. Isolated lipid species from the cells and the medium, except NEFA and cholesterol, were then saponified as described above. Radioactivity of the fatty acids from each lipid species and of cholesterol was counted.

Measurement of the β-oxidation products

To measure the β-oxidation rate, we determined the radioactivity carried by total fatty acids (i.e., the sum of labeled fatty acids remaining in the medium plus total cellular radiolabeled fatty acids and secreted TG fatty acids) after each of the incubation periods.

We also determined the incorporation of ^{14}C from labeled fatty acids into CO_2 and into acid-soluble metabolites after 4 hr incubation. The production of $^{14}CO_2$ was measured as previously described.[26] At the end of the incubation, the cells were harvested using a rubber policeman. Hepatocytes and medium were transferred into a small glass vial containing 250 μL $HClO_4$ (7 mol/L). Each vial was immediately sealed hermetically with a rubber cap fitted with a plastic center well. Hyamine hydroxide (350 μL of a solution at 1 mol/L in methanol; Sigma Chemical Co.) was added to the suspended plastic well by piercing the cap with a syringe. After 2 hr shaking at 37°C, $^{14}CO_2$ trapped in hyamine hydroxide was precipitated with 20 mL barium hydroxide (0.05 mol/L) and 5 mL Na_2CO_3 (0.05 mol/L) as carrier. The resulting precipitate was removed by filtration and washed with 10 mL water, ethanol, and acetone successively. Both the precipitate and the filter paper were dried and put in a scintillation vial to determine their radioactivity, after addition of 10 mL scintillation fluid (Insta-Fluor, Packard).

The production of radiolabeled acid-soluble metabolites was measured according to the method of Mannaerts et al.[27] Hepatocytes and medium of each culture dish were transferred into 250 μL $HClO_4$ (7 mol/L) containing tubes, placed at 0°C for 15 min, and centrifuged (2,500 × g, 10 min, 2°C). The supernatant was collected, washed three times with 2 mL hexane to remove any remaining [1-^{14}C]-labeled fatty acids, and radioactivity was counted.

Individual fatty acid analysis by fatty acid naphthacyl esters preparation and HPLC separation

Fatty acids from each lipid extract were converted to fatty acid naphthacyl esters as recently described.[28] The derivatization procedure was started by the addition of 300 μL 2-bromo-2′-acetonaphthone (0.04 mol/L in acetone) and 300 μL triethylamine (0.1 mol/L in acetone) to the fatty acid extracts (1–3 μmoles). After 15 min in a boiling water bath, 600 μL of acetic acid solution (0.033 mol/L in acetone) was added for an additional 10 min. After evaporation, naphthacyl derivatives were dissolved with 200 μL of a mixture of methanol:dichloromethane (3:1 v/v). Twenty microliters of the derivative solution was used for analysis.

Fatty acid naphthacyl esters were separated on HPLC (Alliance integrated system, Waters, St. Quentin en Yvelines, France) using a Nova-Pak C18 column (4.6 mm × 250 mm, Waters) and a guard column (Nova-Pak C18; 3.9 mm × 20 mm). The separation was performed by elution (1 mL/min) with a linear gradient of methanol:acetonitrile:water starting at 80:10:10 (v/v/v), increasing first to 86:10:4 in 30 min, then increasing to 90:10:0 (v/v/v) in 10 min, holding at 90:10:0 (v/v/v) for 5 min and returning to the initial conditions in 5 min. Elution of naphthacyl derivatives was monitored by ultraviolet absorbance at 246 nm (Tunable absorbance detector 486, Waters). Peaks corresponding to radiolabeled fatty acids were collected (Fraction collector, Waters) and subjected to liquid scintillation counting. Preliminary identification of fatty acid naphthacyl esters was based on retention times obtained for naphthacyl esters prepared from radiolabeled and nonradiolabeled fatty acid standards.[28]

Identification of radiolabeled monounsaturated fatty acids

To identify the radiolabeled monounsaturated fatty acids derived from [1-^{14}C] myristic and palmitic acids, the position of the double bond was determined according to the method of Von Rudloff,[29] adapted in this laboratory for fatty acid naphthacyl esters.[28] Unsaturated fatty acid naphthacyl esters separated by HPLC were collected. They were then submitted to an oxidative cleavage by a mixture of permanganate-periodate during 6 hr. Produced monocarboxylic and dicarboxylic acids were extracted by addition of diethylether, converted to naphthacyl esters as previously described, and separated by HPLC with a first isocratic elution (1 mL/min) of acetonitrile:water 50:50 (v/v) for 10 min, then increasing linearly to 100:0 (v/v) for 40 min. Retention times of monocarboxylic and dicarboxylic acids were previously determined by standards analysis (Fluka, St. Quentin Fallavier, France). Radiolabeled dicarboxylic acids were collected and subjected to liquid scintillation counting. The radioactivity recovered in dicarboxylic acids and the chain length indicate the double bond position on the monounsaturated fatty acid.

