Esterification of free fatty acids in adipocytes: a comparison between octanoate and oleate

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Medium-chain triacylglycerols (MCT) are present in milk, coconut oil and other foods, and are used therapeutically in special diets for certain disorders of lipid and glucose utilization. Recently, it has become apparent that MCT are not only oxidized in the liver, but are also present in lymph and fat tissue, particularly after chronic treatment. To evaluate the influence of MCT on metabolism in fat cells, we compared incorporation of octanoate and oleate into cellular triacylglycerols of 3T3-L1 adipocytes as well as their effects on preadipocyte differentiation. We found that less octanoate than oleate was stored and that more octanoate than oleate was oxidized. Octanoate was esterified to a greater extent at the *sn*-1,3 position of glycerol carbons than at the *sn*-2 position, whereas the opposite was true for oleate. Glycerol release from fat cells pre-treated with octanoate

was also greater than from cells pre-treated with oleate, presumably related to the preferential release of octanoate from the *sn*-1,3 position. Octanoate was not incorporated into lipids in undifferentiated cells and did not induce differentiation in these cells, whereas oleate was readily stored and actually induced differentiation. Incorporation of octanoate into lipids increased as cells differentiated, but reached a maximum of about 10 % of the total stored fatty acids. If these effects *in vitro* also occur *in vivo*, substitution of octanoate for oleate or other long-chain fatty acids could have the beneficial effect of diminishing fat-cell number and lipid content.

Key words: differentiation, lipolysis, triacylglycerol storage.

INTRODUCTION

It has been demonstrated that medium-chain fatty acids (MCFA) are more efficiently oxidized than long-chain fatty acids (LCFA) at the whole-body level [1,2] and in isolated tissue or cells [3–7]. However, little is known about how MCFA are metabolized via pathways alternative to oxidation, and how this affects other metabolic events in cells.

On the other hand, medium-chain triacylglycerols (MCT) have been used as nutrients for patients with disorders of long-chain triacylglycerol (LCT) or glucose metabolism for decades. Several early studies demonstrated that MCT diets prevented weight gain in animals [8–10] without affecting plasma cholesterol or other physiological parameters [11,12]. Feeding MCT early in life influenced adipose-tissue development and resulted in fewer and smaller fat cells with less lipid [10]. Neurotoxicity [13] and ketosis [14] have only been reported after acute, high-dose MCT intravenous infusion in animals. Recent trials have demonstrated that the addition of MCT to human diets is of benefit for certain dyslipidaemic disorders including diabetes [15,16]. The rationale for these therapeutic benefits is not fully understood. The potential applications of MCT in the treatment of obesity have been reviewed in [17,18].

While it is generally accepted that MCFA are absorbed via the portal vein and are oxidized in the liver, a recent study shows that when fed enterally to rats, significant amounts of MCFA relative to LCFA appear in lymph [19]. Chylomicron triacylglycerols (TG) in human subjects consuming MCT diets also contained significant amounts of MCFA [20]. Infants fed MCT-enriched formulae accumulated substantial amounts of MCFA in their adipose tissues [21]. We also found that adipose tissues from young (2-week-old) rats contain levels of MCFA that are more

than 2-fold higher than those in older ones (3-months old; W. Guo and B. E. Corkey, unpublished work). This is most probably due to storage of MCFA acquired from milk in the young rats and their loss after weaning. In pre-term infants, about 27 % [22] to 50 % [23] of the dietary MCT intake was oxidized. On the other hand, up to 82 % and 90 % of octanoate was recovered as CO₂ in fed and fasted rats after 6 h of intravenous infusion of octanoic acid [24]. These studies indicate that MCT may not be exclusively oxidized in the liver, especially after an extended feeding period. The impact of MCFA on metabolism in peripheral tissues may be more significant than previously appreciated.

To determine whether the ability of MCT diet to diminish fat stores could be reproduced *in vitro*, and to study the mechanism, we compared the incorporation of octanoate with that of oleate into lipids using differentiated 3T3-L1 cells as a fat-cell model. We also compared effects of octanoate with oleate on fat-cell differentiation in 3T3-L1 preadipocytes.

MATERIALS AND METHODS

Chemicals

1-¹³C-Labelled free fatty acids (FFA) and deuterated solvent (chloroform) were purchased from Cambridge Isotope (Cambridge, MA, U.S.A.). Other organic solvents were of HPLC grade from Aldrich (Milwaukee, WI, U.S.A.). [1-¹³C]Methyl palmitate was synthesized as described previously [25].

Cell-culture medium, fetal bovine serum, penicillin and streptomycin were purchased from Gibco (Grand Island, NY, U.S.A.). Methylisobutylxanthine, dexamethasone and insulin were purchased from Sigma (St. Louis, MO, U.S.A.).

