Review Article

Identifying Sources of Hepatic Lipogenic Acetyl-CoA Using Stable Isotope Tracers and NMR

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The role of hepatic *de novo* lipogenesis (DNL) in promoting fatty liver disease and hypertriglyceridemia during excessive nutrient intake is becoming firmly established. Certain nutrients such as fructose promote hepatic DNL activity and this has been at least partly attributed to their efficient conversion to the acetyl-CoA precursors of DNL. However, tracer studies indicate a paradoxically low level of fructose incorporation into lipids, which begs the question of what the actual lipogenic acetyl-CoA sources are under these and other conditions. Here, we describe novel approaches for measuring substrate contributions to lipogenic hepatic acetyl-CoA using ¹³C-tracers and ¹³C-NMR analysis of lipids and acetyl-CoA probes. We review and address aspects of hepatic intermediary fluxes and acetyl-CoA compartmentation that can confound the relationship between ¹³C-precursor substrate and lipogenic ¹³C-acetyl-CoA enrichments and demonstrate novel methodologies that can provide realistic estimates of ¹³C-enriched substrate contributions to DNL. The most striking realization is that the principal substrate contributors to lipogenic acetyl-CoA have yet to be identified, but they are probably not the so-called “lipogenic substrates” such as fructose. The proposed methods may improve our insight into the nutrient sources of DNL under various feeding and disease states.

1. Overview

De novo lipogenesis (DNL) is a constitutive pathway that transforms acetyl-CoA into long-chain fatty acids. In humans, DNL occurs primarily in the liver, [1] while, in rodents and other mammals, adipose tissue can also be important contributors [2, 3]. A widely accepted teleological function of this pathway is the conversion of excess nutrient carbons into an inert and energy-dense triglyceride product that can be stored and mobilized at a later time for energy generation in times of nutrient scarcity. DNL activity is highly regulated both allosterically and transcriptionally such that it normally only operates during conditions of nutrient and energy satiety. For the liver, this represents the absorptive and early postabsorptive feeding phase where there are high portal vein levels of simple sugars and amino acids coupled with increased amounts of insulin. The loss of regulation of hepatic DNL flux secondary to excess nutrient consumption may be an important early event in the development of fatty

liver and hypertriglyceridemia, which in turn are harbingers of diabetes and liver disease. Given the steep parallel increases in obesity rates and nonalcoholic fatty liver disease in most Westernized societies, there is a pressing need for a better understanding of the metabolic mechanisms that contribute to lipid overload, of which DNL is likely a key component. However, despite the clear association between elevated DNL activity and the increase in hepatic and systemic triglyceride levels by overnutrition, surprisingly little is known about the sources of acetyl-CoA that fuel this process. For nutrients that are widely held to be “lipogenic,” such as fructose and ethanol, the handful of studies performed to date indicate that their contribution to the lipogenic acetyl-CoA pool is paradoxically low. In reality, the liver has a wide choice of potential acetyl-CoA sources that in addition to dietary carbohydrates and ethanol can also include ketogenic amino acids as well as products of intestinal microflora fermentation such as acetate and butyrate. Moreover, under conditions of dysregulated metabolism, where the tight reciprocal regulation of fat

oxidation and fat synthesis pathways is loosened, endogenous substrates such as fatty acids and ketone bodies that would normally not contribute lipogenic acetyl-CoA may augment the more conventional dietary sources. Profiling the sources of lipogenic acetyl-CoA in the liver is challenging for three principal reasons. First, the diversity of potential acetyl-CoA sources hinders the identification and quantification of the principal contributing substrates by conventional tracer methods. Second, hepatic intermediary metabolism features a number of substrate and metabolite recycling pathways that, among other things, randomize the positional ^{13}C -enrichment during conversion of a ^{13}C -enriched substrate to acetyl-CoA. Thirdly, there is substantial evidence that hepatocytes have distinctive intracellular acetyl-CoA pools that are enriched to different levels by ^{13}C -enriched acetyl-CoA precursors. Thus, for determining ^{13}C -enriched substrate contributions to DNL, it is imperative to sample the lipogenic acetyl-CoA pool.

