Acetyl-L-carnitine flux to lipids in cells estimated using isotopomer spectral analysis

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Abstract Acetyl-L-carnitine is known as a reservoir of activated acetyl units and as a modulator of metabolic function. The objective of this study was to quantify the fate of the acetyl moiety of acetyl-L-carnitine in lipogenic pathways. Lipogenesis was studied in an adipocyte model, differentiated 3T3-L1 cells, and a hepatoma cell, HepG2 cells. Lipogenesis and ketogenesis were examined in rat hepatocytes. Both de novo synthesis and elongation of fatty acids were investigated using gas chromatography/mass spectrometry and [1,2-^{13}C]acetyl-L-carnitine. Comparisons were performed with [^{13}C]glucose and [^{13}C]acetate. Isotopomer Spectral Analysis, a stable isotope method for differentiating between the enrichment of the precursor and the amount of synthesis was used to analyze the data. Acetyl-L-carnitine was generally less effective than acetate as a precursor for de novo lipogenesis. The effects of acetyl-L-carnitine were not identical to those of acetate plus carnitine as expected if acetyl-L-carnitine flux to acetyl CoA is controlled by carnitine acetyl transferase. Acetyl-L-carnitine (2 mM) contributed approximately 10% of the lipogenic acetyl-CoA used for synthesis and elongation as well as 6% of the ketogenic acetyl-CoA. No differences were found between the precursor enrichment for de novo lipogenesis and for elongation of saturated fatty acids. Flux of acetyl-L-carnitine to lipid was increased, not decreased, by the ATP citrate lyase inhibitor, -hydroxycitrate. In contrast, flux of glucose to lipid was dramatically decreased by this inhibitor. These results indicate that flux of acetyl-L-carnitine to lipid can bypass citrate and utilize cytosolic acetyl-CoA synthesis.—**Lligona-Trulla, L., A. Arduini, T. A. Aldaghlas, M. Calvani, and J. K. Kelleher.** Acetyl-L-carnitine flux to lipids in cells estimated using isotopomer spectral analysis. *J. Lipid Res.* 1997. **38:** 1454–1462.

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Acetyl-L-carnitine (ALC) is best known as a reservoir of activated acetyl units in sperm, heart, and insect flight muscle (reviewed in refs. 1, 2). In addition, ALC has been shown to affect a number of key metabolic pathways. It increases phosphocreatine levels in adult and aged rat brain (3) and reverses the age-related decrease in cardiolipin levels in rat heart mitochondria (4). According to the bioenergetic need of the cell, the acetyl moiety of ALC may be converted to acetyl-CoA without utilization of ATP, a reaction catalyzed by carnitine acetyl transferase (CAT). This reaction endows ALC with the potential to provide acetyl units for key metabolic processes when energy levels are compromised, or to relieve the inhibition of pyruvate dehydrogenase by an elevated intramitochondrial acetyl-CoA pressure (5, 6).

Very little is known, however, about the potential role of ALC in modulating and feeding the lipogenic acetyl-CoA pools of the cell. To our knowledge only Farrell, Vogel, and Bieber (7) have provided evidence that the acetyl moiety of ALC readily enters biosynthetic pathways in liver. These investigators found that a large fraction of an injected dose of [1-^{14}C]ALC was incorporated into hepatic lipids in Swiss mice and that [1-^{14}C]ALC was a more effective substrate for de novo lipogenesis than an equal quantity of [1-^{14}C]acetate. The present study was undertaken to evaluate the contribution of ALC to the acetyl-CoA precursor compartments of cultured rat hepatocytes and two lipid-producing cell lines, HepG2 hepatoma cells and 3T3-L1 adipocytes. In addition, the contribution of ALC to the ketogenic acetyl-CoA compartment was investigated in rat hepatocytes. Experiments were conducted comparing the fate of ^{13}C-labeled ALC, acetate, and glucose. The comparison with [^{13}C]acetate was included to determine whether ALC is equivalent to acetate as a lipogenic precursor. The comparison with [^{13}C]glucose was used in conjunction with the ATP citrate lyase inhibitor, (2R,3S)-hydroxycitrate (OHcitrate), to evaluate the requirement

Abbreviations: ALC, acetyl-L-carnitine; CAT, carnitine acetyl transferase; ISA, Isotopomer Spectral Analysis; CoA, coenzyme A; GC/MS, gas chromatography/mass spectrometry; BHB, β-hydroxybutyrate; ACS, acetyl-CoA synthase.

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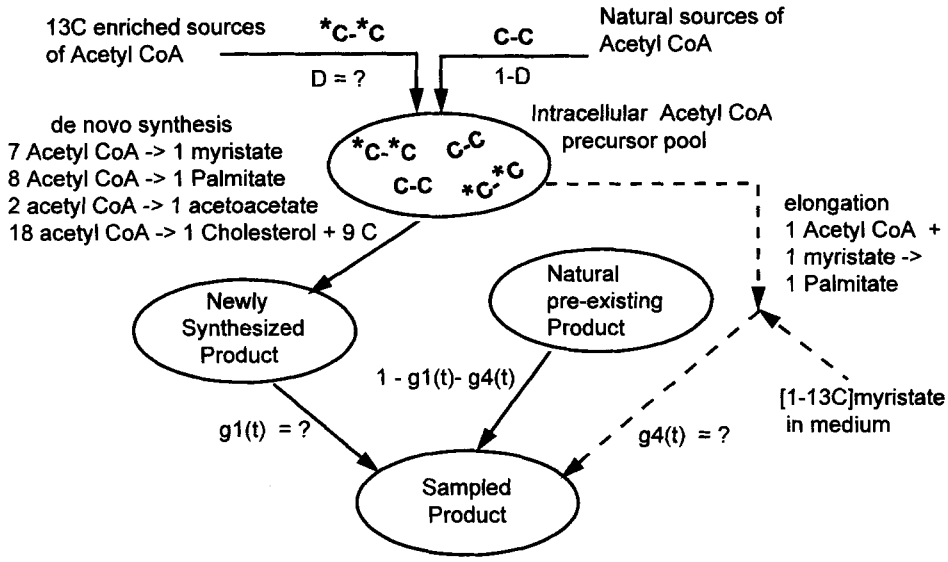