Abbreviations used: TG, triacylglycerols; MCT, medium-chain triacylglycerols; LCT, long-chain triacylglycerols; MCFA, medium-chain fatty acids; LCFA, long-chain fatty acids; FFA, free fatty acids; G3PD, glycerol-3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; MDI, methylisobutylxanthine/dexamethasone/insulin.

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Cell culture

NIH-3T3-L1 cells were cultured in basal medium [Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (10%), penicillin (100 units/ml) and streptomycin (100 µg/ml)] until 2 days post-confluence. To induce differentiation, unless otherwise indicated, cells were exposed to basal medium supplemented with MDI [methylisobutylxanthine (120 µg/ml), dexamethasone (0.39 µg/ml) and insulin (10 µg/ml)]. Cells were washed 2 days later and then exposed to insulin (2.5 µg/ml)-supplemented basal medium. Medium was changed every 2 days. Lipid droplets were visible by phase-contrast microscopy 2 days after MDI treatment.

HepG2 cells were grown in DMEM with 10% fetal bovine serum containing the same concentrations of the above antibiotics and were used 2 days post-confluence.

Fatty acid induced differentiation

Cells were exposed to oleate or octanoate (1 mM in 0.2 mM BSA) and insulin (2.5 µg/ml) in basal medium 2 days after they had reached confluence. The 5:1 molar ratio of FFA/BSA promotes net transfer of FFA from BSA-binding sites to cells [26]. No MDI treatment was applied to these cultures.

Incubation with [1-¹³C]FFA

A stock solution containing [1-¹³C]octanoate or [1-¹³C]oleate (1 mM in 0.2 mM BSA) was added to the cell culture during a regular medium change. At termination, cells were washed three times with an ice-cold solution (pH 7.4) containing sucrose (250 mM), Tris (10 mM) and 2-mercaptoethanol (1 mM), scraped into the above solution containing additional EDTA (0.2 mM) and homogenized. Aliquots of the homogenate were stored at -80 °C for DNA and TG analysis. Aliquots for glycerol-3-phosphate dehydrogenase (G3PD) analysis were centrifuged at 44000 g for 20 min and the supernatants were stored at -80 °C until analysis. The homogenates were then extracted for lipids for NMR studies as described previously [25,27].

Partitioning of [1-¹⁴C]FFA between CO₂ production and TG incorporation

3T3-L1 cells were prepared in T25 culture flasks. After MDI treatment (4 days), cells were exposed to basal medium containing insulin (2.5 µg/ml) and 1 mM fatty acids (in 0.2 mM BSA) as described above but with a trace amount of [1-¹⁴C]octanoate or [1-¹⁴C]oleate (3 µCi). The incubation was terminated after 60 min. ¹⁴CO₂ released from fatty acid oxidation was collected and quantified using a published protocol [4]. The cellular lipids were extracted and separated by TLC. The TG fraction was scraped off and dissolved in Ecosin-A solution for scintillation counting. The d.p.m. values of the reaction products (CO₂, TG) were converted into nmol by referring to the d.p.m. values of known concentrations of the starting materials ([1-¹⁴C]octanoate and [1-¹⁴C]oleate). Cellular DNA was measured in parallel cultures.

Measurement of basal lipolysis

After the MDI treatment (4 days), cells were incubated with [1-¹³C]octanoate or [1-¹³C]oleate for 16 h so that the intracellular TG pool was enriched with the corresponding isotope-labelled fatty acids. Control cultures were terminated at this time, and cells were homogenized for TG and DNA analysis. After aliquots were taken for these analyses, cellular lipids were extracted for NMR and GLC analysis. Parallel cultures were washed three times with PBS solution and incubated with serum-free DMEM

containing 1% BSA, conditions that favour basal lipolysis. At the end of the incubation, media were aspirated and assayed for glycerol (Sigma procedure no. 337A [27]), and the cells were harvested for analysis as described above. The cell morphology examined before and after the incubation showed that more and/or larger lipid droplets appeared after 96 h of incubation. However, there was no microscopically detectable difference between the cells pre-treated with oleate or octanoate.

Quantification of ¹³C label incorporation into cellular TG from the NMR peak intensity

The ¹³C-NMR spectra were obtained at 125 MHz on a Bruker DMX-500 spectrometer with a 5-mm triple resonance probe (Billerica, MA, U.S.A.). Other details have been described in our previous reports [25,27].

