

## Myristic acid increases $\Delta 6$ -desaturase activity in cultured rat hepatocytes

Sophie JAN, Hervé GUILLOU, Sabine D'ANDREA, Stéphanie DAVAL,  
Monique BOURIEL, Vincent RIOUX, Philippe LEGRAND\*

Laboratoire de Biochimie, INRA-ENSA, 65 rue de Saint-Brieuc, CS84215, 35042 Rennes, France

(Received 5 January 2004; accepted 26 January 2004)

**Abstract** – In order to study the effects of saturated fatty acids on  $\Delta 6$ -desaturase activity, rat hepatocytes in primary culture were incubated with lauric (C12:0), myristic (C14:0) or palmitic (C16:0) acids. After optimization, the standard *in vitro* conditions for the measurement of  $\Delta 6$ -desaturase activity were as follows:  $60 \mu\text{mol}\cdot\text{L}^{-1}$   $\alpha$ -linolenic acid (C18:3n-3), reaction time of 20 min and protein content of 0.4 mg. Data showed that cell treatment with  $0.5 \text{ mmol}\cdot\text{L}^{-1}$  myristic acid during 43 h specifically increased  $\Delta 6$ -desaturase activity. This improvement, reproducible for three substrates of  $\Delta 6$ -desaturase, i.e. oleic acid (C18:1n-9), linoleic acid (C18:2n-6) and  $\alpha$ -linolenic acid (C18:3n-3) was dose-dependent in the range  $0.1$ – $0.5 \text{ mmol}\cdot\text{L}^{-1}$  myristic acid concentration.

**myristic acid /  $\Delta 6$ -desaturase / saturated fatty acids / cultured rat hepatocytes**

### 1. INTRODUCTION

The availability of 20- and 22-carbon (n-3 and n-6) polyunsaturated fatty acids (PUFA), which play pivotal roles in a number of biological functions including membrane fluidity, cell signaling and gene regulation, is greatly dependent upon the activity of desaturase enzymes which introduce double bonds between defined carbons of the fatty acyl chains. One of the two limiting steps in the biosynthesis of n-3 and n-6 very long chain PUFA is the  $\Delta 6$ -desaturation of C18:2n-6 and C18:3n-3 to C18:3n-6 and C18:4n-3, respectively. In this biosynthesis, evidence that  $\Delta 6$ -desaturase is also responsible for the conversion of 24:5n-3 to 24:6n-3 was recently demonstrated [1, 2].  $\Delta 6$ -desaturase regulation is now being

explored from different angles and has been found to be associated with hormones, age, diet and various physiological and pathophysiological states including diabetes, atopic dermatitis, cardiovascular disorders and cancer [3].

Fatty acids are known to regulate the expression of many genes involved in lipid metabolism and to modulate the activity of signaling molecules [3]. While dietary PUFA are known to coordinately suppress the activity and expression of a wide variety of lipogenic enzymes including  $\Delta 6$ -desaturase [4–9], the effect of saturated fatty acids has been less explored. Moreover, most previous studies were performed in intact animals and employed complex mixtures of fatty acids under sufficient essential fatty acid supplementation, thereby making it

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\* Corresponding author: philippe.legrand@agrocampus-rennes.fr

difficult to discern the effects of specific saturated fatty acids on desaturase activities [10]. In vitro studies, allowing for the reduction of the number of variables were also conducted but give controversial results with regards to the effect of saturated fatty acids on desaturase activities [11, 12].

The aim of the present study was to investigate the specific effect of myristic acid (C14:0) on  $\Delta 6$ -desaturase activity in cultured rat hepatocytes. The abundance of myristic acid is low in animal lipids but represents 9 to 15% of the total fatty acid content in milk, depending on the mammal [13]. Among the dietary fatty acids, myristic acid is reported to be the most potent cholesterolemia-increasing saturated fatty acid [14], but it is also known to exhibit an important role in cell regulation by acylation of a large number of proteins [15]. It is hence interesting to ask whether exogenous myristic acid could influence the first and limiting step of long chain PUFA biosynthesis in rat hepatocytes, that is  $\Delta 6$ -desaturation.