Fig. 1. General model for ISA biosynthesis. A ^{13}C-enriched precursor of acetyl-CoA (indicated by *) mixes with non-enriched, natural sources of acetyl-CoA in intracellular precursor pools. For each specific biosynthesis the fraction of acetyl-CoA derived from the ^{13}C-enriched precursor is D. A specific equation defines the stoichiometry of each type of de novo synthesis. At the termination of the incubation, the product is sampled. In all cases, the sampled product includes molecules synthesized by de novo synthesis in the presence of the ^{13}C-enriched precursor, g1(t). In some cases the elongation of fatty acids must also be included as indicated by the dashed arrow. For lipogenesis, the sampled product contains pre-existing molecules not enriched in ^{13}C which comprise the balance of the sampled product.

for citrate as an intermediate in the pathway from ALC to the lipogenic acetyl-CoA. A quantitative stable isotope method, Isotopomer Spectral Analysis (ISA) was used along with gas chromatography/mass spectrometry (GC/MS) to quantify these fluxes (8–10).

METHODS

Traditional isotopic estimates of lipogenesis record the amount of a labeled precursor incorporated into the product. These methods do not differentiate between the two factors that influence the label incorporated into product, the enrichment or specific activity of the precursor and the amount of new synthesis. The ISA model (**Fig. 1**) was developed to allow each of these factors to be estimated individually (8). The ISA model used for the de novo synthesis of lipids was identical to that used previously (8, 9). It is expanded here to depict each of the pathways explored with [^{3}C]ALC. For each type of biosynthesis the model estimates parameters related to synthesis of the product. The fractional contribution of the labeled precursor to the acetyl-CoA compartment used for biosynthesis is determined as the $D_{(x)}$ value, where x refers to the source of the ^{3}C label. In this study alternative sources of acetyl-CoA and inhibitors were studied to determine their effect on $D_{(AC)}$ and $D_{(ALC)}$. It is not generally possible by direct sampling to measure the contribution of labeled precursors to the lipogenic compartment of intracellular acetyl-CoA. The D value provided by the ISA analysis provides this information and allows the investigator to study those factors that affect the flux of labeled precursor to lipogenic acetyl-CoA compartment. ISA analysis also provides the fraction of product synthesized de novo during the exposure to ^{13}C-enriched precursors, g1(t), where t indicates the time in hours of the incubation with ^{13}C-enriched precursors. In most studies the balance of the lipid, a fraction equal to 1 − g1(t) represents those product molecules that were synthesized prior to the start of the ^{13}C incubation. In some experiments significant fatty acid synthesis occurred via elongation. In these cases the model included an additional parameter, g4(t), representing that fraction of the specified fatty acid formed by elongation (Fig. 1). Student's *t* test was used to evaluate differences in parameter values.

For the ISA analysis of lipogenesis three cell types were investigated, HepG2 human hepatoma cells, differentiated mouse 3T3-L1 adipocytes, and isolated rat hepatocytes. In all cases the cells were incubated in a serum-free Dulbecco's Minimal Essential Medium (DMEM) containing a serum substitute as described previously (9). Rat hepatocytes were isolated from 24-h fasted animals as described by Berry and Friend (11) and cultured overnight in DMEM containing 10% fetal calf serum. Incubation with ^{13}C-enriched precursors was initiated on the following day. For all cell lines, experi-

mental studies were initiated by changing the tissue culture medium to the serum-free medium containing ^{13}C-labeled substrates. Glucose was included in all incubation media at 25 mM for 3T3-L1 and HepG2 cells and 10 mM for the cultured hepatocyte studies. During the incubation with ^{13}C-labeled precursors, the culture media was changed every 24 h. In some studies fatty acids complexed to bovine serum albumin were included in the incubation medium. At the termination of the experiment cells were washed free of media and cellular lipids were extracted. Excreted lipids in the media were not examined in this study. Cholesterol, triglycerides, and phospholipids were isolated from cells and prepared for ISA analysis for de novo lipogenesis using standard GC/MS techniques as described previously (8, 9). ISA techniques were also used to evaluate the elongation of [1-^{13}C]myristic acid by the addition of ^{13}C-labeled acetyl units as outlined previously (12).

To evaluate the synthesis of ketone bodies, freshly isolated rat hepatocytes (1×10^7 cells) were incubated in 1 ml Krebs-Henseleit bicarbonate buffer for 1 h at 37°C with ^{13}C-labeled precursors. Cells were incubated with carnitine (1 mM) and glucagon (4 μg/ml) in the absence of glucose as described for the study of hepatocyte ketogenesis (13). Experiments were terminated by addition of 0.1 M sodium borohydride in 1 M NaOH which reduced acetoacetate to β-hydroxybutyrate (BHB). After deproteination with sulfosalicylic acid, the supernatant was brought to pH 9–11 with 1 M NaOH and extracted three times with ethyl acetate. The residue was then acidified with 1 M HCl to pH 1–2 and extracted three times. These acid extracts were combined and dried under nitrogen. The dry residue was derivatized overnight at room temperature with 50 μl of N-methyl-N-(tbutyldimethly-silyl)-trifluoroacetamide as described by Des Rosiers et al. (14). One μl was injected on a Hewlett-Packard 5890 GC/MS equipped with a HP-1 column. The temperature program was identical to that used by Des Rosiers et al. (14). Tert-butyl-dimethyl-BHB eluted after 6 min. ISA analysis based on the m/z 275 fragment ($M-tC_4H_9^+$) through m/z 280 assessed the incorporation of ^{13}C-labeled precursors into ketone bodies.