The absolute intensities of ¹³C=O signals were measured by reference to an internal standard [25]. For cells incubated with [1-¹³C]oleate, the ¹³C=O signals from direct incorporation were very intense compared with those from unlabelled carbons (> 90-fold, Figure 1A). Therefore, the measured C=O peak intensity was taken to be equivalent to the [1-¹³C]oleate in TG. For cells incubated with [1-¹³C]octanoate, the signals from direct incorporation of [1-¹³C]octanoate were less intense than the C=O signals from unlabelled TG (Figure 1B; signals from unlabelled saturated acyl chains overlapped those of esterified [1-¹³C]octanoate, but were generally 30–40% of their unsaturated counterparts in control cultures). Therefore, the signal intensity from direct incorporation of [1-¹³C]octanoate was obtained by subtracting the background signal from control. This may not be completely accurate when octanoate incorporation is very limited. Nevertheless, these partial errors do not affect the general conclusions from this study (see below).

Measurement of total cellular DNA, TG and G3PD activity

Duplicate aliquots of homogenate were taken for DNA assays [28] and TG measurement (Sigma procedure no. 337 [27]). G3PD was measured by following the disappearance of NADH during enzyme-catalysed dihydroxyacetone phosphate reduction under zero-order conditions [29].

Methylation of TG and GLC analysis of fatty acyl composition

The lipid components of cell extracts were separated by TLC (hexane/ethyl ether/acetic acid, 70:30:1). Methylation was performed by incubation in BF₃-methanol solution (14%, v/v, BF₃ in methanol) at 60 °C for 30 min. The fatty acid methyl ester was extracted into a hexane solution. The hexane solution was dried with anhydrous sodium sulphate. To avoid the evaporation of methyl octanoate, the hexane solution was used directly for GLC study without further condensation. GLC analysis was performed on a Shimadzu 14A gas chromatograph with a Supelco SP[®]-2380 capillary column with an initial oven temperature of 150 °C, final temperature of 240 °C, heating rate of 4 °C/min, injector temperature of 220 °C and detector temperature of 240 °C. Carrier gas (He) was at 50 kPa, make-up carrier gas (He) at 100 kPa, hydrogen gas at 55 kPa and compressed air at 50 kPa. The sample was injected in 1–1.5 µl with splitting rate of 1:25.

Statistical analysis

Except when indicated otherwise, experiments presented here were repeated 3–6 times. The results were analysed using Microcal Origin (Microcal Software, Northampton, MA, U.S.A.) and are presented as means ± S.E.M. Student's *t* test was performed for

Figure 1 ^{13}C -NMR spectra of lipid extracts after cells were incubated with $[1-^{13}\text{C}]$ fatty acids

(A) Oleate and (B) octanoate in 3T3-L1 fat cells, and (C) octanoate in HepG2 cells. Above the spectra is shown a molecular formula of palmitoleate, a common end product of *de novo* synthesis of LCFA. The spectrum was obtained with 2000 (A) and 4000 (B and C) scans.

selected results, and the *P* values are presented in the corresponding Figures.

RESULTS

Partitioning of exogenous FFA between oxidation and storage

The partitioning of exogenous octanoate and oleate into the metabolic end products of CO_2 and TG is shown in Table 1. Incorporation of either octanoate- or oleate-derived ^{14}C isotopes into other lipid fractions (cholesterol, phospholipids and diglycerides) was much less extensive (results not shown). Our results show that under identical culture conditions, octanoate

partitioned into CO_2 more extensively than TG, but the opposite was found for oleate. The total number of mol of exogenous fatty acids converted into TG plus CO_2 within 60 min was similar. Considering that each octanoyl chain only produces 8 CO_2 molecules (eight times values in Table 1) whereas oleate produces 18 CO_2 molecules (18 times value in Table 1), the actual the amount of CO_2 produced from octanoate was about 3-fold greater than that from exogenous oleate.

It is not possible to determine the endogenous fatty acid pool using this approach. This pool may also contribute to CO_2 production in cells exposed to octanoate and oleate. However, our results show that both octanoate and oleate are actively metabolized in 3T3-L1 adipocytes, and are in accord with previous observations that more octanoate is oxidized than stored in animal cells [1,3–6].

Table 1 Exogenous octanoate and oleate incorporated into the metabolic end products in 3T3-L1 adipocytes detected as CO_2 and TG expressed as nmol of the corresponding fatty acids converted into each product (nmol/h per μg of DNA)

Since part of the acetyl-CoA derived from octanoate may be re-utilized for *de novo* synthesis of LCFA and incorporated subsequently into TG (see below), the amount of isotope recovered in the TG fraction may be more than the actual amount of octanoyl chain esterified into TG. However, since the amount partitioned into the *de novo* synthesis is less than 16% (as detected by ^{13}C -NMR, see below), the results shown here still reflect the amount of $[1-^{14}\text{C}]$ octanoate esterified. Furthermore, the inclusion of *de novo*-synthesized LCFA in TG only supports, rather than averts, the conclusion that octanoate was more oxidized than stored. Means $\pm$ S.E.M. are shown ($n = 3$).