In the first step of the present work, experimental conditions for  $\Delta 6$ -desaturase activity assays in cultured rat hepatocytes were optimized in order to develop a physiological relevant model for studying the regulatory mechanisms of this enzyme. This tool was used to study the effect of myristic acid on enzyme activity, as compared to palmitic and lauric acids.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

Bovine serum albumin (BSA), HEPES, Williams medium E (W 4125), insulin (bovine), dexamethasone, collagenase and cold fatty acids were purchased from Sigma (Saint-Quentin Fallavier, France). The penicillin-streptomycin antibiotic mixture was provided by Gibco BRL (Eragny, France). Fetal bovine serum (FBS) was obtained from J. Boy (Reims, France). [ $1-^{14}\text{C}$ ]-oleic,

-linoleic and - $\alpha$ -linolenic acids were purchased from NEN Life Science Products (Paris, France). Solvent and other chemicals were obtained from Prolabo (Paris, France), Merck (Darmstadt, Germany) or Promega (Lyon, France). High purity reagents for high performance liquid chromatography (HPLC) application came from Fisher (Elancourt, France). Falcon Primaria Petri culture dishes, 60 mm in diameter, were from AES (Combourg, France).

### 2.2. 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, Épinay-sur-Orge, France). They were food-deprived 12 h prior to hepatocyte preparation. The experimental protocol was in compliance with applicable guidelines from The Ministry of Agriculture, France.

### 2.3. Cultured rat hepatocytes

Hepatocytes were obtained by in situ collagenase perfusion, as previously described [16]. The culture medium (Williams medium E) was supplemented with 26 mmol·L<sup>-1</sup> NaHCO<sub>3</sub>, 12.5 mmol·L<sup>-1</sup> HEPES, 15  $\mu\text{mol}\cdot\text{L}^{-1}$  BSA, antibiotic mixture (50 000 IU·L<sup>-1</sup> penicillin, 50 mg·L<sup>-1</sup> streptomycin), 1  $\mu\text{mol}\cdot\text{L}^{-1}$  insulin and 1  $\mu\text{mol}\cdot\text{L}^{-1}$  dexamethasone. The culture medium was supplemented with 7% (vol/vol) FBS for plating only ( $1.5 \times 10^6$  cells/dish). After plating, the cells were maintained in a humidified incubator at 37 °C under 5% CO<sub>2</sub> in air. After 4 h, the plating medium was changed to a serum-free culture medium.

### 2.4. Incubation with fatty acid albuminic complexes

The incubation media containing the fatty acid albuminic complexes were prepared as previously described [17]. Briefly,

each saturated fatty acid (C12:0, C14:0 or C16:0) was incubated for 30 min at 70 °C with 300  $\mu\text{L}$  KOH 2 mol·L<sup>-1</sup> in ethanol. The fatty acid salt obtained was dissolved at pH 10 in Williams medium E containing 0.15 mmol·L<sup>-1</sup> BSA. After 24 h of shaking, the pH was adjusted to 7.35. The solution obtained was used as an incubation medium, after addition of the antibiotic mixture (50 000 IU·L<sup>-1</sup> penicillin, 50 mg·L<sup>-1</sup> streptomycin), 1  $\mu\text{mol}\cdot\text{L}^{-1}$  insulin and 1  $\mu\text{mol}\cdot\text{L}^{-1}$  dexamethasone. The final fatty acid concentration was 0.5 mmol·L<sup>-1</sup> unless indicated otherwise in the figure legends. At 4 h of culture, incubation was initiated by replacing the culture medium with 2 mL of the saturated fatty acid containing medium per dish. The medium was renewed at 17 h of culture. Incubation with saturated fatty acids was carried out for 43 h at 37 °C in a 5% CO<sub>2</sub> atmosphere.