This short review will describe novel approaches for quantifying substrate contributions to the lipogenic hepatic acetyl-CoA pool, with emphasis on ^{13}C isotopic tracers and ^{13}C NMR isotopomer analysis of hepatic acetyl-CoA enrichment patterns. The capacity of this technique to quantify all four possible acetyl-CoA ^{13}C -isotopomers from a variety of reporter metabolites provides a crucial advantage over other methods for resolving ^{13}C -enriched substrate contributions to the lipogenic acetyl-CoA pool. The liver is a principal site for DNL and, as previously mentioned, hepatic intermediary metabolism is characterized by extensive cycling of pyruvate and Krebs cycle intermediates, which causes extensive randomization of ^{13}C derived from glycolytic sources such as glucose and fructose. This results in the generation of multiple acetyl-CoA isotopomers from substrates such as [1- ^{13}C]glucose that in the absence of pyruvate cycling would generate one specific acetyl-CoA isotopomer (in this case [2- ^{13}C]acetyl-CoA). In tissues such as the heart, where pyruvate cycling fluxes are typically low and randomization of ^{13}C at the level of pyruvate is therefore insignificant, contributions of up to four different types of substrates to the acetyl-CoA pool may be determined by selecting a mixture of ^{13}C -enriched substrates that generate each of the four possible acetyl-CoA isotopomers [4–6]. In the liver, the randomization of pyruvate limits the resolution of acetyl-CoA ^{13}C -isotopomers from glycolytic ^{13}C -enriched substrates but can nevertheless provide novel and important information on the contributions of important glycolytic sources such as fructose and glucose to the lipogenic acetyl-CoA pool [7].

2. Measuring Hepatic Acetyl-CoA Enrichment from Tracers of DNL

For measuring the incorporation of isotopically enriched substrates into the hepatic acetyl-CoA precursor pool of DNL, a direct analysis of lipogenic acetyl-CoA enrichment from frozen liver tissue is theoretically possible but this approach has two important limitations. First, acetyl-CoA is a relatively scarce metabolite with a concentration

of ~ 50 nmol/g liver tissue [8]. This precludes ^{13}C or ^2H enrichment analysis by NMR from liver biopsy specimens, or indeed from entire livers in the case of small animal models. Second, the lipogenic acetyl-CoA pool is one of several distinct intracellular acetyl-CoA pools that may exist within the hepatocyte. Since these acetyl-CoA pools may not be equivalently enriched by ^{13}C -precursor substrates, it is important to identify and quantify enrichment of acetyl-CoA that is destined for DNL. With conventional tissue extraction procedures, intracellular acetyl-CoA pools are homogenized and information about the specific enrichment of the lipogenic acetyl-CoA pool is lost.

