Medium-chain fatty acids as short-term regulators of hepatic lipogenesis

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Short-term exposure of isolated rat hepatocytes to short- and medium-chain fatty acids led to an activation of acetyl-CoA carboxylase as measured in digitonin-permeabilized hepatocytes. Up to a certain concentration, typical for each of the fatty acids used, fatty acid-dependent activation of acetyl-CoA carboxylase coincided with an increase in the rate of fatty acid synthesis in intact hepatocytes, as determined by the incorporation of $^3$H from $^3$H$_2$O water into fatty acids. At higher concentrations loss of stimulation of fatty acid synthesis occurred, but not the enhancement of carboxylase activity. With the fatty acids tested (C\textsubscript{8:0}–C\textsubscript{14:0}), the peak in fatty acid synthesis coincided with a peak in the level of malonyl-CoA. The onset of the stimulation of carboxylase activity coincided with the start of the peak in both fatty acid synthesis and malonyl-CoA. The longer the chain length of the fatty acid added, the lower the concentration at which the rate of fatty acid synthesis and the level of malonyl-CoA reached a peak and carboxylase activity started to become elevated. In cell suspensions incubated with increasing concentrations of fatty acids, accumulation of lactate decreased progressively. The latter observation, in combination with the fact that the activity of acetyl-CoA carboxylase is not always related to the rate of fatty acid biosynthesis, suggests that under these conditions not the activity of the carboxylase but the flux through the glycolytic sequence determines, at least in part, the rate of fatty acid synthesis de novo.

INTRODUCTION

Fatty acid biosynthesis in mammalian cells is regulated in several different ways. On a long-term basis, it is controlled by changes in the rate of synthesis of lipogenic enzymes [1]. Short-term modification of the rate of fatty acid synthesis occurs without changes in enzyme levels. This short-term control appears to involve changes in the activity of acetyl-CoA carboxylase, widely held to be one of the major regulatory sites in fatty acid biosynthesis [2]. The changes can be brought about by a variety of mechanisms [2].

Rapid regulation of acetyl-CoA carboxylase is of special interest because the product of the carboxylation reaction, malonyl-CoA, is not only a substrate for fatty acid synthesis but is also an inhibitor of carnitine palmitoyltransferase I [3], an important pace-setting step in fatty acid oxidation. Hence, rapid shifts in carboxylase activity may determine the metabolic fate of hepatic fatty acids by directing them to either the oxidation or the esterification pathway.

In the course of studies on short-term control of fatty acid synthesis, our attention was drawn to fatty acids as potential modulators of the hepatocellular activity of acetyl-CoA carboxylase. Numa et al. [4] reported inhibitory effects of CoA derivatives of fatty acids ranging in chain length from C\textsubscript{10:0} to C\textsubscript{16:0} on the acetyl-CoA carboxylase reaction in vitro. However, in more integrated systems the situation may be different. In isolated hepatocytes incubated with fatty acids of different chain length, the cytosolic enzyme acetyl-CoA carboxylase probably will only encounter CoA derivatives of long-chain fatty acids, since these fatty acids are activated extra-mitochondrially [5,6]. Short- and medium-chain fatty acids, on the other hand, are mainly activated intra-mitochondrially [5,6] and therefore their CoA esters will for the most part be unable to affect cytosolic carboxylase activity. This notion is supported by the finding by Nilsson et al. [7] that the degree of inhibition of fatty acid synthesis in isolated hepatocytes by fatty acids of different chain length paralleled only for the long-chain fatty acids the strength of the inhibitory effect caused by different acyl-CoA derivatives on the acetyl-CoA carboxylase reaction as observed by Numa et al. [4]. The medium-chain fatty acids did not inhibit fatty acid synthesis in the hepatocyte system [7]. Nomura et al. [8] reported even elevated rates of lipogenesis by hepatocytes presented with 1 mM octanoate. This is in line with the observation that diets rich in medium-chain triacylglycerols evoked a marked rise in the plasma concentration of triacylglycerol [9]. Since medium-chain fatty acids do not serve directly for the synthesis of triacylglycerol (cf. [5,6]), the rise in plasma triacylglycerol level suggests accelerated hepatic fatty acid biosynthesis.