Sample	TG	CO_2
Octanoate	0.9 ± 0.1	8.4 ± 1.2
Oleate	7.6 ± 0.8	0.7 ± 0.1

Direct incorporation of exogenous FFA into cellular lipids

The ^{13}C -NMR spectra of lipid extracts from 3T3-L1 adipocytes incubated with $[1-^{13}\text{C}]$ octanoate or $[1-^{13}\text{C}]$ oleate for 24 h are shown in Figures 1(A) and 1(B). For comparison, a spectrum of the lipid extract from HepG2 cells incubated with $[1-^{13}\text{C}]$ octanoate for 24 h is shown in Figure 1(C). The carbonyl signals are shown in the left-hand panels and aliphatic carbon signals in the right-hand panels. Spectra obtained under other incubation conditions had similar general features with different peak intensities.

$[1-^{13}\text{C}]$ oleate and $[1-^{13}\text{C}]$ octanoate were each found to be esterified to TG at the *sn*-1,3 as well as the *sn*-2 positions in fat cells (Figure 1), represented by the peaks arising from the corresponding carbonyl resonances, TG(1,3) and TG(2). Such

Figure 2 Time-dependent incorporation of fatty acids into cellular TG

(A) Incorporation of $[1-^{13}\text{C}]$ oleate and $[1-^{13}\text{C}]$ octanoate into cellular TG as a function of incubation time. (B) The acyl chain-specific esterification of $[1-^{13}\text{C}]$ oleate and $[1-^{13}\text{C}]$ octanoate, as determined by the relative-peak-intensity ratio of the TG(1,3)/TG(2).

direct esterification of $[1-^{13}\text{C}]$ octanoate was not detected in HepG2 cells (Figure 1C), whereas $[1-^{13}\text{C}]$ oleate was directly esterified in HepG2 cells [30] to an extent similar to that in fat cells (results not shown). The amount of $[1-^{13}\text{C}]$ oleate or $[1-^{13}\text{C}]$ octanoate incorporated into phospholipids was insignificant, as evidenced by the lack of corresponding resonances [25].

Utilization of $[1-^{13}\text{C}]$ FFA-derived $[^{13}\text{C}]$ acetyl-CoA in *de novo* synthesis

In principle, the acetyl-CoA derived from the β -oxidation of $[1-^{13}\text{C}]$ FFA can be used for *de novo* FFA synthesis. Any incorporation of $[1-^{13}\text{C}]$ acetyl-CoA into the acyl methylene would be detected by NMR. In previous studies on fat cells treated with oleate or palmitate, partitioning of exogenous fatty acids into this pathway was not detected [25,27,31]. However, for cells incubated with octanoate, we found that the integrated intensities of some methylene peaks representing a single carbon ($\alpha+1$, $\omega-1$, etc.) were about 2-fold more intense than the ωCH_3 peak (Figures 1B and 1C), indicating selective labelling of the aliphatic region with ^{13}C isotope. Peaks for the αCH_2 and $(\alpha+1)\text{CH}_2$ generally were broader or split because of the magnetic shielding from *sn*-1,3 or *sn*-2 carbonyls. Therefore, the peak heights of these signals were lower than the signals arising from the other methylenes even though they may have had the same overall integral intensity.

We then separated the lipid mixtures (of 3T3-L1 or HepG2) by TLC, and examined the TG and phospholipid fractions by NMR. The spectra from the phospholipid fractions were very weak and did not reveal any ^{13}C signal enhancement. The spectra of the TG fractions were essentially the same as those before the separation, indicating that the signals detected in the spectra shown in Figure 1 were from the TG fractions. This also shows that part of the $[1-^{13}\text{C}]$ acetyl-CoA derived from the β -oxidation of $[1-^{13}\text{C}]$ octanoate was used for *de novo* fatty acid synthesis and then stored in cellular TG. Since the NMR signal intensity from each ^{13}C label is equivalent to that from ≈ 100 natural carbons, a 2–3-fold peak intensity corresponds to about 1–2% isotope enrichment. The observation that the $(\omega-1)\text{CH}_2$ peak is more intense than the ωCH_3 peak indicates that $[1-^{13}\text{C}]$ acetyl-CoA can be used as the priming unit for the acyl chains. The peak intensity of ωCH_3 can be used as an intrinsic

reference to detect the partitioning of ^{13}C -labelled substrates in the *de novo* synthesis pathway.

Octanoate was incorporated into TG less extensively than oleate

Within a 24-h period, the accumulation of oleate into TG increased with incubation time, whereas the accumulation of octanoate reached a plateau at ≈ 7 h (Figure 2A). In 24 h, about four times more total $[1-^{13}\text{C}]$ oleate than $[1-^{13}\text{C}]$ octanoate was incorporated into TG.