## 2.5. Desaturase activity assays

After 17 or 43 h of incubation with saturated fatty acids, culture dishes were washed twice with phosphate-buffered saline solution (PBS; 150 mmol·L<sup>-1</sup> NaCl; 5 mmol·L<sup>-1</sup> Na Phosphate, pH 7.4) and the cells were harvested with a rubber policeman in PBS. Pooled cell suspensions were centrifuged at 800 g for 4 min. The supernatant was discarded, the cell pellet was resuspended in 400  $\mu\text{L}$  50 mmol·L<sup>-1</sup> phosphate buffer (pH 7.4) containing 0.25 mol·L<sup>-1</sup> sucrose and sonicated at 20 W for 15 s using a vibra cell VC 50 sonicator (Sonics and Materials, Danbury, CT). The sonicated solution was centrifuged at 10 000 g for 10 min. The resulting post-mitochondrial supernatant was collected and used for  $\Delta 6$ -desaturase assays. Aliquots were stored at -20 °C for protein measurements. Enzymatic activity was determined using a 1 mL assay mixture that contained the following: 100  $\mu\text{L}$  of supernatant, 150 mmol·L<sup>-1</sup> phosphate buffer (pH 7.16), 6 mmol·L<sup>-1</sup> MgCl<sub>2</sub>, 7.2 mmol·L<sup>-1</sup> ATP, 0.54 mmol·L<sup>-1</sup> coenzyme A and 0.8 mmol·L<sup>-1</sup> NADH. The reaction was started with the addition of

various amounts of [1-<sup>14</sup>C]-oleic, linoleic or  $\alpha$ -linolenic acid (740 MBq·mmol<sup>-1</sup>). The incubation was carried out in a shaking water bath at 37 °C for various times. The reaction was stopped by adding 1 mL of 2 mol·L<sup>-1</sup> KOH in ethanol. Each assay mixture was then heated for 30 min at 70 °C. The fatty acids were liberated by acidification, extracted with diethylether and dried. Fatty acids were converted to fatty acid naphthacyl esters as previously described [18] and were separated on HPLC (Alliance integrated system, Waters, St Quentin en Yvelines, France) using a Nova-Pak C18 column (4.6 mm  $\times$  250 mm, Waters) and a guard column (Nova-Pak C18; 3.9 mm  $\times$  20 mm). The peaks corresponding to radiolabeled fatty acids (substrates and products of  $\Delta 6$ -desaturase assays) were collected and subjected to liquid scintillation counting (Packard Tri-Carb 1600 TR, Meriden, CT). Preliminary identification of fatty acid naphthacyl esters was based upon retention times obtained for naphthacyl esters prepared from radiolabeled and non-radiolabeled fatty acid standards [18]. The enzyme activity was determined from the amount of radioactivity found in the products vs. the radioactivity recovered in the substrate. It was expressed as nmol substrate converted to product·min<sup>-1</sup>·mg<sup>-1</sup> protein, as previously described [2].

## 2.6. Protein measurement

Protein in the supernatant used for the desaturase assays was determined by a modified Lowry procedure [19].

## 2.7. Results expression and statistical analysis

All measurements were performed on samples from individual animals and the results are expressed as the mean of three replicates or as means of five to nine animals, as stated in figure legends. *P*-values were calculated using the Student test for two-group comparisons. The differences

