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In rat hepatocytes, myristic acid occurs through lipogenesis, palmitic acid shortening and lauric acid elongation

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The origin of myristic acid in mammalian cells and the regulation of its endogenous cellular low concentration are not known. Another intriguing question is the potential metabolic properties of endogenous myristic acid as compared with exogenous myristic acid. In the present paper, we hypothesised and demonstrated that, in liver cells, in addition to the usual fatty acid synthase (FAS) pathway that produces predominantly palmitic acid and minor amounts of myristic acid, part of endogenous cellular myristic acid also comes from a shortening of palmitic acid, likely by peroxisomal β-oxidation and from lauric acid by elongation. From a nutritional point of view, C16:0 is universally found in natural fats and its shortening to myristic acid could contribute to a non-negligible source of this fatty acid (FA) in the organism. Then, we measured the distribution of endogenously synthesised myristic acid in lipid species and compared it with that of exogenous myristic acid. Our results do not support the hypothesis of different metabolic fates of endogenous and exogenous myristic acid and suggest that whatever the origin of myristic acid, its cellular concentration and lipid distribution are highly regulated.

Keywords: biosynthesis, liver, myristic acid, rats, saturated fatty acids

Introduction

Because of its negative effects on cholesterol metabolism, at high levels (Hayes and Koshla, 1992; Kris-Etherton and Dietschy, 1997), myristic acid (C14:0) has been studied when provided in the diets of animals (Koshla *et al.*, 1997; Salter *et al.*, 1998; Loison *et al.*, 2002) and humans (Hughes *et al.*, 1996; MacDougall *et al.*, 1996; Temme *et al.*, 1997; Dabadie *et al.*, 2006), or when added to the medium of cultured cells (Wang *et al.*, 1992; Rioux *et al.*, 2000 and 2002; Kummrow *et al.*, 2002). Additionally, data have been obtained by studying its positive effect on (n-3) polyunsaturated fatty acid (PUFA) bioavailability (Jan *et al.*, 2004; Dabadie *et al.*, 2005; Rioux *et al.*, 2005) and its specific involvement in protein N-myristoylation (Duronio *et al.*, 1991 and 1992; DeMar and Anderson, 1997; Rioux *et al.*, 2006) or in trypanosome metabolism (Doering *et al.*, 1993). However, none of these studies has determined if endogenous and exogenous myristic acids have equivalent metabolic fates.

In mammalian cells, myristic acid usually accounts for less than 1% of fatty acids (FAs) (Guillou *et al.*, 2002; Rioux *et al.*, 2005). Comparatively, it is particularly abundant (7% to 14% of total FAs) in milk fat (Jensen *et al.*, 1990). In rat mammary glands, this high level has been related to the presence of a cytosolic thioesterase II (Libertini and Smith, 1978) that releases FAs shorter than the highly predominant palmitic acid usually produced by the fatty acid synthase (FAS) complex, which is more specifically associated with thioesterase I. In other organisms, high level of myristic acid and significant activity of cytosolic thioesterase II have also been shown in a pea aphid (Ryan *et al.*, 1982) and in the uropygial gland of some waterfowls (De Renobales *et al.*, 1980).

In tissues other than the lactating mammary gland, however, the origin of myristic acid and the regulation of its endogenous cellular low concentration are not known. Myristic acid can come either directly from the diet, or from *de novo* biosynthesis, or from another tissue in which it has been synthesised and/or stored previously.

In the present paper, we demonstrate that, in liver cells, in addition to the usual FAS pathway that produces predominantly palmitic acid and minor amounts of myristic acid (Christie *et al.*, 1981), part of endogenous cellular myristic acid can also come from a shortening of palmitic acid by peroxisomal β-oxidation and from lauric acid by elongation. The distribution of endogenously synthesised myristic acid in lipid species is analysed and compared with that of exogenous myristic acid.