To determine if the low rate of incorporation of octanoate into TG was related to substrate availability, cells were incubated with various concentrations (1–5 mM) of $[1-^{13}\text{C}]$ octanoate for 24 h. However, the incorporation of $[1-^{13}\text{C}]$ octanoate into lipid did not vary significantly with respect to octanoate concentration, as determined by NMR (results not shown). Hence, substrate availability (within the range investigated) was not rate limiting for the esterification of octanoate, and it is likely that the process was saturated. In other experiments, we incubated cells with $[1-^{13}\text{C}]$ octanoate for longer periods (up to 7 days), and did not find a substantial increase in the incorporation of $[1-^{13}\text{C}]$ octanoate. Such saturation could be either due to limited incorporation or faster turnover of TG that contains octanoate.

Octanoate and oleate differ in the glycerol position to which they are esterified

The extent of $[1-^{13}\text{C}]$ fatty acid esterification at the *sn*-1,3 or *sn*-2 positions on glycerol can be determined by the peak intensity ratio of TG(1,3)/TG(2). A ratio of 2.0 corresponds to random access of exogenous fatty acids to the three glycerol carbons. For cells incubated with oleate, this ratio was lower than 2.0, and decreased with incubation time (Figure 2B), as found previously [27,31]. For cells incubated with octanoate, this ratio was higher than 2.0, and increased with incubation time (Figure 2B). Hence, these two types of fatty acids not only have different overall storage rates, but also have different esterification rates at the three acyl chain positions in TG. The observation that octanoate has a higher preference for *sn*-1,3 positions agrees with the acyl specificity in animal milk [32].

Figure 3 Micrographs (magnification ≈ 200) of 3T3-L1 cells after incubation for 9 days with basal medium containing insulin ($2.5 \mu\text{g/ml}$) in the presence of 1 mM octanoate (A) and oleate (B) in 0.2 mM BSA

These cells were not treated with methylisobutylxanthine or dexamethasone.

Table 2 The cellular TG content and G3PD activity in 3T3-L1 cells with basal medium containing insulin ($2.5 \mu\text{g/ml}$) in the presence of octanoate and oleate (means $\pm$ S.E.M., $n = 3$)

Cells were not treated with methylisobutylxanthine or dexamethasone.

Sample	TG ($\mu\text{g}/\mu\text{g}$ of DNA)		G3PD (nmol/min per μg of DNA)	
	Day 6	Day 9	Day 6	Day 9
Control	0.3 ± 0.05	0.5 ± 0.03	0.12 ± 0.02	0.3 ± 0.02
Octanoate	0.1 ± 0.02	0.6 ± 0.04	0.09 ± 0.01	0.2 ± 0.01
Oleate	1.7 ± 0.2	5.1 ± 0.3	0.45 ± 0.02	1.7 ± 0.1

Octanoate and oleate differ in their effects in inducing fat-cell differentiation

When added to undifferentiated cells (not treated with MDI), lipid droplets began to appear in cells treated with oleate 3 days after the incubation. In octanoate-treated and control cells lipid droplets began to appear 6 days after incubation, but to a much lesser extent than in cells treated with oleate. After 9 days of incubation, about 90 % of the cells contained lipid droplets. The droplets in octanoate-treated cells (Figure 3A) were much smaller than those in oleate-treated cells (Figure 3B). After extended fatty acid incubation, there was about a 20 % cell loss in oleate-treated cultures, as shown by microscopic examination (Figure 3) and corroborated by DNA analysis. Such cell loss was less significant in octanoate-treated cells. Since the cells that lifted off were mostly differentiated fat cells (examined by microscopy), the cell loss was likely to be induced by the propensity of fat-laden cells to float rather than by fatty acid-related toxicity, although the rapid lipid accumulation in the presence of excess oleate may have accelerated this process. When cells were differentiated with MDI treatment and subsequently accumulated lipids mostly by *de novo* synthesis from glucose, cell loss was not significant up to 6 days after MDI treatment.

The total TG accumulated in oleate-treated cells was substantially greater than for cells treated with octanoate (Table 2). The TG thus accumulated contained mostly oleate (> 80 %) in

oleate-treated cells, and mostly palmitate and palmitoleate in control or octanoate-treated cells (results not shown), indicating that the latter accumulate fat via a *de novo* pathway, as well documented in 3T3-L1 cells [33,34].

To confirm that the TG storage was a result of cell differentiation rather than non-specific accumulation of exogenous fatty acids [35], G3PD activity, a commonly recognized differentiation marker, was analysed in cells thus treated. The results showed a close correspondence between G3PD activity and total TG stored (Table 2). We concluded that incubation with oleate (in the presence of insulin) significantly promoted adipocyte differentiation in 3T3-L1 cells, resulting in higher storage of TG, whereas octanoate did not have such an influence.