Protein and DNA measurements

The total cellular protein content of a culture dish was determined by a modified Lowry procedure.[30] The DNA content was measured by a fluorometric method using bisbenzimidazole (Sigma Chemical Co., St. Louis, MO USA).[31]

Results' expression and statistical analysis

The values reported are means ± SD (n = 3). P-values were calculated using the Student's t-test for two-group comparisons. The differences in comparing ^{14}C incorporation from the two labeled fatty acids into one fraction were analyzed after each of the incubation periods. The differences in comparing ^{14}C incorporation from one labeled fatty acid into two fractions were also analyzed at each time point. Finally, the differences in comparing ^{14}C incorporation from one labeled fatty acid into one fraction were analyzed after two incubation periods. The differences were considered significant at a P-value of less than 0.05.

Results

Clearance of [1-^{14}C]-labeled fatty acids from the culture medium and ^{14}C incorporation into fatty acids of cellular and secreted lipids

Figure 1A presents the clearance of [1-^{14}C] myristic acid from the culture medium and the incorporation of ^{14}C from myristic acid into fatty acids of cellular lipids and secreted triglycerides as a function of incubation time with cultured hepatocytes. Only the main radiolabeled lipid species are presented in this figure. The results are expressed as a percent of the initial radioactivity added to the medium and recovered in each fraction.

The decrease of [1-^{14}C] myristic acid in the culture medium showed that myristic acid was rapidly taken up by the cells. After 4 hr of incubation, 13.0 ± 0.9% of the initial precursor was still in the medium. After 12 hr of incubation there was almost no more myristic acid in the medium (0.9 ± 0.5%). The increase of ^{14}C incorporation from myristic acid into total cellular fatty acids was significant during the first 4 hr of incubation. Then the radioactivity of total cellular fatty acids remained at a steady-state level during the remaining 8 hr (approximately 34% of initial radioactivity). The distribution of ^{14}C from myristic acid between the fatty acids of cellular and secreted lipid species initially showed a rapid incorporation into cellular TG