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Material and methods

Chemicals

Bovine serum albumin (BSA), 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES), Williams' medium E, insulin (bovine), dexamethasone, collagenase and FAs were purchased from Sigma (St. Louis, MO, USA). Penicillin–streptomycin antibiotic mixture was provided by Eurobio (Les Ulis, France). Foetal bovine serum (FBS) was obtained from J. Boy (Reims, France). [1-^{14}C]-lauric acid was purchased from Amersham Biosciences (Les Ulis, France). [1-^{14}C]-myristic acid, [9,10-^{3}H]-palmitic acid, [1-^{14}C]-palmitic acid and [^{3}H]-acetate were from Perkin Elmer Life Sciences (Le Blanc Mesnil, France). [16-^{14}C]-palmitic acid was from CEA (Saclay, France). Solvents and other chemicals were obtained from VWR International (Fontenay-sous-Bois, France). High-purity reagents for HPLC application came from Fisher Labosi (Elancourt, France). Falcon Primaria Petri culture dishes were used (AES, Combourg, France).

Cultured rat hepatocytes

The experimental protocol was in compliance with European Union guidelines for animal care and use. Sprague–Dawley male rats (250 g body weight) were obtained from the breeding centre R. Janvier (Le Genest, St. Isle, France) and were food-deprived 12 h prior to hepatocyte preparation. Hepatocytes were obtained after perfusion of liver *in situ* with collagenase, as previously described (Rioux *et al.*, 2000). The culture medium (Williams' E) was supplemented with 26 mmol/l $NaHCO_3$; 12.5 mmol/l HEPES; 15 μmol/l BSA; 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% (v/v) FBS. After plating, the cells were maintained in a humidified incubator at 37°C under 5% CO_2 in air. After 4 h, the plating medium was changed to a serum-free culture medium.

Incubation with radiolabelled acetate and FAs

Hepatocytes were incubated with [1-^{14}C]-lauric acid, [1-^{14}C]-myristic acid and [1-^{14}C]-palmitic acid. [1-^{14}C]-FA/albumin complexes were prepared as previously described (Rioux *et al.*, 2000). The final FA concentration in serum-free Williams' medium was 100 μmol/l with a specific activity of 183 MBq/mmol. After 24 h of culture, the medium was replaced by 2 ml of [1-^{14}C]-FA-containing medium per dish. Incubation with [9,10-^{3}H]-palmitic acid was initiated at the same time of culture (Rioux *et al.*, 2002) by replacing the culture medium by serum-free fresh medium containing the tritiated FA (4 μmol/l, 925 MBq/mmol). In parallel, hepatocytes were incubated with [^{3}H]-acetate (500 μmol/l, 732 MBq/mmol).

Lipid extraction and lipid species separation

At the end of the incubation, the medium was taken off, the cells were washed and harvested in phosphate-buffered saline solution (150 mmol/l NaCl; 5 mmol/l Na phosphate; pH 7.4). After centrifugation, lipids were extracted from the cell pellet with hexane/isopropanol (3:2 v/v) as previously described (Rioux *et al.*, 2000). Total lipids were saponified for 30 min at 70°C by 1 ml of 2 mol/l KOH in ethanol. FAs were liberated by acidification and extracted with diethylether. Alternatively, lipid species from the cells and medium were separated by thin-layer chromatography (TLC) using a mixture of hexane:diethylether:acetic acid (80:20:0.5 v/v/v). Phospholipids (PL), triglycerides (TG) from the hepatocytes and TG secreted in the medium were collected and saponified as described above.