The following ^{13}C-labeled compounds were obtained from Cambridge Isotope Laboratories, Andover MA: [U-^{13}C]glucose, [U-^{13}C]acetate, [U-^{13}C]acetyl chloride, [U-^{13}C]palmitic acid, and [1-^{13}C]myristic acid. The abundance of ^{13}C at each labeled carbon was at least 90%. [1,2-^{13}C]acetyl carnitine was synthesized by *o*-acetylation of l-carnitine. One gram of l-carnitine was solubilized at 0°C with 1 ml trifluoracetic acid. To this solution 0.5 ml [1,2-^{13}C]acetyl chloride (99% ^{13}C) was added and the mixture was left at room temperature for 4 h. The reaction product was precipitated with 10 ml ethyl ether, recovered on filter paper, and dried overnight at 30°C. The reaction yield was 92% and the purity of [1,2-^{13}C]acetyl carnitine tested with HPLC and ^{3}H-NMR was 99%. ALC solutions were freshly prepared every 24 h for each batch of new culture media. The stability of ALC in DMEM tissue culture media was evaluated by incubating media with ALC for 24 h in the tissue culture incubator followed by assay of the acetate concentration of the medium. Free acetate was estimated with a radiometric assay using [^{14}C]acetate and acetyl-CoA synthetase based on the method of Rougham and Ohlrogge (15). The assay indicated that the free acetate concentration of the medium, approximately 0.15 mM, was not increased by incubation with 2 to 10 mM ALC. Thus ALC added to the medium was not converted to free acetate prior to cellular metabolism. Except for [^{13}C]glucose, all isotopes were used in the media without dilution. When used at 25 mM, [U-^{13}C]glucose was diluted 1.5-fold with unlabeled glucose to conserve isotope. This dilution was accounted for in the ISA calculations. The ATP citrate lyase inhibitor OHcitrate was isolated from *Garcinia cambodia*. Biochemicals and tissue culture media were obtained from Sigma Chemical Co., St. Louis, MO. Derivatizing agents for GC/MS were obtained from Pierce Chemical Co., Rockford, IL. Acetyl-D-carnitine was supplied by Sigma Tau S.p.A., Pomezia, Italy.

RESULTS AND DISCUSSION

ISA reveals flux of [^{13}C]ALC to lipids

Isotopomer spectra obtained from the GC/MS analysis of lipid products indicated the utilization of ^{13}C-labeled ALC provided in the medium for the lipogenesis. A typical isotopomer spectrum (**Fig. 2,** solid bars) indicated that ^{13}C-labeled precursors were incorporated into lipids because mass isotopomers greater than mass +1 were observed in significant amounts. Very small amounts of these mass isotopomers are observed in the absence of ^{13}C-labeled precursors. If a treatment increases the contribution of the ^{13}C-enriched precursors to the lipogenic acetyl-CoA, the isotopomer spectrum displays an increase in fractional abundance of isotopomers at mass +4 and +6 as shown for OHcitrate comparing Figs. 2, A and B.

To quantify the information available in the isotopomer spectrum, the ISA nonlinear regression procedure matches the experimental spectrum with a simulation of pre-existing and new synthesis of the product to produce the best fit match of model to data (Fig. 2). For the analysis of palmitate or cholesterol synthesis in

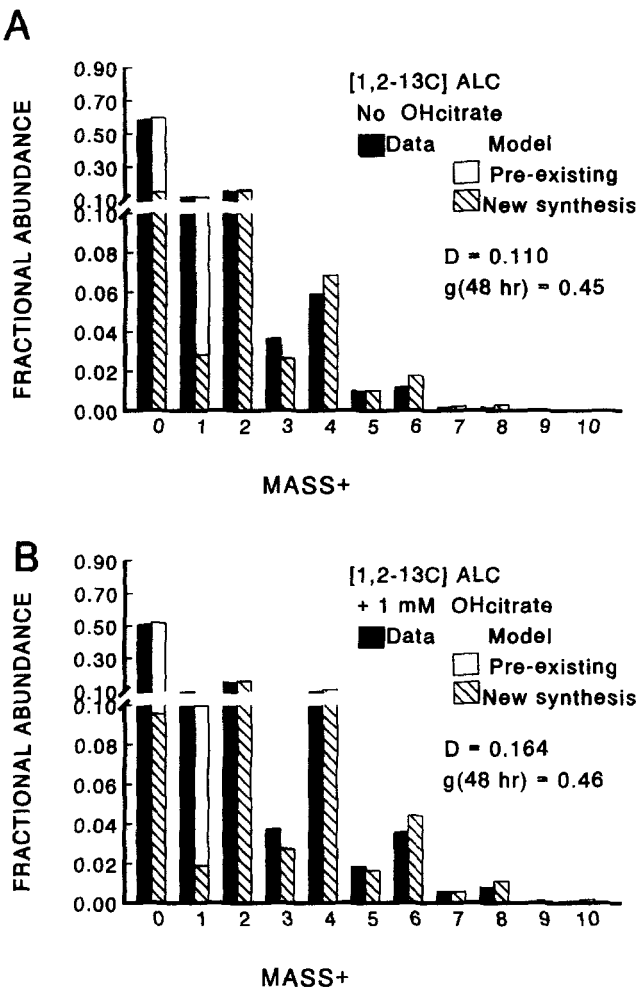

Fig. 2. Sample isotopomer spectra obtained from rat hepatocytes incubated for 48 h in 2 mM ALC in the presence (A) or absence (B) of the ATP citrate lyase inhibitor, OHcitrate. The model solution plotted at the right of the data represents the best fit solution using the indicated parameter estimates.

the presence of a single ^{13}C-enriched precursor of acetyl-CoA, the model solved for two parameters, D and g1(t) as shown in Fig. 1. The parameter g4(t) (Fig. 1) was set equal to 0 because palmitate synthesis by elongation of shorter chain fatty acids was negligible in the absence of added myristate.