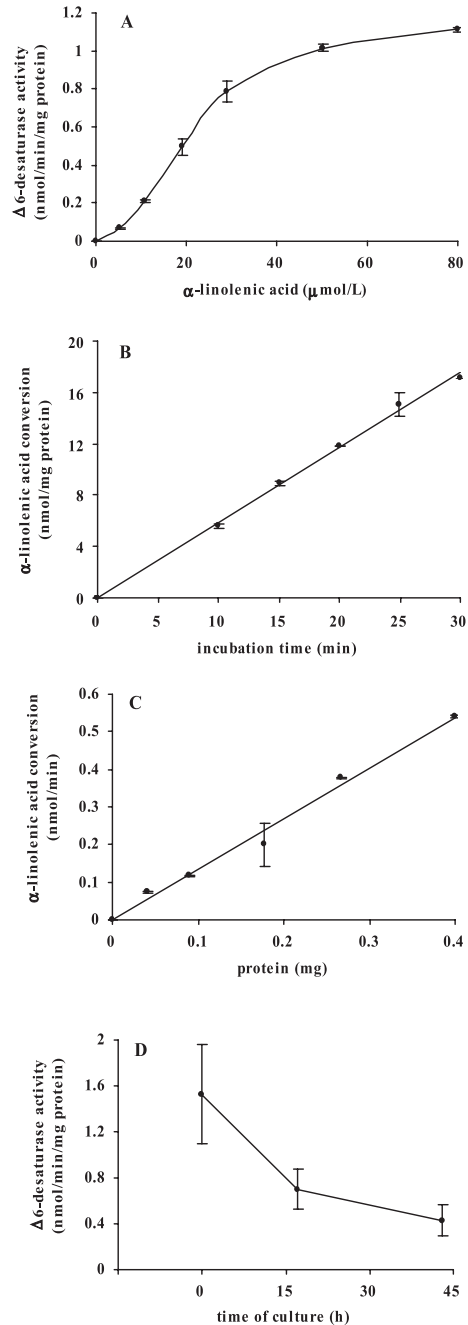
were considered significant at a *P*-value of less than 0.05.

### 3. RESULTS

#### 3.1. Characterization of experimental conditions for $\Delta 6$ -desaturase activity assays

Initial studies were carried out to determine what concentration of substrate ( $\alpha$ -linoleic acid C18:3n-3), time of reaction and protein content in the post-mitochondrial supernatant were required to optimize  $\Delta 6$ -desaturase activity assays (Fig. 1). The results in Figure 1A show that  $\Delta 6$ -desaturase activity was optimized with C18:3n-3 concentrations ranging from 50 to 80  $\mu\text{mol}\cdot\text{L}^{-1}$ . As shown in Figure 1B and Figure 1C,  $\Delta 6$ -desaturase activity was linear up to 30 min of incubation time and for a protein content up to 0.4 mg. The following conditions were then used for subsequent experiments: 60  $\mu\text{mol}\cdot\text{L}^{-1}$  of C18:3n-3 and a reaction time of 20 min. Five culture dishes were treated giving rise to a protein content in the range of 0.2–0.4 mg depending on the cellular density per dish. Similar results were obtained with the two other substrates of  $\Delta 6$ -desaturase i.e. oleic acid (C18:1n-9) and linoleic acid (C18:2n-6) (data not shown).

**Figure 1.** Measurement of  $\Delta 6$ -desaturase activity in the post-mitochondrial supernatant of cultured rat hepatocytes as a function of (A)  $\alpha$ -linolenic acid (C18:3n-3) concentration (incubation time: 20 min, protein content in the post-mitochondrial supernatant: 0.24 mg and culture time: 17 h) (B) Incubation time (C18:3n-3 concentration: 60  $\mu\text{mol}\cdot\text{L}^{-1}$ , protein content in the post-mitochondrial supernatant: 0.4 mg and time in culture: 17 h) (C) protein content in the post-mitochondrial supernatant (C18:3n-3 concentration: 60  $\mu\text{mol}\cdot\text{L}^{-1}$ , incubation time: 20 min and culture time: 17 h), and (D) culture time (C18:3n-3 concentration: 60  $\mu\text{mol}\cdot\text{L}^{-1}$ , incubation time: 20 min and protein content in the post-mitochondrial supernatant: 0.4 mg). Values are means  $\pm$  SD,  $n = 3$ .