In vitro *elongation and shortening assays on subcellular organelles*

Peroxisomes were purified from rat liver by using the peroxisome isolation kit (Sigma), according to the procedure supplied by the producer. Microsomes were obtained from rat liver by a first centrifugation at 10 000 × **g** (10 min, 4°C) followed by a second centrifugation at 100 000 × **g** (1 h, 4°C). The resulting peroxisomal and microsomal fractions were used for palmitic acid shortening assay and lauric acid elongation assay, respectively. A 1-ml assay mixture containing 200 μl of peroxisomes (1 mg protein), 150 mmol/l phosphate buffer (pH 7.16), 6 mmol/l $MgCl_2$, 7.2 mmol/l ATP, 0.54 mmol/l CoA and 0.8 mmol/l NADPH was incubated at 37°C for 30 min with 60 nmol of [16-^{14}C]-palmitic acid (740 MBq/mmol). For elongation assay, the incubation was made with [1-^{14}C]-lauric acid in a similar mixture containing microsomes (5 mg protein) and malonic acid (200 μmol/l). The reactions were stopped by adding 1 ml of 2 mol/l KOH in ethanol. After 30 min at 70°C, the FAs were liberated by acidification and extracted with diethylether. Control assays were run by stopping the reactions before addition of the labelled substrates.

FA analysis

FAs from each lipid extract were converted to FA naphthacyl esters as previously described (Rioux *et al.*, 1999) and separated on HPLC (Alliance, Waters, France) with a linear gradient of methanol:acetonitrile:water, starting at 80:10:10 (v/v/v) and increasing to 90:10:0 in 40 min. Elution of naphthacyl derivatives was monitored by UV absorbance at 246 nm. Peaks corresponding to radiolabelled FAs were collected (Fraction collector, Waters), diluted with scintillation liquid (InstaFluor Plus, Perkin Elmer Life Sciences) and counted (Packard Tri-Carb 1600TR, Meriden, CT, USA).

Results expression and statistical analysis

The values reported are mean ± s.d. ($n = 3$). Results are expressed as the per cent of the radioactivity initially added to the culture and recovered in each fraction. The cellular protein content of cell cultures, determined by a modified Lowry procedure (Bensadoun and Weinstein, 1976), was found to be homogenous for all the treatments, and is indicated in each figure legend. *P*-values were calculated using the Student *t*-test

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for two-group comparison. The differences were considered significant at a *P*-value of less than 0.05.

Results

Potential precursors for myristic acid biosynthesis

Several potential precursors for myristic acid production were assayed with cultured rat hepatocytes (Figure 1). We first quantified the level of myristic acid produced from acetyl-CoA through the activity of FAS, by using [^{3}H]-acetate as a precursor (Figure 1a). A linear incorporation of the radioactivity was shown in the cell FA fraction, as a function of the incubation time (0 to 4 h) and as a function of the initial concentration of acetate (50 to 500 μmol/l, data not shown). Five radiolabelled FAs were identified (C14:0, C16:0, C18:0, C16:1 n-7 and a mixture of C18:1 n-9 and n-7) but only the saturated FA are presented in Figure 1a. For all the incubation times, linear regression analyses showed that myristic acid and palmitic acid represented 10.4 ± 3.4% and 72.5 ± 3.2% of the radiolabelled FAs, respectively.

When the potential biosynthesis of myristic acid was measured from [1-^{14}C]-lauric acid elongation (Figure 1b), the radioactivity was rapidly and strongly recovered onto longer saturated FAs, i.e. myristic, palmitic and stearic acids. Depending on the incubation time, endogenous myristic acid represented 30.8 ± 1.6% of the newly synthesised FAs after 30 min of incubation, and only 8.8 ± 3.5% after 12 h, then being elongated to endogenous palmitic acid (Figure 1b).

Finally, we measured the potential biosynthesis of myristic acid from palmitic acid shortening. Figure 1c shows that [9,10-^{3}H]-palmitic acid incubation with cultured rat hepatocytes led to the biosynthesis of radiolabelled myristic acid, with other FAs obtained by elongation and desaturation of the precursor. For all the incubation times (between 3 and 12 h), myristic acid arose for 9.4 ± 1.5% of the newly synthesised FAs.