The present study was undertaken to gain further insight into fatty acids as potential short-term regulators of the hepatocellular activity of acetyl-CoA carboxylase, and hence of hepatic fatty acid synthesis. For that purpose we studied the effects of different fatty acids on (i) the rate of fatty acid synthesis by monitoring the incorporation of $^3$H from $^3$H$_2$O into fatty acids in intact hepatocytes and (ii) the activity of acetyl-CoA carboxylase as measured in digitonin-permeabilized hepatocytes. The latter method is the method of choice for detecting changes in the activity of the carboxylase in isolated hepatocytes [10,11]. In a one-step procedure digitonin permeabilizes the plasma membrane of the cells, and at the same time a coupled assay measures enzyme activity. The use of this assay largely overcomes ambiguities in other procedures used with non-purified preparations of this enzyme [12,13].

MATERIALS AND METHODS

Sources of materials

$^3$H$_2$O (5 Ci/ml) was supplied by Amersham International (Amersham, Bucks., U.K.); [1-14C]acetyl-CoA (54.3 Ci/mol) and [$^{3}H$]acetyl-CoA (3.9 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.); digitonin, collagenase type I, pyruvate, lactate, citrate, fatty acids, acetyl-CoA, malonyl-CoA and butyryl-CoA were purchased from Sigma (St. Louis, MO, U.S.A.); NADPH, NADH, lactate dehydrogenase, malate dehydrogenase and citrate lyase were from Boehringer (Mannheim, Germany); other chemicals were from Baker (Deventer, The Netherlands).
Animals

Male Wistar rats (225–250 g) were used throughout this study. The animals were fed on a standard pelleted diet and had free access to water. The rats were subjected to a 12 h-light (06:00–18:00 h)/12 h-dark cycle.

Isolation and incubation of hepatocytes

Hepatocytes were harvested at 09:00 h by the method of Seglen [14], with modifications described previously [15]. In order to minimize glycogenolysis, 20 mM glucose was added to the perfusion buffers and to all buffers subsequently employed in the isolation [16]. Isolated cells were incubated in Krebs–Henseleit bicarbonate buffer (1 g wet wt. of cells/20 ml) supplemented with 10 mM glucose in the absence or presence of the indicated fatty acids. Fatty acids were added in the presence of 3.5% (w/v) BSA which had been charcoal-treated [17] and extensively dialysed [18]. Incubations were conducted at 37 °C in 25 ml Erlenmeyer flasks in a metabolic shaker (80 strokes/min). Flasks were continuously gassed with O₂/CO₂ (19:1) during the incubation. Two sets of incubation flasks were run simultaneously. In the first set, 3H₂O (0.5 mCi/ml) was included for measuring the rate of fatty acid synthesis. These cell incubations were run for 30 min. From the second set, samples were removed for measuring the activity of acetyl-CoA carboxylase and the levels of cellular metabolites. Since measurement of carboxylase activity reflects a single point in time, and synthesis of fatty acids is a time integral, carboxylase activity was determined at an earlier time point than the rate of fatty acid synthesis. Therefore, the radioisotope-free hepatocyte incubations lasted 20 min.

Analytical procedures

Lipogenesis was terminated by addition of chloroform/methanol (1:1, v/v). Lipids were extracted as described by Sundler et al. [19]. Total lipids were saponified at 75 °C for 2 h with 0.3 M NaOH in 90% (v/v) methanol. The saponifiable fraction was extracted with light petroleum (b.p. 40–60 °C) and counted for radioactivity.

The activity of acetyl-CoA carboxylase was measured in a coupled assay in digitonin-permeabilized hepatocytes exactly as described previously [10].

Neutralized HClO₄ extracts of the hepatocyte incubations were analysed for lactate and ketone bodies by standard enzymic spectrophotometric procedures [20]. In portions of such extracts, intracellular malonyl-CoA levels were measured as described by Beynen et al. [21]. Fatty acid synthase, utilized in determining the activity of acetyl-CoA carboxylase and in the malonyl-CoA assays, was isolated and purified from rat liver as described by Linn [22], and stored at −80 °C.