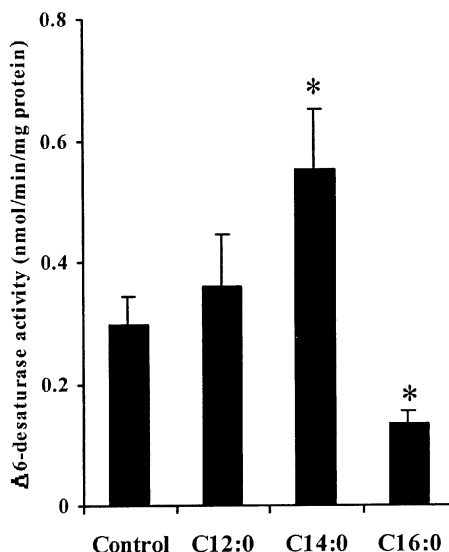
$\Delta 6$ -desaturase activity was also studied as a function of culture time (Fig. 1D). While significant  $\Delta 6$ -desaturase activity was observed at time zero of culture (isolated hepatocytes), a time-dependent decrease was observed thereafter but the cultured hepatocytes still exhibited  $\Delta 6$ -desaturase activity up to 43 h of culture without FBS addition to the medium. After 43 h of culture,  $\Delta 6$ -desaturase activity toward C18:3n-3 still attained 0.43 nmol/min/mg protein.

### 3.2. Comparative effect of lauric acid, myristic acid and palmitic acid on $\Delta 6$ -desaturase activity

On the basis of the above results, the effect of the addition of 0.5 mmol·L<sup>-1</sup> lauric acid (C12:0), myristic acid (C14:0) or palmitic acid (C16:0) to the cell culture medium was studied on  $\Delta 6$ -desaturase activity towards  $\alpha$ -linolenic acid C18:3n-3 (Fig. 2). As compared to the control, the  $\Delta 6$ -desaturase activity, measured on 6 independent hepatocyte cultures, was significantly increased by the 43 h treatment with 0.5 mmol·L<sup>-1</sup> myristic acid while 0.5 mmol·L<sup>-1</sup> lauric acid had no significant effect. On the contrary, 0.5 mmol·L<sup>-1</sup> palmitic acid supplementation of the culture medium induced a significant decrease in  $\Delta 6$ -desaturase activity.

The effect of 0.5 mmol·L<sup>-1</sup> myristic acid was further tested on 9 independent hepatocyte cultures using either C18:1 n-9, C18:2 n-6 or C18:3 n-3 as the  $\Delta 6$ -desaturase substrate (Fig. 3). Whatever the substrate used for  $\Delta 6$ -desaturase assays, the addition of 0.5 mmol·L<sup>-1</sup> myristic acid in the culture medium resulted in significant higher activities as compared to the controls.

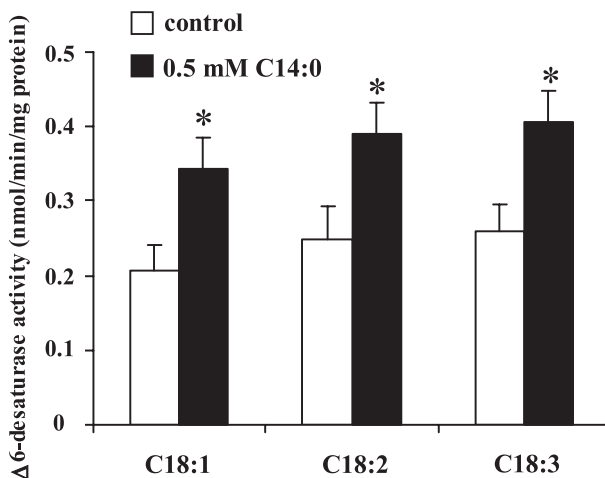
Finally, the effect of myristic acid on  $\Delta 6$ -desaturase activity was studied as a function of its concentration in the culture medium for 5 independent cell cultures (Fig. 4). The results show that the increasing effect of myristic acid on  $\Delta 6$ -desaturase activities was dose-dependent, whatever the substrate used for the enzymatic assays.



**Figure 2.** Effect of 0.5 mmol·L<sup>-1</sup> lauric acid (C12:0), myristic acid (C14:0) or palmitic acid (C16:0) on  $\Delta 6$ -desaturase activity assayed with  $\alpha$ -linolenic acid (C18:3n-3) on the post-mitochondrial supernatant of 43 h cultured rat hepatocytes. Values are means  $\pm$  SEM ( $n = 6$ ). \* Values significantly different from the control ( $P < 0.05$ ).