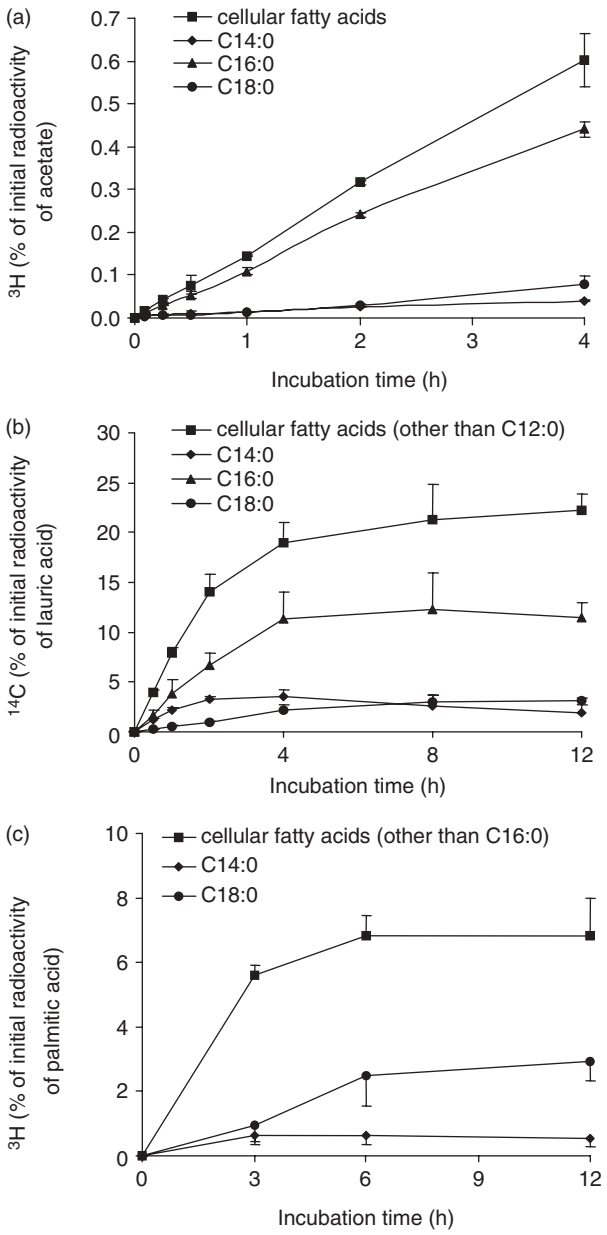

Figure 1 Endogenous biosynthesis of myristic acid from (a) acetyl-CoA, (b) lauric acid and (c) palmitic acid in cultured rat hepatocytes. Results are expressed as the per cent of the initial radioactivity added to the culture medium and recovered in cellular fatty acids. Each value is the mean ± s.d. from three different cell cultures. The protein content was 1.46 ± 0.25 mg per dish for experiments with acetate, 1.46 ± 0.36 mg/dish for experiments with C12:0 and 1.25 ± 0.15 mg per dish for experiments with C16:0.

In vitro *evidence for lauric acid elongation and palmitic acid shortening to myristic acid*

In order to confirm the shortening of C16:0 to C14:0 and elongation of C12:0 to C14:0 in specific subcellular fractions from a crude rat liver, [1-^{14}C]-lauric acid was incubated with the microsomal fraction (Figure 2a) and [16-^{14}C]-palmitic acid with the peroxisomal fraction (Figure 2b). Figure 2 shows the radiolabelled FA separation profiles obtained by HPLC after these incubations. Evidence is presented that lauric acid elongation to myristic acid occurred in the microsomes and that palmitic acid shortening to myristic acid occurred in the peroxisomes.