ALC contribution to de novo synthesis of fatty acids and ketone bodies

For each cell type and lipid class investigated, a contribution of ALC to the lipogenic acetyl-CoA was detected as summarized in **Table 1.** The D values for ALC (D_{ALC}) indicated that the fractional contribution of this compound to the lipogenic acetyl-CoA was approximately 0.1 in each of the cell types investigated. This indicates that ALC contributed about 10% of the acetyl-

TABLE 1. D and g(48 h) values for lipid and ketone synthesis in three cell types

Cell Type	$D_{(X)}$	g1 (48 h)
HepG2 hepatoma	$D_{(ALC)}$ = 0.096 ± 0.006	0.199 ± 0.007
Triglyceride palmitate	$D_{(AC)}$ = 0.398 ± 0.006	0.435 ± 0.005
	$D_{(GLU)}$ = 0.47 ± 0.006	0.400 ± 0.001
HepG2 hepatoma	$D_{(ALC)}$ = 0.101 ± 0.006	0.222 ± 0.007
Phospholipid palmitate	$D_{(AC)}$ = 0.419 ± 0.006	0.515 ± 0.005
	$D_{(GLU)}$ = 0.48 ± 0.006	0.503 ± 0.005
HepG2 hepatoma	$D_{(ALC)}$ = 0.115 ± 0.007	0.400 ± 0.009
Triglyceride myristate	$D_{(AC)}$ = 0.453 ± 0.006	0.567 ± 0.012
	$D_{(GLU)}$ = 0.517 ± 0.004	0.533 ± 0.005
HepG2 hepatoma	$D_{(ALC)}$ = 0.106 ± 0.004	0.383 ± 0.013
Phospholipid myristate	$D_{(AC)}$ = 0.419 ± 0.003	0.495 ± 0.003
	$D_{(GLU)}$ = 0.463 ± 0.006	0.677 ± 0.004
HepG2 hepatoma	$D_{(ALC)}$ = 0.096 ± 0.006	0.13 + 0.007
Cholesterol	$D_{(AC)}$ = 0.35 ± 0.006	0.19 + 0.005
	$D_{(GLU)}$ = 0.37 ± 0.006	0.20 + 0.001
3T3-L1 mouse adipocytes	$D_{(ALC)}$ = 0.086 ± 0.006	0.590 ± 0.01
Triglyceride palmitate	$D_{(AC)}$ = 0.212 ± 0.006	0.508 ± 0.005
Rat hepatocytes	$D_{(ALC)}$ = 0.111 ± 0.006	0.492 ± 0.007
Triglyceride palmitate	$D_{(AC)}$ = 0.292 ± 0.006	0.420 ± 0.005
	$D_{(GLU)}$ = 0.124 ± 0.006	0.419 ± 0.001
Rat hepatocytes	$D_{(ALC)}$ = 0.058 ± 0.009	1.0
Ketogenesis	$D_{(AC)}$ = 0.288 ± 0.02	1.0

All cells were incubated in DMEM containing glucose as described in Methods. [^{13}C]acetate or ALC was added to this medium at 2 mM to estimate $D_{(AC)}$ and $D_{(ALC)}$. To estimate parameters for [U-^{13}C]glucose, unlabeled glucose in the medium was replaced by ^{13}C-enriched glucose as described in Methods. Reported values are mean ± SEM for n ≥ 3.

CoA used for de novo lipogenesis. For rat hepatocyte triglyceride palmitate, the D value for glucose, $D_{(GLU)}$, and $D_{(ALC)}$ were nearly equal indicating that ALC at 1 mM supplies approximately the same fraction of lipogenic acetyl-CoA as does glucose which was present in the medium at a much higher concentration (10 mM). Each ^{13}C-labeled precursor (X) produced different values for $D_{(X)}$ depending on the ability of that precursor to contribute to the lipogenic acetyl-CoA. However, within each cell type, similar values for $D_{(X)}$ were obtained for each type of lipid synthesized. For example, $D_{(ALC)}$ was approximately 0.1 for all hepatoma cell lipids consistent with a common pool of lipogenic acetyl-CoA that is used for the de novo synthesis of all lipids.

Table 1 also indicates that the $D_{(ALC)}$ was less than that for acetate, $D_{(AC)}$, in all three cell types. To separate the effects of ALC from that of carnitine produced by flux of ALC through CAT, the effects of carnitine on the $D_{(AC)}$ and g1(48 h) values was investigated. In all cases, addition of carnitine (1–5 mM) had no effect on the ISA parameters found using [^{13}C]acetate (data not shown). These results demonstrate that the results observed with [^{13}C]ALC could not be duplicated by carnitine plus [^{13}C]acetate consistent with distinct metabolic pathway for the conversion of acetate to acetyl-CoA via acetyl-CoA synthase (ACS) and for conversion of ALC to acetyl-CoA via CAT.