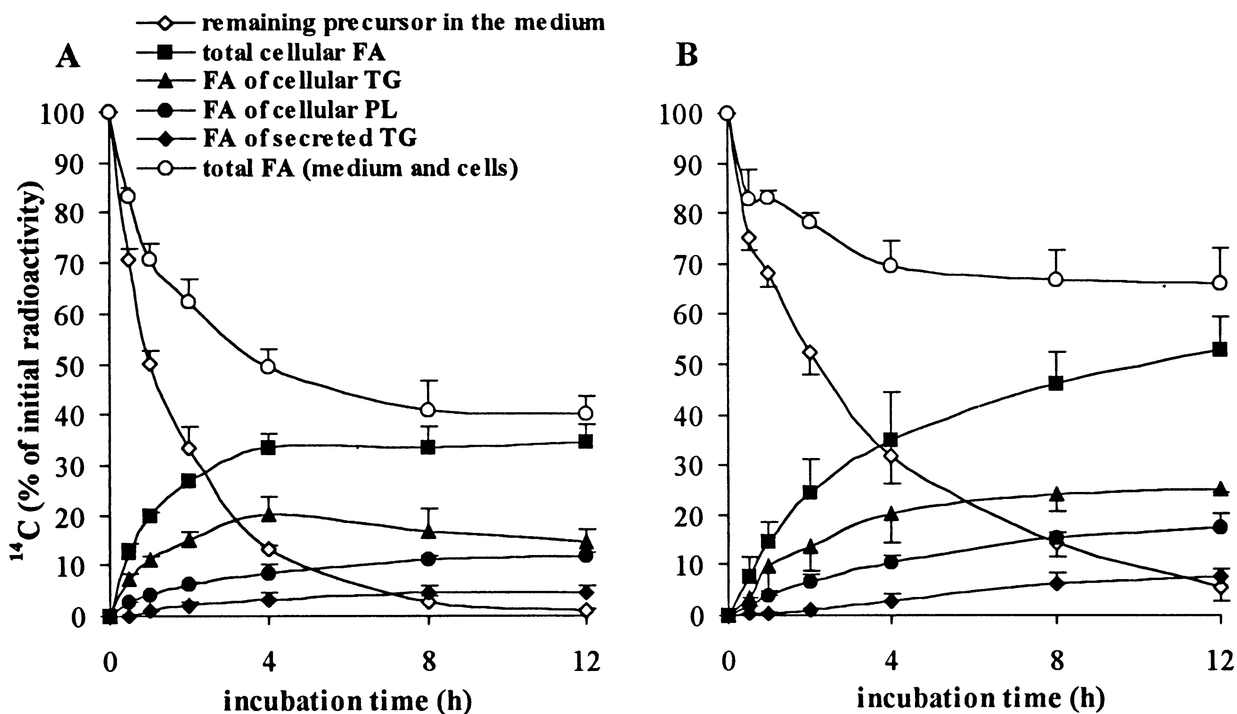

Figure 1 Clearance of (A) [1-^{14}C] myristic acid and (B) [1-^{14}C] palmitic acid from the culture medium and ^{14}C incorporation into fatty acids (FA) of cellular and secreted lipids as a function of incubation time with cultured rat hepatocytes. [1-^{14}C]-Labeled FA were incubated for 12 hr with 24-hr cultured rat hepatocytes. Only the main radiolabeled lipid species are presented. The other lipid species (nonesterified FA, mono- and diglycerides, cholesterol esters, and cholesterol) are described in the text. Results are expressed as the percent of the initial radioactivity added to the culture medium and recovered in each fraction. Each value is the mean ± SD of measurements (two samples per time point) from three different cell cultures. Protein content was 1.26 ± 0.40 mg/dish for C14:0 experiments and 1.13 ± 0.31 mg/dish for C16:0 experiments. DNA content was 26.50 ± 7.16 μg/dish for C14:0 experiments and 25.33 ± 6.01 μg/dish for C16:0 experiments. TG, triglycerides; PL, phospholipids.

(20.1 ± 3.8% after 4 hr). The initial incorporation of ^{14}C into fatty acids of cellular PL was significantly slower (8.4 ± 1.6% after 4 hr). In the 4- to 12-hr interval, the radioactivity of cellular TG fatty acids decreased slowly, whereas that of PL fatty acids was still increasing. The incorporation of ^{14}C into fatty acids of secreted TG was detectable only after 2 hr of incubation. Radioactivity recovered in secreted TG then increased steadily with time, reaching 4.7 ± 1.2% of the initial radioactivity after 12 hr. Incorporation of ^{14}C from myristic acid into fatty acids of other lipid species did not represent more than 4% of the initial radioactivity at each time of incubation. Incorporation into NEFA and fatty acids of MDG remained relatively constant (approximately 0.5% and 2%, respectively). The incorporation into fatty acids of CE was very low and increased steadily to reach 0.5% of the initial radioactivity after 12 hr. New synthesized cholesterol from [1-^{14}C] myristic acid (after a putative β-oxidation producing radiolabeled acetyl-CoA) was very limited (0.02%).

Figure 1B shows the results of similar experiments realized with [1-^{14}C] palmitic acid. The comparison between myristic acid and palmitic acid (*Figures 1A* and *1B*) exhibits several differences. First, the clearance of [1-^{14}C] palmitic acid from the culture medium was slower than that of [1-^{14}C] myristic acid. Indeed, 31.7 ± 5.7% of the initial C16:0 remained in the culture medium after 4 hr of incubation and 5.7 ± 2.8% after 12 hr of incubation. The initial uptake of C16:0 by cultured hepatocytes and ^{14}C incorporation into total cellular fatty acids and lipid species fatty acids were consequently lower than the corresponding values with C14:0 as precursor. However, in the 4- to 12-hr interval, ^{14}C incorporation into total cellular fatty acids was still increasing and reached 52.6 ± 6.8% of the initial radioactivity after 12 hr. This value was significantly higher than the corresponding one with C14:0. As for myristic acid, the bulk of radiolabeled fatty acids was incorporated into cellular TG, PL, and secreted TG. ^{14}C incorporation into these three lipid species exceeded the corresponding values obtained with C14:0 as precursor in the 4- to 8-hr interval and were significantly higher ($P < 0.05$) after 12 hr. The incorporation of ^{14}C from palmitic acid into NEFA, fatty acids of MDG and CE, and cholesterol was very low and not significantly different from the corresponding values using C14:0, during any incubation period.