## 4. DISCUSSION

In this study, the optimization of the conditions for  $\Delta 6$ -desaturase activity assays in rat hepatocytes in primary culture provides a reliable tool for investigating the effect of an individual parameter on its regulation. Cultured hepatocytes have already been described as a useful physiological model for the investigation of the mechanisms of lipid metabolism regulation [20–22]. In vitro studies of mammalian  $\Delta 6$ -desaturase have also been developed in a large number of cell lines [23–25] and the cloning of the gene has recently offered a new opening in exploring regulatory mechanisms of  $\Delta 6$ -desaturation via transfected cells [5]. In the

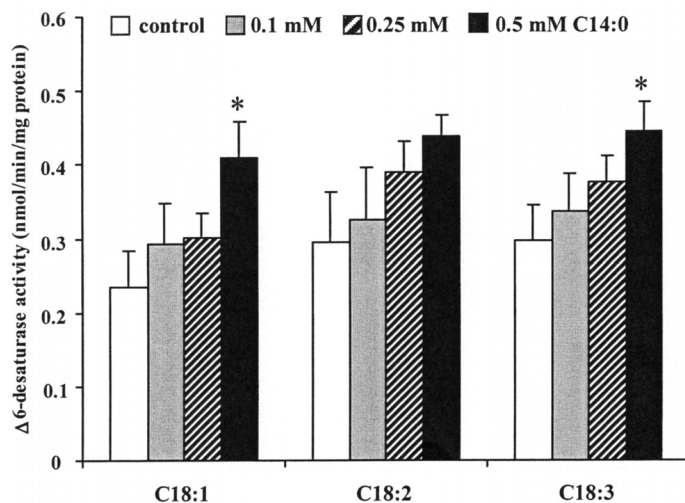


**Figure 3.** Effect of 0.5 mmol·L<sup>-1</sup> myristic acid (C14:0) on  $\Delta$ 6-desaturase activity assayed with oleic acid (C18:1n-9), linoleic acid (C18:2n-6), or  $\alpha$ -linolenic acid (C18:3n-3) on the post-mitochondrial supernatant of 43 h cultured rat hepatocytes. Values are means  $\pm$  SEM ( $n = 9$ ). \* Values significantly different from the control ( $P < 0.05$ ).

first part of the present study,  $\Delta$ 6-desaturase activity assays were shown to be optimized under the following experimental conditions: 60  $\mu$ mol·L<sup>-1</sup> of C18:3n-3, reaction time of 20 min and protein contents ranging from 0.2 to 0.4 mg (Fig. 1). Under these experimental conditions, two other substrates of  $\Delta$ 6-desaturase, i.e. C18:1n-9 and C18:2n-6 were also shown to be desaturated (Figs. 3 and 4). As already shown in freshly extracted microsomes from the rat liver [11], the highest enzyme activity was obtained with C18:3n-3 followed by C18:2n-6 and C18:1n-9. These results were also consistent with those of Geiger et al [26] who showed that there is always preferential  $\Delta$ 6-desaturation of the fatty acids of the n-3 family. On the basis of the above experimental conditions,  $\Delta$ 6-desaturase activities in cultured rat hepatocytes were consistent with the values obtained with freshly extracted microsomes from rat liver [10, 26–30].

Since non-treated cells still exhibited sufficient  $\Delta$ 6-desaturase activities after 43 h

of culture, the effect of lauric acid (C12:0), myristic acid (C14:0) or palmitic acid (C16:0) was investigated 43 h after addition to the culture medium. Exogenous fatty acids were solubilized with albumin in the physiological range of fatty acid binding to albumin molar ratio and in the physiological range of fatty acid supply (from 0.1 to 0.5 mmol·L<sup>-1</sup>). Under similar experimental conditions, rapid intake of these fatty acids by cultured rat hepatocytes has already been demonstrated [17, 31]. According to our results, the addition of myristic acid in the culture medium was shown to increase  $\Delta$ 6-desaturase activity significantly (Figs. 2 and 3) and in a dose-dependent manner (Fig. 4), while this effect was not observed with lauric and palmitic acids (Fig. 2), whose chain lengths differ from those of myristic acid by only two carbons. Hence, there was no correlation between  $\Delta$ 6-desaturase activity and chain length of the saturated fatty acid, as could be assumed from previous observations where their metabolic fate (uptake,  $\beta$ -oxidation and elongation) was shown to be a function of carbon