For each lipid class, ISA estimated g1(48 h), the fraction of lipid found at the end of the 48 h incubation produced by de novo synthesis during the 48-h exposure to ^{13}C. Differences among the g1(48 h) values reflect any changes in the rates of de novo synthesis or turnover due to the composition of the medium. For example, comparing acetate with ALC indicated that ALC produced higher g1(48 h) values for hepatocyte triglyceride palmitate but lower g1(48 h) values for hepatoma cells. The reason for these differences is not clear; however, this comparison does indicate an important feature of ISA. Unlike ^{14}C isotopic flux measurements which only provide a measure of the amount of isotope incorporated into product, ISA provides estimates of the two key parameters governing the flow of isotope to product, the enrichment of the precursor pool and the fraction of the product that was produced during the isotopic incubation.

ISA analysis of ketogenesis was performed using the same principles as for fatty acid synthesis except that pre-existing ketone bodies were negligible in freshly isolated hepatocytes so that the model contained one unknown, D_x, the fractional contribution of the labeled precursor to the ketogenic acetyl-CoA and g1(t) was set equal to 1. The flux of ALC to ketogenic acetyl-CoA was detected by an analysis of the labeling of ketone bodies in isolated rat hepatocytes after incubation with [U-^{13}C]ALC (Table 1) and found to be 0.058 or 5.8% of this flux. Thus, flux of ALC to the ketogenic acetyl-CoA is present in rat hepatocytes suggesting a role of mitochondrial CAT in ketogenesis from ALC. Table 1 also indicates that ratios of the D values (triglyceride palmitate/ketone bodies) for acetate (1.0) versus ALC (1.9) are not identical as expected if hepatocytes metabolize ALC and acetate by separate enzyme systems. Furthermore, these ratios provide an estimate of the relative ability of each precursor to contribute to the acetyl CoA compartment for lipogenesis and ketogenesis. The larger ratio for ALC suggests that the CAT pathway more readily contributes to de novo lipogenesis than to ketogenesis when compared to the ACS pathways used by acetate.

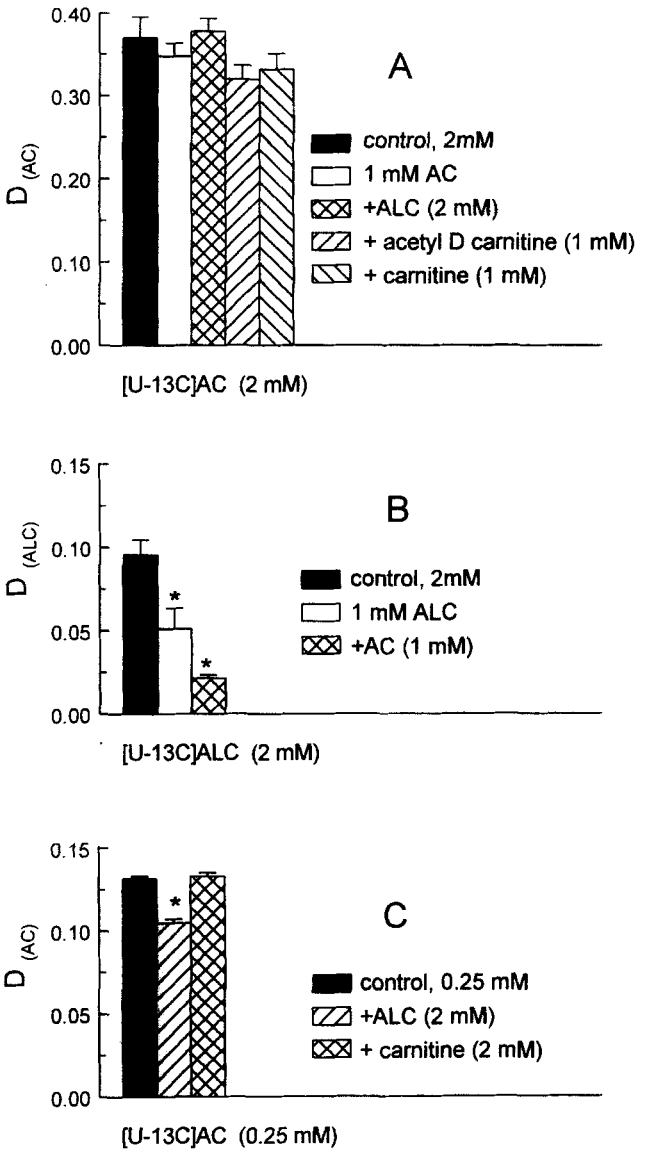

Fig. 3. Effect of additions to media on the contribution of labeled precursors (D values) for triglyceride palmitate synthesis from acetate (AC) and ALC. Control refers to the ^{13}C-labeled precursor used at the concentration listed. All conditions use the ^{13}C-labeled precursor at the concentration listed on the abscissa except the open bars presenting results for 1 mM of the ^{13}C-labeled precursor. Data shown are mean ± SEM for at least three determinations in HepG2 hepatoma cells. * indicates significant difference at $P \leq 0.05$.

Effects of metabolites on flux of acetate and ALC to lipogenic acetyl-CoA

Fatty acid synthesis was further investigated in HepG2 cells to determine the effect of inhibitors or additional sources of acetyl units on the $D_{(AC)}$ and $D_{(ALC)}$ (**Fig. 3**). These experiments provide insight into the processes regulating the flux of compounds into the lipogenic acetyl-CoA compartment. Acetate, at 2 mM, makes a strong contribution to lipogenic acetyl-CoA ($D_{(AC)}$ = 0.38) that was not significantly affected by any of the conditions tested including addition of the reversible CAT inhibitor, acetyl D carnitine (Fig. 3A). The pathway from extracellular acetate to intracellular lipogenic acetyl-CoA appeared to be saturated by 1 mM extracellular acetate because no change in $D_{(AC)}$ resulted when the [^{13}C]acetate concentration was reduced from 2 mM to 1 mM. The fact that 2 mM ALC was not effective in altering the $D_{(AC)}$ value was consistent with the fact that acetate is a more effective precursor for lipogenesis than ALC as indicated by the larger $D_{(AC)}$ versus $D_{(ALC)}$