β-*Oxidation*

The decrease of the radioactivity carried by total fatty acids (*Figure 1*) was significantly sharper with myristic acid than with palmitic acid (49.5 ± 3.4% and 69.6 ± 4.9%, respectively, of the initial radioactivity after 4 hr of incubation), which suggests that myristic acid was more rapidly β-oxidized than was palmitic acid. After a 4-hr incubation period, the production of $^{14}CO_2$ and radiolabeled acid-soluble metabolites from [1-^{14}C] myristic acid represented 0.4 ± 0.1% and 14.5 ± 2.0%, respectively, of initial

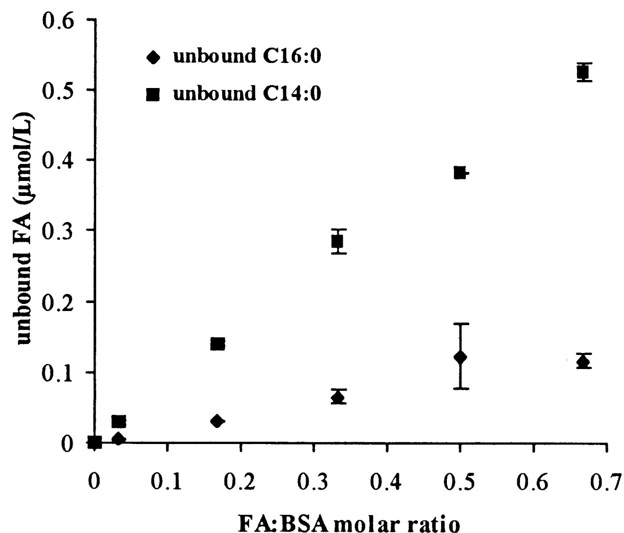

Figure 2 Levels of unbound myristate and palmitate at different fatty acid (FA):bovine serum albumin (BSA) molar ratios in the incubation medium. Determination of unbound FA concentration is described in Methods and materials. BSA concentration was 0.15 mmol/L in all assays.

radioactivity. The values obtained with [1-^{14}C] palmitic acid as substrate were significantly lower (0.1 ± 0.0% and 2.2 ± 0.6%, respectively). Total myristic acid oxidation (14.9 ± 2.2%) was then approximately sixfold higher than palmitic acid oxidation (2.3 ± 0.6%).

Total recovery of the initially added radioactivity from the two precursors (including the β-oxidation products, lipids from the medium and from the cells), however, was not statistically different after 4 hr of incubation (64.5 ± 3.6% from the initially added [1-^{14}C] myristic acid and 71.8 ± 4.7% from the initially added [1-^{14}C] palmitic acid were recovered).

Effect of albumin binding on fatty acid uptake

The different rates of [1-^{14}C] myristic acid and [1-^{14}C] palmitic acid uptake by cultured rat hepatocytes led us to measure the albumin binding capacity of both fatty acids in the fatty acid albumin complex containing media. *Figure 2* presents the unbound fatty acid concentration in the incubation medium as a function of the fatty acid:BSA molar ratio for the two substrates comparatively. The results show that the unbound fatty acid concentration depended on the fatty acid:BSA molar ratio. Whatever the fatty acid:BSA molar ratio, less myristic acid than palmitic acid was bound to albumin. For the 0.67 molar ratio (*Figure 2*), there was 4.5 times more unbound myristic acid than unbound palmitic acid. The different rates of uptake between the two substrates could then be readily explained by the difference in unbound fatty acid concentration.

Conversion to other fatty acids

The radioactivity of the individual fatty acids in cellular total lipids, cellular TG, PL, and secreted TG was determined as a function of incubation time with each precursor (*Figure 3*). Only the main radiolabeled fatty acids are presented in *Figure 3*. It shows that the incorporation of myristic acid itself increased during the first 4 hr of incubation in all lipid fractions (*Figures 3A–D*), then myristic acid decreased during the remaining 8 hr in all cellular lipids (11.3 ± 2.5% of the initial [1-^{14}C] myristic acid was recovered in total cellular fatty acids after 12 hr of incubation), but not in secreted TG (*Figure 3D*). Radiolabeled palmitic acid increased to reach 12.2 ± 0.8% in total cellular fatty acids after 12 hr of incubation (*Figure 3A*). The rapid appearance of new synthesized palmitic acid demonstrated that myristic acid was elongated. Radiolabeled stearic acid (especially in PL, *Figure 3C*) and arachidic acid also were identified, but in small amounts (2.1% and 1.9% of the radioactivity after 12 hr). Monounsaturated radiolabeled fatty acids were identified as C14:1, C16:1, and C18:1 (0.2%, 1.0%, and 0.6%, respectively, in total cellular fatty acids after 12 hr of incubation). No detectable radiolabeled polyunsaturated fatty acid was found.