Since extensively dialysed BSA still contains citrate [23], incubations intended for citrate determination were stopped by transferring a 0.9 ml portion to an Eppendorf microtube containing 200 µl of ice-cold 2 M HClO₄ and about 400 µl of a mixture of bromododecane/bromodecane (3:1, v/v) [24] on top of this layer. The tube was spun for 30 s at full speed in a modified Eppendorf 3200 centrifuge with swing-out rotor. Citrate was determined in samples of the neutralized-HClO₄ bottom layer by a standard enzymic technique [20].

Separation of cytosolic and mitochondrial fractions was performed by the digitonin cell-fractionation procedure originally described by Zuurendonk and Tager [25]. For this purpose 0.7 ml portions of cell incubations were transferred to an Eppendorf microtube containing 200 µl of ice-cold 2 M HClO₄.

RESULTS

In short-term incubations with isolated rat hepatocytes, 1 mM of long-chain fatty acids inhibited acetyl-CoA carboxylase in the order C₁₈:₀ > C₁₆:₀ > C₁₆:₁ > C₁₈:₁ > C₂₀:₄ (Table 1), which is about the same order as the strength of the inhibitory effect caused by their acyl-CoA derivatives on partly purified acetyl-CoA carboxylase, as reported by Numa et al. [4]. In the latter study, short- and medium-chain acyl-CoA derivatives were also found to inhibit acetyl-CoA carboxylase. Much to our surprise, short- and medium-chain fatty acids stimulated rather than inhibited hepatocellular carboxylase activity (Table 1). Inclusion of the fatty acids in the assay mixture without prior incubation of the cells with fatty acids did not significantly affect carboxylase activity in control cells (Table 1). For reasons of interest we chose to conduct all the following experiments using medium-chain fatty acids.

The effect of 1 mM of these fatty acids on carboxylase activity was time-dependent. It disappeared faster for the shorter chains than for the longer ones (Figure 1). These results suggested that

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of the presence of 1 mM of different fatty acids on acetyl-CoA carboxylase activity in cell incubations or in enzyme assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid added</td>
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</tr>
<tr>
<td></td>
<td>In cells</td>
</tr>
<tr>
<td>None</td>
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</tr>
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<td>C₂₀:₀</td>
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<tr>
<td>C₁₆:₀</td>
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</tr>
<tr>
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<td>C₁₆:₁</td>
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</tr>
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<tr>
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<tr>
<td>C₂₀:₄</td>
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</tr>
<tr>
<td>C₁₆:₀</td>
<td>0.14 ± 0.02*</td>
</tr>
<tr>
<td>C₁₆:₁</td>
<td>0.30 ± 0.07</td>
</tr>
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</table>

Significantly different from the corresponding incubations without added fatty acid.
Figure 1  Time course of the effects of addition of 1 mM of different medium-chain fatty acids on the activity of acetyl-CoA carboxylase

Symbols: ○, control; △, C_{8:0}; ●, C_{10:0}; □, C_{12:0}; ▲, C_{14:0}. Results are expressed as means ± S.D. *Significantly different from control at that time point.

Figure 2  Time course of the formation of ketone bodies in the absence or presence of octanoate

Symbols: ○, control; △, 0.5 mM C_{12:0}; ■, 1.0 mM C_{12:0}. Results are expressed as means ± S.D.; the latter are indicated by error bars.

Figure 3  Time course of the effect of addition of 1 mM octanoate or laurate on the activity of acetyl-CoA carboxylase (a) and on fatty acid synthesis (b)

Symbols: ○, control; ●, C_{8:0}; ▲, C_{12:0}. Results are expressed as means ± S.D. *Significantly different from control at that time point.

Figure 4  Effect of the concentration of different fatty acids on the rate of fatty acid synthesis measured after 30 min incubation (a) and on the cellular level of malonyl-CoA measured after 20 min incubation (b)

Symbols: ●, C_{8:0}; □, C_{10:0}; ▲, C_{12:0}; ▼, C_{14:0}. For fatty acid synthesis the 100% values were 9.59, 7.20, 8.29 and 7.98 nmol/30 min per mg of protein, and for malonyl-CoA these values were 51.0, 18.4, 31.0 and 21.3 pmol/mg of protein, in the experiments with C_{8:0}, C_{10:0}, C_{12:0} and C_{14:0} respectively. *Significantly different from the corresponding control with no fatty acid added.

metabolism of the fatty acids is needed to sustain the effect. For instance, the metabolism of 1 mM octanoate by the amount of cells used in these experiments was complete within 40 min, as evidenced by cessation of ketone-body formation (Figure 2). At that time, the effect of octanoate on carboxylase activity was lost (cf. Figure 1). Ketone bodies by themselves did not have an effect. Acetoacetate or 3-hydroxybutyrate at 5 mM affected neither the rate of fatty acid synthesis nor the activity of acetyl-CoA carboxylase (results not shown).