Comparison of endogenous and exogenous myristic acid distribution between lipid species

The second purpose of this work was to compare the distribution of endogenously synthesised myristic acid in lipid species, with that of exogenous myristic acid. Figure 3a shows the incorporation of myristic acid, endogenously synthesised from acetyl-CoA, in the cellular lipids and secreted TG as a function of the incubation time (0 to 4 h). Most of endogenous C14:0 stayed in the cells during the incubation, i.e. 88.6 ± 2.0% of total C14:0 over 4 h of incubation. A detectable incorporation was however shown in secreted TG (11.4 ± 2.0%). In the cellular lipids, endogenous myristic acid was predominantly incorporated into

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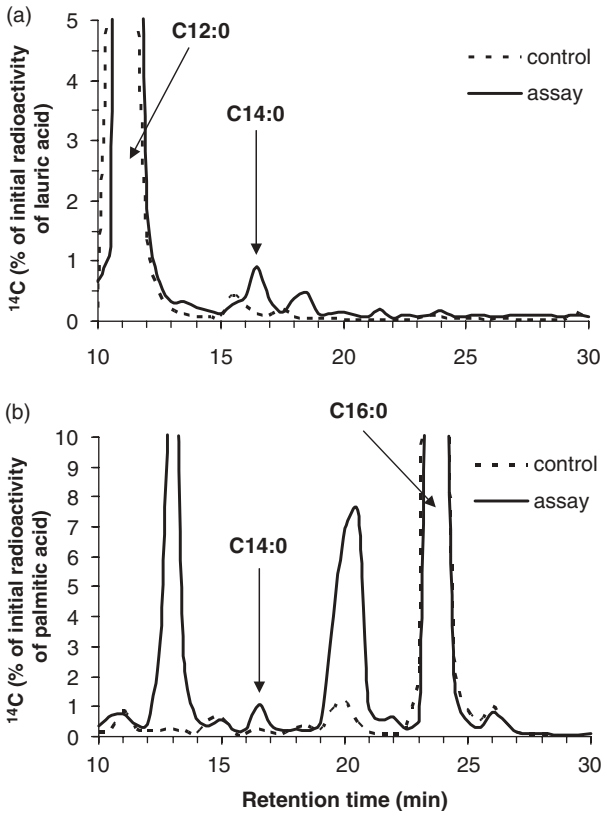

Figure 2 HPLC separation profiles of radiolabelled fatty acids obtained after (a) incubation of [1-^{14}C]-lauric acid with microsomes and after (b) incubation of [16-^{14}C]-palmitic acid with peroxisomes purified from a crude rat liver. A control assay was also run by stopping the reactions before addition of the labelled substrates. After extraction and derivatisation as naphthacyl esters, radiolabelled fatty acids were identified using their retention times compared with standard fatty acids.

the TG fraction (59.4 ± 8.5%) and less into the PL fraction (19.2 ± 2.1%).

Figure 3b presents a similar analysis using exogenous [1-^{14}C]-myristic acid directly incubated with cultured rat hepatocytes. The distribution in lipid species, analysed over the 4-h incubation, showed that cellular myristic acid represented 90.8 ± 4.7% of total myristic acid. A detectable incorporation was also shown in secreted TG (9.2 ± 4.7%). In hepatocytes, exogenous myristic acid was predominantly incorporated into the TG fraction (56.5 ± 3.3%) and less into the PL fraction (19.3 ± 3.6%). All these values of incorporation, measured over the 4 h of incubation, are remarkably close and statistically similar to those obtained with endogenous myristic acid (Figure 3a).

When the lipid distribution of endogenous C16:0 originating from acetyl-CoA was studied (Figure 3c), palmitic acid represented 95.6 ± 2.6% in cellular lipids and 4.4 ± 2.6% in secreted TG, after 4 h of incubation. In cellular lipids, 42.0 ± 8.0% of endogenous palmitic acid was incorporated in TG and 33.0 ± 1.2% in PL. Figure 3d presents a similar pattern using exogenous [1-^{14}C]-palmitic acid directly incubated with cultured rat hepatocytes. When analysed over the 4 h of incubation, palmitic acid in cells represented 93.5 ± 3.6% and palmitic acid in secreted TG 6.5 ± 3.6% of total C16:0. In cellular lipids, 47.2 ± 2.7% of exogenous palmitic acid was incorporated into the TG fraction and 25.4 ± 1.7% into the PL one. Comparison between the lipid distribution of endogenous and exogenous palmitic acid showed a significant difference ($P < 0.05$) in the PL incorporation (Figure 3c and d).