**Figure 4.** Dose effect of myristic acid (C14:0) on  $\Delta 6$ -desaturase activity assayed with oleic acid (C18:1n-9), linoleic acid (C18:2n-6), or  $\alpha$ -linolenic acid (C18:3n-3) on the post-mitochondrial supernatant of 43 h cultured rat hepatocytes. Values are means  $\pm$  SEM ( $n = 9$ ). \* Values significantly different from the control ( $P < 0.05$ ).

chain length [17, 31]. The significance of the decreasing effect of palmitic acid on  $\Delta 6$ -desaturase activity was not confirmed in a second set of experiments, whereas myristic acid still significantly increased  $\Delta 6$ -desaturase activity as compared to the control (data not shown). Hence, among other saturated fatty acids, myristic acid displayed a specific effect on  $\Delta 6$ -desaturase activity.

Our results were in agreement with a recent study in humans treated during 5 weeks with a diet rich in myristic acid (1.2% of energy vs. a diet containing 0.6%). A significant enhancement of docosahexaenoic acid (DHA) concentration was observed in the cholesteryl ester fraction, suggesting a myristic acid-dependent increase in  $\Delta 6$ -desaturase activity *in vivo* [32].

In studies mentioning the effect of myristic acid on enzymes involved in lipid metabolism, myristic acid was described as

the most potent inducer of peroxisomal fatty acid oxidation in hepatoma cells as compared to other saturated fatty acids [33]. In cultured hepatocytes, retinoid X receptor (RXR $\alpha$ ) [34] and peroxisomal proliferator activated receptor (PPAR $\alpha$ ) [35] mRNA and protein levels were shown to be synergistically up-regulated by myristic acid and dexamethasone and down-regulated by the simultaneous treatment with myristic acid and insulin, suggesting a coupling between hormonal control and the steady-state level of the dietary fatty acid in the cells. Hence, the possibility that myristic acid may control  $\Delta 6$ -desaturase via PPAR $\alpha$  can not be discarded, since  $\Delta 6$ -desaturase emerges as a PPAR $\alpha$  target gene [7, 36].

Since protein myristoylation has already been shown to be involved in several biological functions such as signal transduction pathways, vesicular trafficking, and structural roles [15], another hypothesis is

that myristic acid added to the culture medium contributes to increase the overall level of cellular protein myristoylation, which may govern  $\Delta 6$ -desaturase expression [37–40] and/or activity. Considering the enzyme activity, direct acylation of  $\Delta 6$ -desaturase by myristic acid could also occur, since rat  $\Delta 6$ -desaturase exhibits a potential site of myristoylation ( $\text{NH}_2$ -terminal glycine). Myristoylation of the  $\text{NH}_2$ -terminal glycine of the NADH cytochrome  $b_5$  reductase could also account for the change in the activity of the whole complex of  $\Delta 6$ -desaturation [41].

Finally, it might also be speculated that myristic acid treatment of the cell culture affects  $\Delta 6$ -desaturase activity through changes in the biophysical properties of the membrane lipid environment in which the desaturation complex is embedded [4, 42].

Elucidating the mechanism of  $\Delta 6$ -desaturase activation by myristic acid in rat hepatocytes will help us to further raise questions as to whether different types of dietary saturated acids can modulate PUFA biosynthesis in vivo.

## ACKNOWLEDGMENTS

The authors are indebted to Arilait Recherches (France) and Conseil Régional de Bretagne for constructive scientific discussion and financial support. We thank A. Leborgne and K.L. Cung for able technical assistance and animal care.

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