The increase in carboxylase activity caused by 1 mM octanoate (C_{8:0}) (Figure 3a) was reflected in the cumulative increments in fatty acid synthesis per 0–15 min, 0–30 min, 0–45 min and 0–60 min (Figure 3b). At 15 min the effect of 1 mM octanoate on fatty acid synthesis was not yet apparent, probably because the difference in accumulation of radioactivity in fatty acids between incubations with and without added fatty acid was not yet large enough. At 60 min, on the other hand, when carboxylase activity was no longer stimulated in incubations with 1 mM octanoate fatty acid synthesis was enhanced. The stimulatory effect of 1 mM laurate (C_{12:0}) on enzyme activity, however, was not

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mirrored by an increase in fatty acid synthesis. On the contrary, this fatty acid even caused some inhibition of fatty acid synthesis. The data in Figure 4(a) show that at certain concentrations the stimulatory effect of a given fatty acid on carboxylase activity (cf. Table 1 and Figure 1) was paralleled by an increased synthesis of fatty acids, whereas at higher concentrations it was not. This was rather unexpected.

The loss of stimulation of lipogenesis by the higher concentrations of added fatty acids (cf. Figure 4a) may have resulted from inhibition of fatty acid synthase. This, however, was not the case. Fatty acid synthase, measured like acetyl-CoA carboxylase in digitonin-permeabilized hepatocytes [10], was not affected by the concentrations of the different fatty acids used in the present study (results not shown). Alternatively, the loss of stimulation of lipogenesis by the higher concentrations of fatty acid may be an artefact. A decrease in the rate of glycolysis (cf. Figure 6) will make less $^3$H available in the aldolase reaction for subsequent incorporation into fatty acids synthesized de novo, leading to a fictitious decrease in the rate of fatty acid synthesis. Determination of the cellular concentration of malonyl-CoA, an index of lipogenic activity, showed that this assumption was not correct. The fatty acid-determined profile of fatty acid synthesis (Figure 4a) precisely matched the profile of the fatty acid-induced level of malonyl-CoA (Figure 4b). In addition, the peak of both parameters was found to shift towards lower concentrations of added fatty acid with increasing chain length (Figure 4).

It was decided to determine whether the activity of acetyl-CoA carboxylase followed the same fatty acid-influenced profile as the level of malonyl-CoA did. Comparison of Figure 4(b) with Figure 5 shows that this was not the case. Therefore the decrease in fatty acid synthesis was not due to a decreased activity of acetyl-CoA carboxylase. At all concentrations of fatty acid tested, beyond the onset stimulatory concentration, the activity of the carboxylase was stimulated.

Changes in the generation of citrate, an allosteric modulator of enzyme activity, during the rapid metabolism of the fatty acids may be responsible for the phenomena observed. The data in Figure 6 illustrate that indeed at certain fatty acid concentrations citrate accumulated in the cells. We also determined the cytosolic level of this intermediate. It followed the same pattern as total citrate. Percentage-wise, however, the increase in the cytosolic level was largest (Figure 6).