The results obtained using [1-^{14}C] palmitic acid as substrate (*Figures 3E–H*) indicated that a small amount of C16:0 was converted to other fatty acids when compared with C14:0. In all lipid fractions and whatever the incubation time, palmitic acid always represented the bulk of radiolabeled fatty acids (reaching 37.8 ± 2.7% of the initially added radioactivity, in total cellular fatty acids after 12 hr). Elongations to form radiolabeled stearic and arachidic acids and desaturations to produce radiolabeled C16:1 and C18:1 were identified but these fatty acids always represented a small percentage of the initial radioactivity in cellular lipids (*Figure 3E*) and secreted TG (*Figure 3H*).

In addition, *Table 1* presents the distribution of radiolabeled identified fatty acids after 12 hr of incubation with either [1-^{14}C] myristic or palmitic acid. Radiolabeled cellular and secreted saturated fatty acids were significantly higher with palmitic acid than with myristic acid.

Identification of the monounsaturated fatty acids

Characterization of the radiolabeled monounsaturated fatty acids derived from the two precursors were done after 12 hr of incubation. After the oxidative cleavage (see Methods and materials), radioactivity was recovered in the dicarboxylic acids but not in the monocarboxylic acids (*Table 2*). For all the monounsaturated fatty acids, the main labeled dicarboxylic acid was C9 dicarboxylic acid. C14:1 derived from [1-^{14}C] myristic acid was then essentially myristoleic acid C14:1 (n-5). C16:1 derived from [1-^{14}C] myristic acid was mainly C16:1 (n-7) (palmitoleic acid) and, to a lesser extent, C16:1 (n-5) (elongation of the C14:1 (n-5)). C18:1 was a mixture of C18:1 (n-9) (oleic acid) and C18:1 (n-7) (elongation of the C16:1 (n-7)). C16:1 derived from [1-^{14}C] palmitic acid was essentially C16:1 (n-7) (palmitoleic acid) and C18:1 was a mixture of C18:1 (n-9) (oleic acid) and C18:1 (n-7) (elongation of the C16:1 (n-7)).

Discussion

The aim of this work was to investigate myristic acid metabolism in rat hepatocytes in primary culture and to compare it to that of another long-chain saturated fatty acid,