When comparing their lipid distribution, no significant difference was shown between exogenous myristic and palmitic acids, whereas significant differences for every value were noticed between endogenous myristic and palmitic acids.

Discussion

This work was designed to study several possible pathways for the biosynthesis of myristic acid in liver cells and to compare the metabolism of endogenous and exogenous myristic acid. The first part of the present work was focused on the molecules that are potential precursors for myristic acid biosynthesis. We hypothesised that, in liver cells, part of endogenous cellular myristic acid could come from the shortening of palmitic acid by peroxisomal β-oxidation, and from the elongation of lauric acid by FA elongases. Figure 1 shows that incubation of cells with the three precursors led to the biosynthesis of cellular myristic acid. In this model, myristic acid coming from acetate represented 10% of the endogenous pool of newly synthesised FAs (Figure 1a) and palmitic acid represented more than 70%. It has already been shown that palmitoyl-CoA is the main product of mammalian FAS (Chirala and Wakil, 2004). Our results are in agreement with those obtained by Lin and Smith (1978) showing that the activity of thioesterase I in liver was associated with FAS and was 10-fold less with myristoyl-CoA than with palmitoyl-CoA. In other tissues than liver, using [1-^{14}C]-acetate, Christie *et al.* (1981) have also shown that small amounts of myristic acid are synthesised by the FAS system in adipocytes (about 8% of the endogenous FAs) and higher amounts in the mammary gland (about 20% of the endogenous FAs), because of the presence of the so-called additional cytosolic type II thioesterase (Libertini and Smith, 1978).

Figure 1b shows that endogenous myristic acid was also produced by elongation of lauric acid. Figure 2a demonstrates further that microsomes from rat liver were able to catalyse *in vitro* the elongation of lauric acid to myristic acid. In a previous study (Rioux *et al.*, 2003), lauric acid elongation was suggested to produce endogenous myristic acid used for protein N-myristoylation and S-acylation. However, myristic acid coming from lauric acid did not stay in this form in cells since it was rapidly elongated to palmitic and stearic acid (Figure 1b). In addition, from a nutritional point of view, lauric acid is available only in a small number of dietary fats, like copra and palm oils (39% to 54% and 44% to 51% of FAs).

Palmitic acid was finally investigated as a potential precursor for myristic acid in cultured rat hepatocytes (Figure 1c).