The level of lactate was determined as indicator of the glycolytic flux. Accumulation of this compound was strongly inhibited in hepatocytes incubated in the presence of medium-chain fatty acids (Figure 6). Remarkably, glycolysis was already maximally inhibited at a concentration of fatty acid also inducing maximal stimulation of fatty acid synthesis. This is a paradoxical situation. By-passing the block in glycolysis by adding a mixture of pyruvate and lactate did not abolish the stimulatory effect of 1 mM octanoate either on lipogenesis or on the cellular content of malonyl-CoA, although the rate of fatty acid synthesis as well as the level of malonyl-CoA was much higher (Table 2).
DISCUSSION

Stimulation of the activity of acetyl-CoA carboxylase by short- and medium-chain fatty acids was a major finding of this study. It appears that with an increase in chain length of the added fatty acids the stimulation of acetyl-CoA carboxylase reached a maximum with C_{12}. With higher chain length the stimulation decreased, and from C_{16} on enzyme activity became inhibited (Table 1, Figures 1 and 5). In search for the mechanism underlying this rather unexpected observation, we have measured a variety of cellular parameters as function of the concentration of the fatty acids used: (i) total cellular and cytosolic levels of citrate, since this intermediate serves not only as precursor for fatty acid synthesis but also as allosteric modulator of relevant key enzymes like phosphofructokinase [27] and acetyl-CoA carboxylase [2], (ii) accumulation of lactate as index of glycolytic activity, and (iii) the cellular level of malonyl-CoA both as indicator of the flux through the sequence of the fatty acid biosynthetic pathway and as regulator of carnitine palmitoyltransferase I, an important regulatory enzyme in fatty acid oxidation [3].

From our studies it is clear that the glucose/fatty acid cycle, which has recently been shown to occur not only in muscle but also in liver, using palmitate as the fatty acid [28], can be extended to short- and medium-chain fatty acids. This was unexpected, as the site of activation of the latter fatty acids is quite different. The data in Figure 6 showed that addition of fatty acid inhibited lactate accumulation, indicating a decrease in glucose utilization, and therefore sparing of glucose. At 1 mM octanoate, for instance, the accumulation fell to a maximum of about 50% of the accumulation in the absence of added fatty acid.

Citrates are an allosteric activator of acetyl-CoA carboxylase in vitro. It is still a matter of debate whether this is of physiological relevance in an intact hepatocyte (cf. [2]). Attempts to correlate levels of this intermediate with the activity of the enzyme in homogenates or with the rate of fatty acid synthesis in intact cells have met with limited success. These efforts were inconclusive for a number of reasons: (i) problems in assessing the activity of acetyl-CoA carboxylase in crude tissue extracts [10,12], and (ii) measurement of the total cellular citrate level, rather than the amount in the cytosolic compartment in which the carboxylase is located. Due to a mitochondrial/cytosolic gradient of 10 or more [29], the small changes sometimes observed in total cellular level (cf. [2]) are not informative as far as the cytosolic level is concerned. Since only extra-mitochondrial citrate can serve as effector of phosphofructokinase or acetyl-CoA carboxylase, we have also determined the cytosolic content of citrate. It was found that the changes in that compartment were most pronounced (Figure 6). It should be noted that the changes in the cytosolic level of citrate induced by exogenously added medium-chain fatty acids did not exactly coincide with those in the activity of acetyl-CoA carboxylase as measured in digitonin-permeabilized hepatocytes. With increasing concentrations of added fatty acid, carboxylase activity became elevated at lower concentrations than the onset in the increase in the cytosolic level of citrate. The peak of cytosolic citrate did not shift towards lower concentrations of added fatty acid with increasing chain length (cf. Figures 5 and 6). This suggests that the increase in cytosolic citrate was not instrumental in the fatty acid-evoked activation of carboxylase activity. However, the elevated level of citrate may sustain the enhanced activity of acetyl-CoA carboxylase when the rate of fatty acid synthesis dropped upon further increasing the concentration of added fatty acid.

The reason for the decrease in the rate of fatty acid synthesis, as well as in the level of malonyl-CoA, above a certain concentration of added fatty acid (cf. Figure 4) is not clear. The fall observed in both parameters is not only the result of fatty acid-induced inhibition of glycolysis. This notion is derived from the observation that by-passing the block in glycolysis by adding pyruvate plus lactate to the incubated cells did not prevent either the decrease in the rate of fatty acid synthesis or that in the level of malonyl-CoA at higher concentrations of added fatty acids (cf. Table 2). It is tempting to speculate that the fall in both parameters, despite enhanced activity of acetyl-CoA carboxylase (cf. Figure 5), resulted from a decrease in ATP available for activation of fatty acids. A decrease in adenine nucleotide content of hepatocytes by incubation with octanoate has been demonstrated [30]. The absence of effects of fatty acids such as octanoate in the concentration range used in this study on cholesterologenesis (results not shown) argues against the level of ATP being responsible for the discrepancy in the rate of fatty acid synthesis and the activity of acetyl-CoA carboxylase. The lack of effect of the added fatty acids on cholesterol biosynthesis also argues against the tricarboxylic acid carrier having a role in the phenomena observed. Another possible explanation for the decrease of fatty acid synthesis at higher concentrations of added fatty acid, when acetyl-CoA carboxylase is still activated, is a lack of glycerol for further processing of fatty acids synthesized de novo in the esterification pathway, due to inhibition of glycolysis.