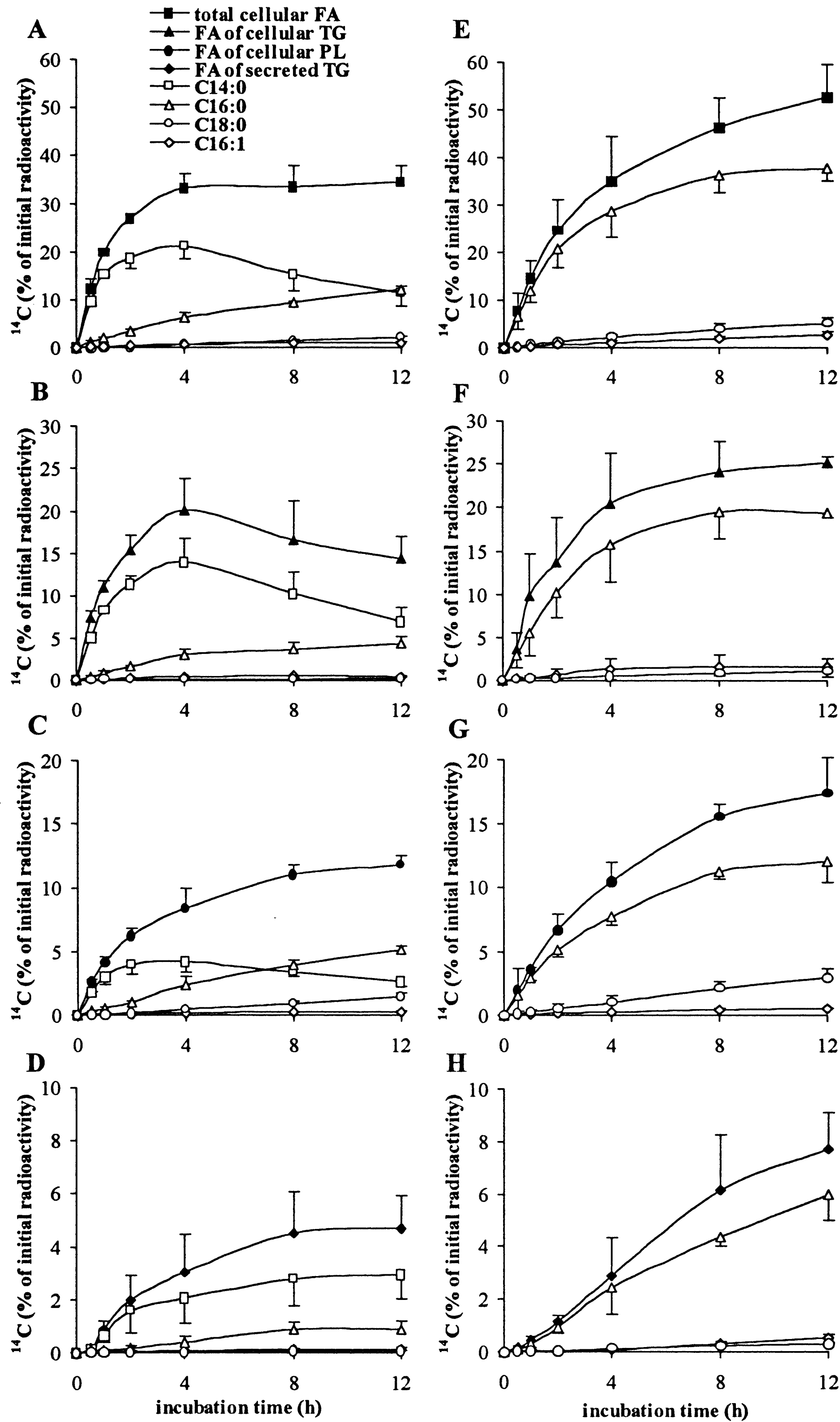

Figure 3 Elongation and desaturation of (A–D) [1-^{14}C] myristic acid and (E–H) [1-^{14}C] palmitic acid, and incorporation of the new synthesized radiolabeled fatty acids (FA) into different lipid fractions as a function of incubation time with cultured rat hepatocytes. (A,E) Total cellular lipids; (B,F) cellular triglycerides (TG); (C,G) cellular phospholipids (PL); (D,H) secreted TG. [1-^{14}C]-Labeled FA were incubated for 12 hr with 24-hr cultured rat hepatocytes. Only the main radiolabeled FA (C14:0, C16:0, C18:0, and C16:1 with myristic acid and C16:0, C18:0, and C16:1 with palmitic acid) are presented. The other radiolabeled FA are described in the text. Results are expressed as the percent of the initial radioactivity added to the culture medium and recovered in each FA. Each value is the mean ± SD of measurements (two samples per time point) from three different cell cultures. Protein and DNA contents were the same as in *Figure 1*.

Table 1 Distribution of radiolabeled fatty acids in cell lipids and secreted triglycerides (TG) after 12 hr of incubation with [1−^{14}C] myristic acid or [1−^{14}C] palmitic acid as substrate

	[1-^{14}C] Myristic acid (% of initial substrate radioactivity)		[1-^{14}C] Palmitic acid (% of initial substrate radioactivity)	
	Cell lipids	Secreted TG	Cell lipids	Secreted TG
Myristic acid	11.3 ± 2.5	3.0 ± 0.9		
Palmitic acid	12.2 ± 0.8	0.9 ± 0.3	37.8 ± 2.7	6.0 ± 1.0
Saturated fatty acids	27.5 ± 3.2[1]	4.0 ± 1.0[1]	43.3 ± 4.1	6.3 ± 1.0
Monounsaturated fatty acids	1.8 ± 0.4[1]	0.3 ± 0.1	3.9 ± 1.0	0.7 ± 0.2
Total fatty acids	34.6 ± 3.3[1]	4.7 ± 1.2[1]	52.6 ± 6.8	7.7 ± 1.4

Results are expressed as percent of the initial radioactivity added to the culture medium and recovered in each fraction. Each value is the mean ± SD of measurements (two samples per time point) from three different cell cultures. Protein and DNA contents were the same as in *Figure 1*.
[1]Significant difference between C14:0 and C16:0 ($P < 0.05$).

palmitic acid, which is universally found in natural fats. Incubating [1-^{14}C] myristic or palmitic acid with cells at 24 hr of culture, we determined the time-course of uptake of these two substrates by the cells, ^{14}C incorporation into cellular and secreted lipids, and β-oxidation. We also determined the distribution of radioactivity in individual fatty acids in the lipids, which allowed an assessment of the extent of conversion of each precursor to longer or unsaturated derivatives during the incubations. The results show that myristic acid, unlike palmitic acid, was rapidly metabolized in cultured rat hepatocytes. Myristic acid was indeed rapidly β-oxidized and converted to other fatty acids. As a result, it disappeared quickly in the cell whereas palmitic acid was not transformed in the same way in the cell and remained mainly in its initial form.