Octanoate incorporation increased as differentiation progressed

Incorporation of [$1\text{-}^{13}\text{C}$]octanoate was nearly undetectable in undifferentiated cells. A gradual increase in direct esterification of [$1\text{-}^{13}\text{C}$]octanoate occurred as cells progressed to later stages of differentiation, using G3PD as a marker of differentiation (Figure 4). The incubation with octanoate in this experiment was performed from day 0 to day 6 after MDI treatment. Incorporation of octanoate at the *sn*-2 position reached a plateau early in differentiation whereas incorporation at the *sn*-1,3 position continued to increase, resulting in an increase in the TG(1,3)/TG(2) ratio (Figure 4, inset).

Glycerol release from cells pre-treated with oleate or octanoate

To compare the effects of oleate and octanoate on TG hydrolysis, cells pre-treated with the corresponding fatty acids were incubated with lipid-free DMEM (1 % BSA). The total DNA values per culture assayed at 0, 7, 48 and 96 h were essentially unchanged ($139 \pm 4 \mu\text{g/culture}$), and there was no difference between cells treated with oleate or octanoate. Total cellular TG continued to increase, and slightly more than doubled in 96 h (Figure 5A). The slightly higher TG storage in oleate-treated cells was likely to be a result of more extensive storage of exogenous oleate than octanoate during the 16-h pre-treatment period. The steady rate at which stored TG increased thereafter indicates that *de novo* synthesis of LCFA was not affected differently by octanoate or oleate pre-treatment, and was not hampered by serum deprivation during the time course.

Figure 4 Quantitative incorporation of [$1\text{-}^{13}\text{C}$] octanoate into *sn*-1,3 [TG(1,3)], *sn*-2 [TG(2)] and total TG [TG(1,2,3)] as a function of cellular G3PD activity

Inset: TG(1,3)/TG(2) ratio as a function of G3PD activity.

Figure 5 The total cellular TG before and after lipolysis (A) and the amount of glycerol released during the incubation (B)

Cells were pre-treated with oleate or octanoate as indicated. The data points are connected by lines purely for visual examination of the results.

Despite a slightly lower cellular TG content, cells treated with octanoate released more glycerol than cells treated with oleate after 48 and 96 h of incubation (Figure 5B). Glycerol release was linear with incubation time, and comparable with the reported values in basal lipolysis [33].

Octanoate turnover is faster than oleate during lipolysis

The turnover rate of [$1\text{-}^{13}\text{C}$]FFA incorporated in TG can also be measured by NMR. As shown in Figures 6(A) and 6(B), the total intensity of the [$1\text{-}^{13}\text{C}$]oleate signal was similar before and after the 96-h incubation with DMEM. This indicates that most of the [$1\text{-}^{13}\text{C}$]oleate remained esterified to TG (including unhydrolysed

and hydrolysed but re-esterified oleate), as reported previously [27]. Some of the [$1\text{-}^{13}\text{C}$]oleate released from the *sn*-1,3 positions may have been re-esterified at the *sn*-2 position, because the ratio of TG(1,3)/TG(2) decreased from about 1.2 (Figure 6A) to less than 1.0 (Figure 6B). Based on the integrated signal intensities, over $99 \pm 3.3\%$ ($\pm$ S.E.M.) of the [$1\text{-}^{13}\text{C}$]oleate remained in TG ($n = 3$).

On the other hand, incorporation of [$1\text{-}^{13}\text{C}$]octanoate was initially lower than oleate, but the absolute amount of incorporation was significant compared with the control (Figure 6C). After the 96-h incubation with DMEM, signals from [$1\text{-}^{13}\text{C}$]octanoyl chains were largely decreased at the *sn*-1,3 position, and completely depleted at the *sn*-2 position (Figure 6D). About

Figure 6 ^{13}C -NMR spectra (carbonyl region) of cellular lipids before (left) and after (right) 96 h of basal lipolysis of cells pre-treated with $[1-^{13}\text{C}]$ oleate (A, B) and $[1-^{13}\text{C}]$ octanoate (C, D)

The aliphatic regions (results not shown) of spectra (C) and (D) were both similar to that shown in Figure 1(B), implying no significant changes in the utilization of $[1-^{13}\text{C}]$ octanoate for *de novo* synthesis of LCFA.

Table 3 Fatty acid composition of cellular TG before and after 96 h of incubation with DMEM (1% BSA) in cells pre-treated with oleate and octanoate

Results are shown as percentages (means $\pm$ S.E.M., $n = 3$). The appearance of odd-number chain-length fatty acids is typical in 3T3-L1 fat cells as a result of *de novo* synthesis [50]. Other fatty acids, including $\text{C}_{18:2}$ and $\text{C}_{18:3}$, were also detected, but to a lower extent.