(Fig. 3 A vs. B). Another difference between these two substrates was that $D_{(ALC)}$ decreased when the ALC concentration was decreased from 2 mM to 1 mM, indicating that the lipogenic pathway for acetyl carnitine involved some step(s) that were not saturated at 1 mM extracellular ALC. As saturation was not observed, the $D_{(ALC)}$ measured here at 2 mM may not represent the maximum value. Addition of 1 mM acetate decreased $D_{(ALC)}$ indicating that 1 mM acetate was able to displace ALC at 2 mM as a carbon source for lipogenesis. Because [^{13}C]acetate at 1–2 mM appeared to saturate the acetate pathway, $D_{(AC)}$ was evaluated at 0.25 mM (Fig. 3C). This concentration of extracellular acetate did not saturate the pathway as indicated by the lower $D_{(AC)}$ value at 0.25 mM versus 1 or 2 mM. At this reduced acetate concentration, a small but significant effect of ALC (2 mM) competing for the lipogenic acetyl-CoA was observed. It is significant that carnitine alone produced no effect, demonstrating that the effect of ALC is due to competition for the lipogenic acetyl units and not related to carnitine generated by the actions of CAT. Comparing both flux pathways below saturation (Figs. 3B and 3C) indicates that acetate was more effective than ALC at reducing the contribution of the competing substrate as a precursor for lipogenic acetyl-CoA. Because the D value represents the enrichment of lipogenic acetyl-CoA, these differences between acetate and ALC reflect differences between the capacity and control of the ACS pathway and the CAT pathway to produce lipogenic acetyl-CoA. In summary, although ALC can produce a significant fraction of lipogenic acetyl-CoA in HepG2 cells, the contribution of the CAT pathway is heavily affected by flux from acetate to lipogenic acetyl-CoA.

ALC reaches lipogenic acetyl-CoA via a cytosolic route

In theory the flux of both acetate and ALC to acetyl-CoA for lipogenesis could involve either a mitochondrial or cytosolic route. ACS, the enzyme producing acetyl-CoA from acetate, is found in both the mitochondria and cytosol. CAT, the enzyme responsible for the production of acetyl-CoA from ALC, has been shown to occur in mitochondria, endoplasmic reticulum, and peroxisomes of rat liver (reviewed in ref. 2). Using the mitochondrial route, acetyl units reach the lipogenic acetyl-CoA compartment via citrate transport forming acetyl-CoA after ATP citrate lyase cleavage. A cytosolic route is also possible as the active site of rat liver microsomal CAT is partly facing the cytosolic compartment (16). A portion of the acetate moiety of ALC could be directly transferred to CoA in the cytoplasm without going through the citrate pathway. To distinguish between these two routes, hepatocytes were treated with the ATP citrate lyase inhibitor, OHcitrate (1 mM). ISA analysis performed on cells incubated in media containing either [^{13}C]glucose or [^{12}C]glucose plus either [^{13}C]acetate or [^{13}C]ALC. The results (**Fig. 4**) indicated that glucose flux to lipogenic acetyl-CoA, $D_{(GLU)}$, was reduced 50% by the addition of OHcitrate. A decrease was expected because glucose flux to acetyl-CoA involves the mitochondrial generation of acetyl-CoA. A different result was observed when either [^{13}C]acetate or ALC was included in the medium. $D_{(AC)}$ and $D_{(ALC)}$ increased by 30 and 54%, respectively, in the presence of OHcitrate (Figs. 2 and 4). This indicated that mitochondrial metabolism of acetate and ALC was not required to generate lipogenic acetyl-CoA. The increased $D_{(AC)}$ and $D_{(ALC)}$ may represent enzyme induction or activation in the presence of OHcitrate. Mechanisms by which OHcitrate may lead to increased enzyme activity include decreased cytosolic acetyl-CoA concentration or elevated citrate.

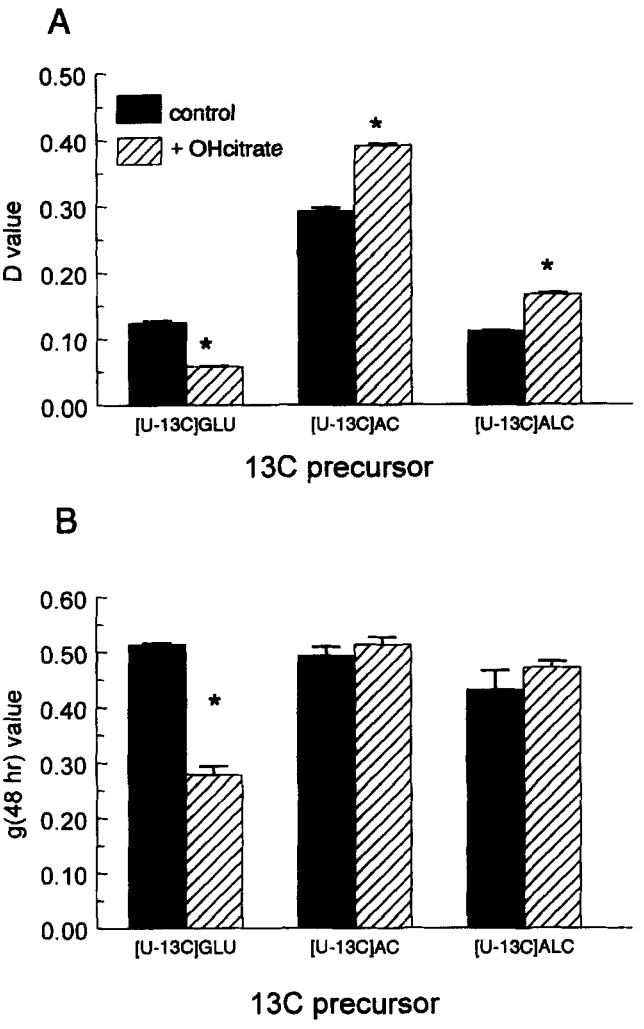

Fig. 4. Effect of 1 mM OHcitrate on D and g(48 h) values for three acetyl-CoA precursors (mean ± SEM) using at least three determinations in cultured rat hepatocytes. * indicates significant difference at $P \leq 0.01$.