The present study emphasizes several major differences between myristic and palmitic acid utilization by cultured hepatocytes. First, myristic acid was taken up by the cells more rapidly than was palmitic acid. Although our goal was not to focus on the mechanism of transport of long-chain fatty acids across mammalian cellular membranes, which is still being discussed,[32] conducting a study of the metabolism of each precursor in intact cells inevitably involves taking the first step (i.e., the uptake) into account. In our experiments, exogenous fatty acids were solubilized with albumin in the culture medium in the physiologic range of fatty acid binding to albumin molar ratio (from 0.5 to 2.0). The binding of free fatty acids to BSA has been extensively investigated.[33–36] In the majority of studies on fatty acid uptake, the unbound fatty acid fraction is commonly calculated using published association constants for fatty acid binding to BSA.[37] In accordance with previous results,[38,39] we showed that unbound fatty acid concentration was dependent both on the fatty acid and on the fatty acid: albumin molar ratio (*Figure 2*). Albumin binding capacity for myristic acid was always smaller than for palmitic acid. Then our results showed that the rate of uptake depended on the unbound fatty acid concentration (*Figure 2*). We hypothesized that a two-carbon decrease in the fatty acid chain length favors the entry into the cells either by simple diffusion[40,41] or by a membrane-associated protein involved in the permeation process.[42,43] However, possible differences in the affinities of such a plasma membrane fatty acid binding protein ($FABP_{pm}$) according to the substrate, if existing, have not yet been demonstrated.[44] The utilization after short-term incubation is greatly influenced by the initial rate of uptake. The utilization of the two precursors was then compared when a similarly large amount of each fatty acid was taken up by the cells (i.e., long-term incubation), so that the remaining substrate in the medium was negligible. Therefore, we demonstrated, for ^{14}C incorporation into lipids, oxidation, elongation, and desaturation, that the major differences between myristic and palmitic acids are not related to their respective medium depletion rate.

Table 2 Identification by oxidative cleavage of the radiolabeled monounsaturated fatty acids derived from [1-^{14}C] myristic acid and [1-^{14}C] palmitic acid

	Oxidative cleavage substrate	Dicarboxylic acid produced	Percent radioactivity	Identified structure
[1-^{14}C] Myristic acid	14:1	C9	80	14:1 (n−5)
	16:1	C9	53	16:1 (n−7)
		C11	19	16:1 (n−5)
	18:1	C9	54	18:1 (n−9)
		C11	29	18:1 (n−7)
[1-^{14}C] Palmitic acid	16:1	C9	85	16:1 (n−7)
	18:1	C9	60	18:1 (n−9)
		C11	29	18:1 (n−7)

After 12 hr of incubation with [1-^{14}C] myristic acid or [1-^{14}C] palmitic acid, radiolabeled cellular monounsaturated fatty acids were submitted to an oxidative cleavage (see Methods and materials). The radioactivity recovered in dicarboxylic acid and the chain length indicate the double bond position on the monounsaturated fatty acid. Results are expressed as percent of the total radioactivity recovered in dicarboxylic acids.

A second difference between the two substrates lies in the ^{14}C incorporation into the fatty acids of cellular and secreted lipids. Because no significant amounts of radioactivity were found in cholesterol (*Figure 1*), it may be inferred that de novo lipid biosynthesis from labeled acetyl residues must have been very marginal. Interestingly, we found only small amounts of labeled NEFA whatever the substrate and the incubation period, both substrates (and derivatives) being readily incorporated mainly into TG and PL and, to a lesser extent, into secreted TG. In this respect, myristic acid was incorporated more rapidly than was palmitic acid during the shorter incubation periods (up to 2 hr, *Figure 1*), which can be explained by its faster uptake rate from the medium, as has been pointed out above. However, during the longer incubation periods (4–12 hr), palmitic acid (and derivatives) was more thoroughly incorporated than myristic acid: approximately 53% of the initial radioactivity was found in cell lipids after 12 hr of incubation with palmitic acid as substrate (35% with myristic acid). Using the same incubation time, substrate depletion in the medium was 99% for C14:0 and 94% for C16:0, showing that both precursors have almost fully been taken up by the cells. Our results may be compared with those already reported in the literature. Normann et al.[45] showed that myristic acid can be converted to acyl-CoA as efficiently as palmitic acid by rat liver microsomes, which is consistent with the near absence of labeled NEFA in our cells during incubation with myristic or palmitic acid. In L1210 murine leukemia cells,[46] myristic acid was preferentially incorporated into TG, which is also the case in our experiments. In BC_3H1 muscle cells,[19] myristic acid was equally distributed between neutral lipids and phospholipids. The poor incorporation of either precursor into MDG and CE is in agreement with previous studies using various cell cultures.[19,46] However, when myristate was introduced as chylomicrons in rats, it remained largely unesterified in the liver during the early stage of its metabolism and was poorly utilized for lipid synthesis.[47] This emphasizes possible differences between results obtained in vivo and in cultured hepatocytes in vitro. In our culture system, Williams' medium E contains 11 mmol/L glucose and 1 μmol/L insulin, all conditions that presumably favor lipid esterification of fatty acids rather than oxidation.