Acyl chains	Oleate		Octanoate	
	Before	After	Before	After
8:0	0	0	10.33 $\pm$ 0.3	0.99 $\pm$ 0.05
14:0	2.84 $\pm$ 0.09	3.28 $\pm$ 0.02	3.74 $\pm$ 0.04	3.88 $\pm$ 0.21
14:1	4.75 $\pm$ 0.08	5.48 $\pm$ 0.03	5.56 $\pm$ 0.29	5.78 $\pm$ 0.11
15:0	1.02 $\pm$ 0.04	1.36 $\pm$ 0.02	1.43 $\pm$ 0.14	1.63 $\pm$ 0.17
15:1	0.81 $\pm$ 0.01	1.24 $\pm$ 0.09	1.4 $\pm$ 0.14	1.41 $\pm$ 0.09
16:0	20.01 $\pm$ 0.36	23.65 $\pm$ 0.03	24.62 $\pm$ 0.35	24.45 $\pm$ 0.03
16:1	27.34 $\pm$ 0.29	38.65 $\pm$ 0.2	38.61 $\pm$ 0.2	47.64 $\pm$ 0.34
17:0	0.82 $\pm$ 0.03	0.75 $\pm$ 0.03	0.9 $\pm$ 0.03	0.85 $\pm$ 0.096
17:1	3.31 $\pm$ 0.26	3.98 $\pm$ 0.07	3.89 $\pm$ 0.1	4.73 $\pm$ 0.12
18:0	0.38 $\pm$ 0.04	0.41 $\pm$ 0.02	0.49 $\pm$ 0.02	0.51 $\pm$ 0.16
18:1 n9	36.4 $\pm$ 0.4	19.71 $\pm$ 0.02	6.69 $\pm$ 0.09	6.2 $\pm$ 0.29
18:1 n11	1.35 $\pm$ 0.07	1.51 $\pm$ 0.12	2.34 $\pm$ 0.05	1.95 $\pm$ 0.13

21 $\pm$ 1.2 % ($\pm$ S.E.M.) of the $[1-^{13}\text{C}]$ octanoate remained esterified in TG ($n = 3$).

Table 3 shows the acyl chain composition in cellular TG fractions determined by GLC. For cells pre-treated with $[1-^{13}\text{C}]$ oleate, the percentage of fatty acyl chains in cellular TG was in the order: oleate > palmitoleate > palmitate. The predominance of oleate corresponds to the rapid uptake and storage of exogenous $[1-^{13}\text{C}]$ oleate. After the 96-h incubation with DMEM, this order changed to palmitoleate > palmitate > oleate. The proportion of oleate declined by ≈ 50 %. Since the amount of total TG was approximately doubled during this period of time, this result indicates that the absolute amount of oleate in TG did

not change significantly. Instead, the reduction in the proportion of oleate was due to increases in palmitoleate and palmitate.

For cells pre-treated with $[1-^{13}\text{C}]$ octanoate, the acyl chains of TG were predominantly palmitoleate and palmitate (Table 3). Octanoate incorporation amounted to 10 ± 0.9 % ($n = 3$) of total acyl composition. After the 96-h incubation with DMEM, the proportion of octanoate in total stored fatty acids was reduced to about 1 ± 0.1 % ($n = 3$). Considering that the total TG content doubled during this period (96 h), the absolute amount of octanoate that remained in TG was about 20 % of the original amount (10 %), a figure that agrees with the NMR results (Figures 6C and 6D).

To determine whether the released octanoate accumulated in the medium, the fatty acid composition of the incubation medium was analysed. The fatty acids detected were mainly palmitate and palmitoleate, with no detectable octanoate and only an insignificant amount of oleate. Together, these results suggested that when deprived of exogenous lipid supply, $[1-^{13}\text{C}]$ oleate was largely conserved in the cellular TG [27], whereas $[1-^{13}\text{C}]$ octanoate was largely dissipated, probably through oxidation.

DISCUSSION

Fatty acids of different chain lengths have different effects on cellular processes. Whereas the pathological roles of saturated compared with unsaturated LCFA are well established [36], the effects of MCFA have received far less attention. Goals of this study were to determine how much octanoate was stored, how it perturbed the molecular structure of TG, and how it affected cell differentiation. Our data document major differences between octanoate and oleate in their oxidation, esterification and release from TG and their influence on adipocyte differentiation.

It has been widely accepted that MCFA are mainly oxidized in cells through the carnitine-independent pathway whereas LCFA may be stored or oxidized depending on the economy of other fuels, as we have also found [37]. This argument has been used to explain the low storage rate of MCFA in fat cells [17]. However, there is evidence that MCFA can be esterified in TG in the liver [38] and fat cells [21,39], which we confirmed in this study.