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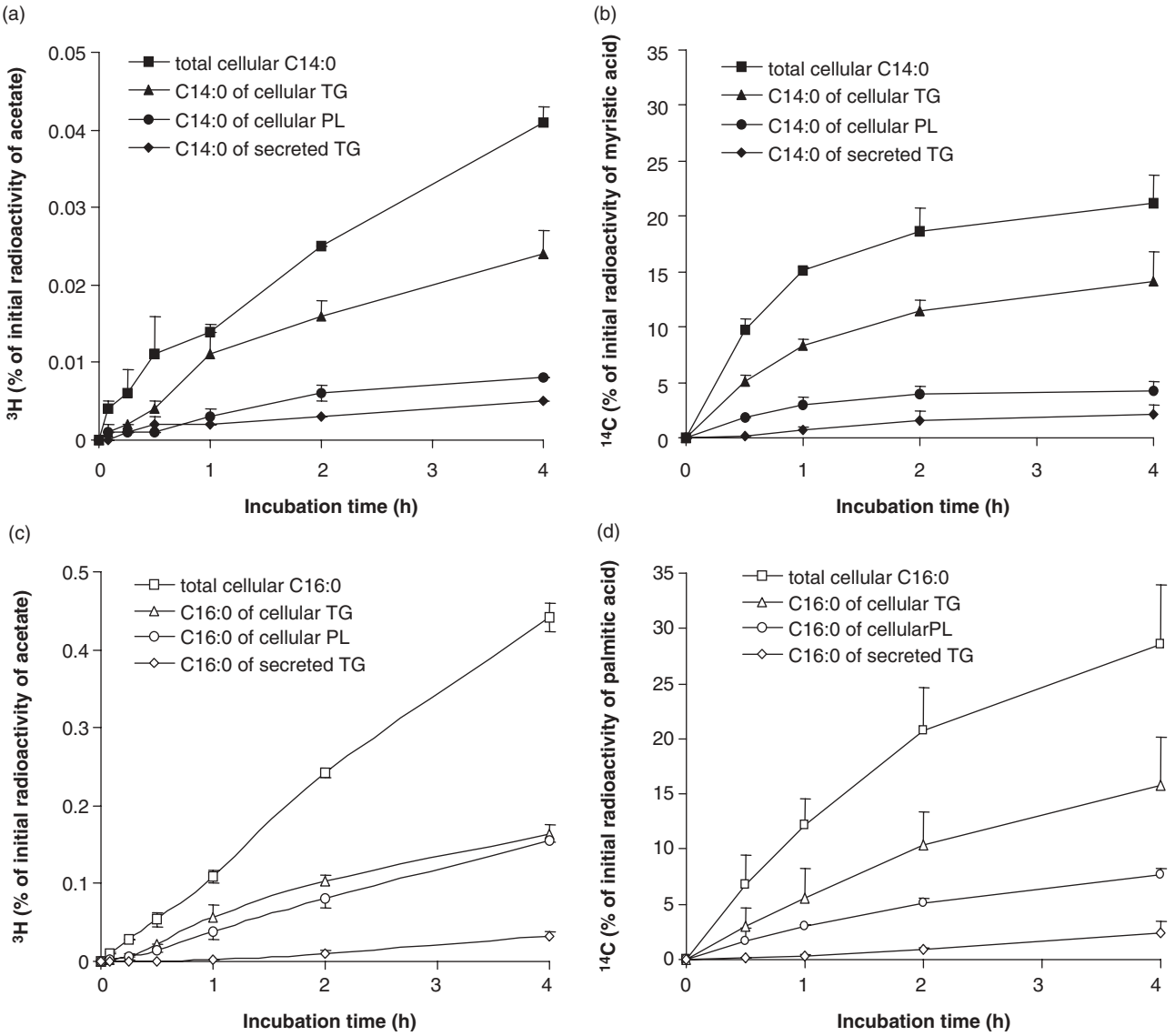

Figure 3 Distribution of radiolabelled endogenous myristic acid synthesised from (a) [^{3}H]-acetate and (b) of exogenous [1-^{14}C]-myristic acid into cellular and secreted lipid species of cultured rat hepatocytes. Comparison is made with endogenous palmitic acid synthesised from (c) [^{3}H]-acetate and (d) [1-^{14}C]-palmitic acid incubated directly with the hepatocytes. Results are expressed as the per cent of the initial radioactivity added to the culture and recovered in each fraction. Each value is the mean ± s.d. from three different cell cultures. The protein content was 1.46 ± 0.25 mg per dish for experiments with acetate, 1.26 ± 0.40 mg per dish for experiments with C14:0, and 1.13 ± 0.31 mg per dish for experiments with C16:0.