Alternatively, these increased D values may indicate that ACS and CAT operate below maximum rates when normal activity of ATP citrate lyase is present. A possible mechanism for this less than maximum flux through ACS and CAT in the presence of normal ATP citrate lyase is the competition for CoA provided by the ATP citrate lyase. However, regardless of the mechanism, these findings clearly indicated that increased flux of both acetate and ALC to lipid can occur when citrate flux to acetyl-CoA is decreased by the inhibitor.

The g(48 h) value reflects the synthesis of lipids during the experimental period. OHcitrate produced a decrease in the g(48 h) value for glucose consistent with a decrease in de novo lipogenesis (Fig. 4B). However, when the medium was supplemented with either acetate or ALC, the g(48 h) value was not affected by OHcitrate. These results indicate that the addition of acetate or ALC to the medium replaces some of the glucose-derived acetyl-CoA so that lipogenesis is maintained. Thus both acetate and ALC were able to replace, in part, the carbon lost when flux of citrate to lipogenic acetyl-CoA was blocked. A decrease in the g1(48 h) value when glucose was the labeled precursor indicated that hepatocytes could not continue normal lipogenesis in the absence of citrate derived from mitochondrial metabolism. At 1 mM OHcitrate the inhibition of glucose flux to lipogenic acetyl-CoA was not 100%. Previous work by Brunengraber, Boutry, and Lowenstein (17) indicated that 0.5–2 mM OHcitrate inhibited total lipid synthesis approximately 60% in perfused rat liver. Although the level of OHcitrate used here did not totally inhibit glucose flux to lipid, it did produce significant alterations in both the D and g1(48 h) values allowing the cytosolic pathway for ALC to be identified.

ALC contribution to fatty acid elongation

The ISA method was used to assess the role of ALC in the elongation of fatty acids. The synthesis of palmitic and stearic acid was studied in HepG2 cells and 3T3-L1 adipocytes incubated in the presence of [1-^{13}C]myristic acid plus [1,2-^{13}C]ALC. An additional parameter, g4(t), was required to represent the elongation of added myristic acid (Fig. 1) (12). ISA analysis of triglyceride palmitate indicated that ALC contributed to the acetyl-CoA compartment for elongation (**Fig. 5**). The model fit the data only if it included elongation of myristate by an acetyl-CoA pool corresponding to a $D_{(ALC)}$ of 0.093. De novo synthesis of palmitate also occurred along with elongation of myristate. The $D_{(ALC)}$ = 0.093 also fit the de novo synthesis profile (Fig. 5). Thus, no differences in the $D_{(ALC)}$ value for synthesis and elongation were observed consistent with a common acetyl-CoA pool for both processes as diagrammed in Fig. 1. ISA analysis of stearate synthesis in this sample demonstrated that

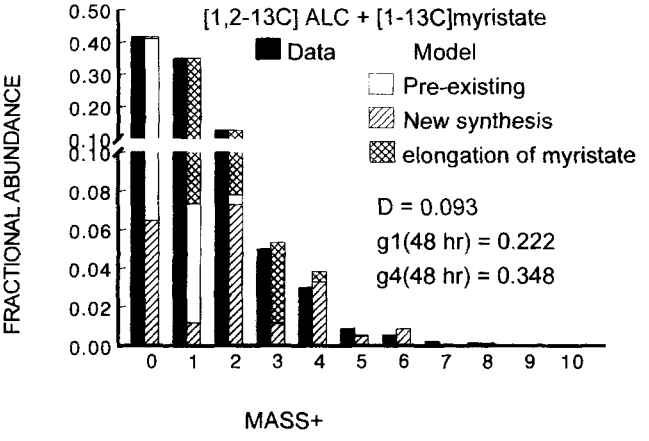

Fig. 5. Isotopomer spectrum and ISA fit for triglyceride palmitate from HepG2 cells incubated simultaneously with both ^{13}C-labeled precursors. Elongation of myristate to palmitate is included in the model as described in Fig. 1. Best fit ISA solution parameter values are shown.

some myristate was elongated by the addition of two acetyl-CoA units with a D value of approximately 0.09 (data not shown). Furthermore, the D value for de novo synthesis in the presence of [1-^{13}C]myristate was not different from that found without added myristate, $D_{(ALC)}$ = 0.096 (Table 1) indicating that the addition of 0.3 mM myristate to the medium did not alter the fractional contribution of ALC to the lipogenic acetyl-CoA. Similar results were observed using 3T3-L1 adipocytes. These data demonstrated that ALC is readily used for both de novo synthesis and elongation in common cell models of lipogenesis. These studies were not designed specifically to distinguish between a mitochondrial and microsomal elongation system. However, in accord with the OHcitrate studies, if ALC feeds a cytosolic acetyl-CoA for the de novo synthesis of fatty acids, this pool of acetyl-CoA appears also to give rise to malonyl-CoA, the primer of the first reaction in the microsomal elongation pathway (18).