As has been already pointed out, ^{14}C incorporation into all lipids after 12 hr of incubation was significantly higher with palmitic acid than with myristic acid as substrate. This was likely to mean that the latter had been catabolized at a faster rate during the incubation. We then measured the β-oxidation rate of the two fatty acids. The results showed a highly significant difference between them, myristic acid being catabolized at a much faster rate than palmitic acid into both CO_2 and acid-soluble products, after 4 hr of incubation. Using the same incubation time, substrate depletion in the medium was 87% for C14:0 and 69% for C16:0, showing a depletion ratio of 1.3. The difference between this depletion ratio and the sixfold higher oxidation rate of myristic acid suggests that palmitic acid was stored in glycerolipids or secreted as TG.

Other studies have suggested that myristic acid could be β-oxidized at a faster rate than palmitic acid. In humans, oxidation of dietary [1-^{13}C] myristic acid exceeded that of [1-^{13}C] palmitic acid.[48,49] Wang and Koo[47] suggested that myristic acid was oxidized at a faster rate than was stearic and linoleic acids in rat liver. Pai and Yeh[50] also reported that the saturated fatty acids were β-oxidized at different rates in cultured rat hepatocytes. Finally, by studying [9,10-^{3}H] myristic acid utilization in Chinese hamster ovary cells, it was suggested that myristic acid, in contrast to palmitic acid, was converted by peroxisomal oxidation to several lipid-soluble metabolites such as lauric (C12:0) and decanoic (C10:0) acids.[51] These labeled metabolites, however, could not be identified in our incubations with [1-^{14}C] myristic acid, because the first cycle of oxidation produces [^{14}C] acetyl-CoA.

Identifying radiolabeled fatty acids of cellular and secreted lipids showed that both precursors, mainly myristic acid, were converted to longer or unsaturated derivatives and that amounts of the derivatives increased with the incubation time (*Figure 3*). Successive elongations and Δ^9 desaturations may explain the conversions of [1-^{14}C] myristic and palmitic acids to the identified radiolabeled saturated and monounsaturated fatty acids (*Table 2*). Desaturation of stearate and palmitate by Δ^9-desaturase has been demonstrated in cultured rat[25,52] and in cultured chicken hepatocytes.[53] The presence of myristoleic acid C14:1 (n-5) provides evidence that myristic acid was also converted by Δ^9-desaturase (*Table 2*). The elongation of myristate to palmitate was described in a variety of cells,[19,22,46,54] but the rate of this conversion in our culture system was surprisingly high when compared with the elongation of palmitic acid to stearic acid. As already discussed for ^{14}C incorporation into lipids and oxidation, the significant difference in elongation between the two substrates cannot be explained by the difference in uptake. After 12 hr of incubation, the elongation index of [1-^{14}C] myristic acid to palmitic acid (i.e., ratio between labeled product and substrate) was 11-fold higher than the elongation index of [1-^{14}C] palmitic acid to stearic acid. Using the same incubation time, the substrate depletion ratio was only 1.1. Differences observed in the distribution of incorporated radiolabeled fatty acids in the lipid species confirmed the specificity of glycerophosphate-acylating enzymes. Radiolabeled stearate derived from either [1-^{14}C] myristic acid or [1-^{14}C] palmitic acid was, for example, preferentially incorporated into PL (*Figures 3C* and *G*). The distribution of radiolabeled fatty acids between cellular and secreted TG was different (*Figures 3B* and *F*). In cellular TG, myristic acid was rapidly elongated to palmitic acid. By contrast, myristic acid always represented the bulk of radiolabeled fatty acids in secreted TG. This finding supports the view of the existence of two pools of TG in the cells: a storage pool and a secretory pool.[55] The latter seemed to incorporate the fatty acid taken up by the cells preferentially, without important conversions.

Our results provide evidence that myristic acid and palmitic acid do not have the same fate in cultured rat hepatocytes: we found that myristic acid was readily oxidized, little secreted as TG, and quickly elongated to other fatty acids (especially palmitic acid). Palmitic acid was not transformed in the same way and was stored in glycerolipids or secreted as TG. *Table 1* supports the view that these important utilizations of myristic acid in cultured hepato-

cytes result in a rapid disappearance of the precursor. There was then more radiolabeled cellular total and saturated fatty acids but also more radiolabeled secreted total and saturated fatty acids after 12 hr of incubation with palmitic acid than with myristic acid. The rapid transformation of myristate may explain the small amounts of this fatty acid in animal tissues.

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