Table 2  Effect of addition of pyruvate plus lactate on the rate of fatty acid synthesis and on malonyl-CoA levels of hepatocytes incubated in the presence or absence of different concentrations of octanoate

Incubations intended for measuring the rates of fatty acid synthesis and levels of malonyl-CoA were conducted for 30 min and 20 min, respectively. Cells were incubated in the absence or presence of 1 mM pyruvate plus 10 mM lactate. Results are presented as means ± S.D. of 3 incubations of one hepatocyte preparation. This experiment has been repeated twice with similar results.

*Significantly different from the corresponding incubations without added octanoate.

<table>
<thead>
<tr>
<th>Octanoate added (mM)</th>
<th>Pyruvate+lactate...</th>
<th>Malonyl-CoA</th>
</tr>
</thead>
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<td>Absent  Present</td>
<td>Absent  Present</td>
</tr>
<tr>
<td>0</td>
<td>4.27±0.06 8.47±0.13</td>
<td>18.9±0.6 29.5±1.0</td>
</tr>
<tr>
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<td>7.97±0.23 13.92±1.55</td>
<td>46.4±3.9 51.7±2.5</td>
</tr>
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<td>1.5</td>
<td>5.69±0.12 11.17±0.06</td>
<td>13.3±0.6 78.0±0.3</td>
</tr>
<tr>
<td>2.0</td>
<td>4.95±0.09 7.95±0.24</td>
<td>15.9±1.0 34.5±0.6</td>
</tr>
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</table>

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Although the precise mechanism underlying medium-chain fatty acid-determined increases in carboxylase activity remains to be established, the effects that were observed on enzyme activity presumably resulted from covalent alteration of the enzyme rather than from allosteric regulation by non-covalently bound effectors such as citrate. This notion is derived from the observations (i) that changes in the cytosolic level of citrate induced by the added fatty acids did not coincide with those in the activity of acetyl-CoA carboxylase, and (ii) that the fatty acid-induced effects on the acetyl-CoA carboxylase were observed in cells permeabilized with digitonin which is accompanied by dilution of effectors. Covalent modification of acetyl-CoA carboxylase is known to occur through AMP-activated protein kinase, an enzyme entity that is part of a protein kinase cascade inactivating not only the carboxylase but also several other key enzymes of lipid metabolism [31]. Apart from adenine nucleotides, this cascade is also sensitive to low concentrations of palmitoyl-CoA [31]. The metabolism of medium-chain fatty acids very likely interacts with the protein kinase cascade.

Does the observation of this study that medium-chain fatty acids stimulated fatty acid synthesis have relevance for the situation in vivo? An affirmative answer to this question is suggested by results from a study in which rats were fed on a diet rich in medium-chain triacylglycerols leading to a marked increase in the plasma concentration of triacylglycerol [9]. Unlike long-chain fatty acids, their medium-chain counterparts are mainly activated inside mitochondria, and therefore they do not serve directly for triacylglycerol synthesis. Thus the increase in plasma triacylglycerol concentration suggests enhanced fatty acid biosynthesis. Medium-chain triacylglycerols are used extensively to treat patients having impaired absorption of long-chain triacylglycerol in traditional diets. They have also been promoted for other uses, such as in enteral and parenteral nutritional support and appetite control [32,33]. Also in the human situation diets high in medium-chain triacylglycerols produce dramatic increases, up to 3-fold, in fasting serum triacylglycerol levels [34]. The results of the present investigation may help to explain the observations in rat [9] and human [34] dietary studies.

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