First, we showed that octanoate was stored in differentiated fat cells but not in undifferentiated preadipocytes (Figure 4), although oleate can also be stored in undifferentiated fat-cell precursors [27]. Storage of octanoate increased as cells became more differentiated until a maximum level was reached. Whether the storage rate would continue to increase with further fat-cell enlargement (as found in obesity) remains to be determined. It is well known that MCFA have a low affinity for cytosolic acyl-CoA synthase [40], but can be readily activated within the mitochondrial matrix for oxidation. Both factors might lead to relatively low cytosolic substrate availability for esterification. However, these may not be the sole reasons for the low rate of MCFA storage, because increasing the substrate concentration of octanoate (up to 5-fold) and extending the incubation period did not increase the proportion of octanoate in stored fat. Another related factor may be the pool of carnitine, which is needed to transport octanoyl-CoA from the mitochondria to the cytosol, and the pool of CoA, which increases with differentiation (A. M. Richard and B. E. Corkey, unpublished work) and could lead to an increase of octanoyl-CoA concentration in the cytosol.

Secondly, we found that when stored, octanoate was mostly esterified at the *sn*-1,3 positions. Esterification of a medium chain to the *sn*-2 position may be thermodynamically unfavourable. Monoacylglycerol acyltransferase may have a higher affinity for LCFA than MCFA so that most of the acyl chains delivered to the *sn*-2 position are from LCFA, especially unsaturated LCFA

[41]. In addition, the incorporation of MCFA might be mostly accomplished via de-acylation/re-acylation of existing TG molecules, whereas incorporation of LCFA may also be accomplished via the synthesis of new TG molecules directly from free fatty acids and α -glycerolphosphate. This is particularly relevant since we found that MCFA are not esterified in cells that have not acquired a suitable amount of TG, whereas the storage of LCFA does not have such a pre-requisite. Since the turnover of *sn*-1,3 chains in TG is more active than *sn*-2 chains [31], it is not surprising that more MCFA are esterified at the *sn*-1,3 positions.

Our observation that octanoate incorporation in fat cells becomes saturated and accounts for about 10% of total fatty acids is consistent with previous studies *in vivo* [21]. When converted to a molar scale, this accounts for $\approx 20\%$ of the total acyl chains stored in fat cells, an amount that would be predicted to have substantial effects on cellular metabolism. In contrast to our observation that octanoate turnover was much faster than that of oleate (Figure 6), storage of MCFA in infant subcutaneous fat was rather stable and remained unchanged 1 week after switching to a MCT-free diet [21]. This is probably because subcutaneous-fat turnover rate is intrinsically lower than that in visceral fat since it serves mainly as thermal insulation and mechanical cushioning [42]. Furthermore, visceral fat is affected more by dietary modulation than peripheral fat.

Thirdly, an interesting finding in our study was that fat cells pre-treated with octanoate have a significantly enhanced TG hydrolysis (Figure 5). To our knowledge, such a finding has not been reported before. It may be argued that less TG storage in cells pre-treated with octanoate results in smaller fat droplets and thus a larger surface area of lipids. However, the cells we used were well differentiated before they were treated with octanoate, and there were no microscopic differences in cell morphology or fat droplet size examined by phase contrast microscopy. The difference in total stored TG was rather small between cells pre-treated with octanoate or oleate, and could not account for the difference in glycerol release (Figure 5). Instead, our results suggested that incorporation of octanoate may facilitate TG hydrolysis. Since the hydrolysis product MCFA diffuses away from the site of reaction more rapidly than LCFA, the lipase efficiency is higher for MCT than LCT [43]. Furthermore, hydrolysis at the *sn*-1,3 position is the rate-limiting step in TG lipolysis [44]. Hence, our findings of the preferential incorporation of octanoate at the *sn*-1,3 position and the increased glycerol release in octanoate pre-treated fat cells fit together logically.

Finally, another important observation was that octanoate, in contrast to oleate, did not stimulate differentiation after an extended incubation period. The eventual lipid accumulation was similar to that seen in control cells as a result of limited spontaneous differentiation in the presence of insulin and glucose [34]. In contrast, incubation with oleate rapidly induced differentiation with characteristic marked TG accumulation and increased G3PD activity. This is consistent with previous reports that LCFA induce the expression of genes involved in fatty acid metabolism [45–47]. This may explain the observation that weaning rats on MCT diets have lower fat-cell numbers as adults compared with their littermates on LCT diets [10]. Furthermore, long-chain CoA esters, but not short- or medium-chain CoA esters, are potent modulators of metabolic enzymes and signal transduction [48,49]. The fact that LCFA induces preadipocyte differentiation *in vitro* may also correlate with the observations that animals and humans on high-fat diets usually acquire more fat cells than controls [50–52]. Hence, replacement of part of the LCFA in conventional high-fat diets with MCFA at critical

times in development may provide a means to control cell number and decrease lipid accretion.

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