Myristic acid was shown to represent constantly about 10% of the newly synthesised FAs coming from palmitic acid. In addition, Figure 2b demonstrates that peroxisomes from rat liver were able to catalyse *in vitro* this shortening activity. We had previously shown (Rioux *et al.*, 2000) in the same model that only 2% of C16:0 was β-oxidised after 4 h incubation. Our experimental conditions (11 mmol/l glucose and 1 μmol/l insulin in the medium) presumably favor lipid esterification rather than oxidation, and it is therefore unlikely that radiolabelled C14:0 (Figure 1c) comes from total β-oxidation of [9,10-^{3}H]-palmitic acid followed by *de novo* biosynthesis from radiolabelled acetyl-CoA. In other cell lines (CHO, BC3H1, 3T3 and PC12), incubation of [9,10-^{3}H]-palmitic acid did not lead to the identification of radiolabelled myristic acid (Wang *et al.*, 1991 and 1992; Olson *et al.*, 1985), except in the IM-9 cultured human lymphocytes, in which Hedo *et al.* (1987) have shown radiolabelled C14:0 linked to the insulin receptor, after incubation with tritiated C16:0. Our results suggest that palmitic acid, because of its importance in natural fats, is shortened to myristic acid in the liver, which would contribute to a non-negligible portion of the synthesis of the latter in the organism, depending on the physiological status.

From a nutritional point of view however, whatever the precursors and pathways used for endogenous myristic acid biosynthesis, the amount of C14:0 produced (some hundred μg at the most, when considering a whole liver) is of far less extent relative to dietary myristic acid. Indeed, the level of myristic acid from dairy fat has been estimated up to 4 g/day (4.6 g/100 g total fat) in a Swedish population (Wolk *et al.*, 2001). In the same study, it has been shown that adipose

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tissue C14:0 level (3.6% of FAs) is a valid biomarker for dairy fat intake in men. In the rat, tissue myristic acid was also shown to be highly correlated with dietary myristic acid, especially in adipose tissue but also in the plasma and liver (Rioux *et al.*, 2005). Under the experimental conditions used, our results suggest that *de novo*-synthesised myristic acid can be considered as negligible when compared to exogenous myristate.

One can therefore wonder whether endogenous and exogenous myristic acids are regulated the same way. Indeed, different metabolic pathways of exogenous and endogenous FAs, and different physiological influences as a function of their origins, have been demonstrated for oleate (Legrand *et al.*, 1997), leading to the major conclusion that endogenous oleic acid coming from *in situ* Δ9-desaturation of stearate and exogenous dietary oleate are not equivalent in terms of TG secretion (Sampath and Ntambi, 2005). The second purpose of this work was therefore to compare the distribution of endogenously synthesised myristic acid between lipid species, with that of exogenous myristic acid. In this investigation, we studied endogenous myristic acid coming from acetyl-CoA and assumed that C14:0 coming from C16:0 shortening or C12:0 elongation would behave in the same way. The results showed that similar percentages of both types of myristic acid were preferentially incorporated into cellular TG, and less into cellular PL and secreted TG (Figure 3a and b). In the pea aphid in which myristic acid seemed to be almost exclusively synthesised from acetate, C14:0 was also preferentially incorporated into the TG fraction (Ryan *et al.*, 1982). Our results do not support the hypothesis of different metabolic utilisations of endogenous and exogenous myristic acid. On the contrary, a massive uptake of exogenous myristic acid and a small *de novo* synthesis were metabolised in exactly the same way (Figure 3a and b). By contrast, results obtained with palmitic acid (Figure 3c and d) indicated that this latter was more incorporated into the PL fraction when coming from the endogenous pathway. In addition, the lipid distribution of exogenous myristic and palmitic acids was similar (Figure 3b and d), whereas significant differences in the lipid incorporation were found between endogenous myristic and palmitic acids (Figure 3a and c). Therefore, compared with palmitic acid, our results suggest that the metabolism and concentration of myristic acid are highly regulated in the liver.

In conclusion, we show that in addition to the usual lipogenesis pathway, cellular myristic acid also comes from a shortening of palmitic acid by peroxisomal β-oxidation and from lauric acid by elongation. Our results suggest that whatever the origin of myristic acid, its cellular concentration and lipid distribution are highly regulated.

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