Effects of acetate and ALC on cholesterol synthesis from [U-^{13}C]palmitate

HepG2 cells incubated in lipid-free media actively synthesize new fatty acids de novo as described above. However, if [U-^{13}C]palmitate is added to this medium, some of the [^{13}C]palmitate will be oxidized to [^{13}C]acetyl-CoA and reused for de novo lipogenesis. This pathway was investigated by examining the isotopomer profile of cholesterol synthesized in the presence of [^{13}C]palmitate and either acetate or ALC as alternative sources of lipogenic acetyl-CoA. Flux of palmitate to lipogenic acetyl-CoA was demonstrated by finding significant D values for cholesterol synthesis when [U-^{13}C]palmitate was the only enriched precursor present

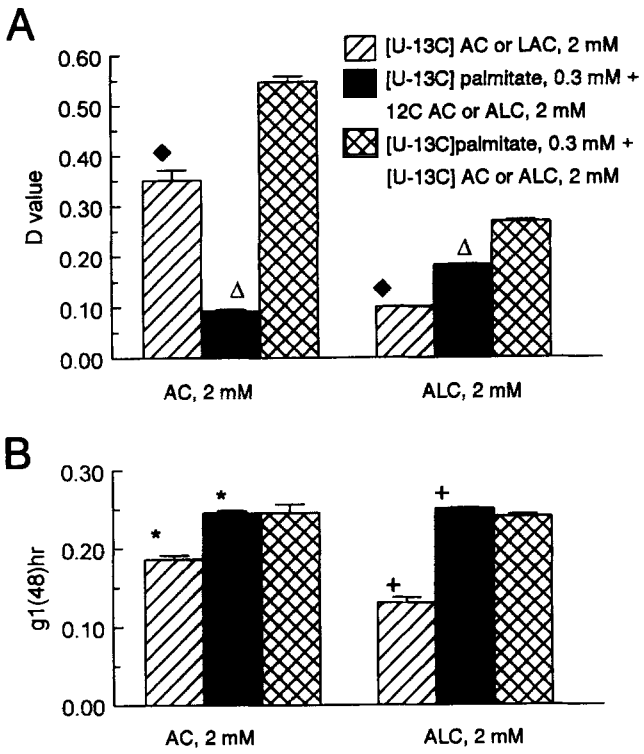

Fig. 6. D and g1(48 h) values for de novo cholesterol synthesis in HepG2 cells incubated in DMEM plus either AC or ALC at 2 mM. D values estimated using [U-^{13}C]palmitate; $D_{(PAL)}$ (solid bars) were based on the labeling of the acetyl moiety produced after β-oxidation of palmitate to acetyl-CoA. When two ^{13}C-labeled precursors were used simultaneously (crosshatched bars), the two labeled precursors were very similar in ^{13}C enrichment and thus D represents the sum of the contribution of each labeled precursor in the incubation. Symbols above bars indicate groups compared. All comparisons indicated significant differences at $P \leq 0.05$.

(**Fig. 6A,** solid bars). The differences in the D values observed in the presence of acetate versus ALC (Fig. 6A) demonstrated an interesting difference in the metabolic behavior of these two substrates. Although the D value for acetate was larger than for ALC in the absence of palmitate, ALC was more effective than acetate in promoting flux of [U-^{13}C]palmitate to lipogenic acetyl-CoA. The $D_{(PAL)}$ was larger in the presence of ALC (Fig. 6A, solid bars). When the media contained both [^{13}C]palmitate and either [^{13}C]acetate or [^{13}C]ALC, the D value calculated for the combined ^{13}C precursors, approached the sum of the two D values for each precursor alone. The larger $D_{(PAL)}$ in the presence of ALC versus acetate (solid bars) indicates that acetyl units derived from β-oxidation of palmitate are more effective in supplying acetyl units for cholesterol synthesis in the presence of unlabeled ALC versus acetate. A possible explanation for this result is that carnitine, released as CAT converts ALC to acetyl-CoA, may enhance β-oxidation. In addition, carnitine produced from ALC may modulate a reservoir of intracellular acyl carnitines (19). In keeping with the role of carnitine in β-oxidation, any effect of carnitine released by CAT on fatty acid oxidation is not shared by acetate as carnitine had no effect on $D_{(AC)}$ (Fig. 3). The addition of palmitate to the medium also increased the g1(48 h) value (Fig. 6B) suggesting a stimulation of cholesterol synthesis by extracellular palmitate.

Conclusions

In past decades investigations of ALC have focused on the role of ALC and/or CAT in the pathophysiological context of cellular bioenergetics (1, 20, 21). Most hypotheses for the mechanism of action of ALC involve some form of intracellular metabolism. Thus, this study analyzed the metabolic fate of the acetyl moiety of ALC in lipogenesis. The first step in the metabolism of ALC is governed by a CAT, an enzyme whose subcellular distribution and catalytic properties are distinct from those of other acetyl-CoA-producing enzymes such as ATP citrate lyase, pyruvate dehydrogenase, and ACS. While it is recognized that specific properties of each enzyme may affect the acetate trafficking and the contribution of acetyl-CoA to biosynthetic pathways, quantitative methodologies have not been previously applied to the investigation of these issues. ISA was used to quantify the contribution of ALC to lipogenesis, demonstrating that ALC contributed about 10% of the lipogenic acetyl-CoA pool used both for de novo synthesis and for elongation in commonly used models of cellular lipogenesis. ISA analysis also indicated that metabolism of the acetyl moiety of ALC is not identical to acetate either in its fractional contribution to lipogenic acetyl-CoA pools or in its effects on the metabolism of other substrates. Using the inhibitor OHcitrate, it was shown that the capacity of ALC to supply acetyl units for de novo lipogenesis could be increased significantly. Because ALC provides acetyl units for metabolic activities without the cost of ATP hydrolysis, this increased capacity may represent a mechanism by which ALC can provide acetyl units in metabolically compromised situations.

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