3. Isotopic Studies of DNL and Acetyl-CoA Enrichment

With isotopic tracers, the fraction of hepatic or VLDL lipid that was synthesized via DNL can be measured. Although beyond the scope of this review, it should be reminded that for conversion of fractional synthetic rates into absolute synthetic rates, it is necessary to independently quantify VLDL secretion rates and changes in hepatic lipid levels. Since hepatic lipid levels are relatively invariant over the interval of a typical tracer measurement, measurement of this parameter may not be necessary. In humans, adipose tissue triglyceride can also be biopsied for tracer incorporation but its enrichment level was shown to be miniscule compared to that of plasma triglyceride indicating that adipose tissue DNL activity was insignificant compared to that of the liver [1]. The principal tracer approaches for quantifying fractional DNL rates have involved carbon-based tracers such as ^{13}C - or ^{14}C -acetate as well as labeled water ($^2\text{H}_2\text{O}$ or $^3\text{H}_2\text{O}$). Carbon-based tracers result in obligatory enrichment of the acetyl-CoA precursor pool followed by passage of the carbon label through subsequent intermediates of the DNL pathway. In the case of the labeled hydrogens of water (which from this point on will focus on the stable ^2H isotope), these are partially incorporated at the level of acetyl-CoA or precursor substrates as well as in the subsequent hydration and reduction steps of fatty acyl synthase (FAS). The terminal methyl hydrogens of synthesized fatty acyls are directly descended from the initial acetyl-CoA molecule that binds to FAS and therefore reflects the isotopic enrichment of acetyl-CoA at the moment of recruitment by DNL. Enrichment of these hydrogens from $^2\text{H}_2\text{O}$ can be resolved and quantified by ^2H NMR spectroscopy of isolated lipids [9, 10]. It has been previously assumed that acetyl-CoA enrichment is equivalent to that of body water; that is, exchange of acetyl-CoA and water hydrogens is complete at the point of DNL [9, 10]. However from analysis of hepatic glutamine hydrogen 4 to hydrogen 3 enrichments from $^2\text{H}_2\text{O}$ in humans, the estimated acetyl-CoA ^2H -enrichment was only 66% of its theoretical value [11] while the same methodology applied to *in situ* and perfused rat livers revealed enrichments that were 71% and 53% of theoretical values, respectively [12]. On the other hand, analysis of lipogenic acetyl-CoA enrichment via *N*-acetyl *p*-amino benzoic acid (*N*-acetyl-PABA) in feeding mice revealed enrichments that were equivalent to that of

body water [13]. Interestingly, GC-MS analyses of fatty acid enrichment from $^2\text{H}_2\text{O}$ also indicate enrichment levels that are substantially below theoretical values [14–16]. Since GC-MS does not resolve fatty acid ^2H -enrichment sites, it does not reveal to what degree the different mechanisms of ^2H incorporation (i.e., reductive NADPH transfer, hydration, and acetyl-CoA incorporation) contribute to the incomplete stoichiometry of fatty acyl ^2H enrichment.

It is not currently known to what extent the enrichment of lipogenic acetyl-CoA methyl hydrogens from $^2\text{H}_2\text{O}$ reflects the contributions of different precursor substrates. Pyruvate methyl hydrogens are extensively enriched with $^2\text{H}_2\text{O}$ as a result of exchange catalyzed by alanine aminotransferase [17] hence the enrichment of acetyl-CoA derived from pyruvate is expected to approach that of body water. Acetyl-CoA derived via β -oxidation will theoretically have two hydrogens out of three derived from body water, that is, a theoretical enrichment that is 67% of body water. Similar considerations apply to other acetogenic pathways, such as the formation of acetate by intestinal fermentation of complex carbohydrates or the hepatic oxidation of ketogenic amino acids. In addition to enrichment of precursor substrate hydrogens prior to acetyl-CoA formation, it is also possible that acetyl-CoA hydrogens are subsequently enriched from body water ^2H during transport from mitochondria to cytosol *via* the citrate shuttle and/or via reversible binding to enzymatic active sites and via exchange with malonic acid. Hence, the enrichment of lipogenic acetyl-CoA at the time of binding to FAS may include factors above and beyond the contributions of different precursor substrates. This, in addition to the diversity of possible acetyl-CoA contributors, diminishes the prospects of directly relating acetyl-CoA ^2H -enrichment to contributions from any given substrate, such as fructose. However, there is a possibility that a given level of lipogenic acetyl-CoA ^2H -enrichment relative to that of body water might be associated with a particular prevalence of nutrient precursors, for example, ketogenic amino acids *versus* carbohydrates. However to date, no such association has been described in any animal model or in humans.

4. Determining Lipogenic Acetyl-CoA ^{13}C -Isotopomer Distribution from ^{13}C -Enriched Substrates

Acetyl-CoA is the common precursor for all lipogenic carbons hence the fractional contribution of a particular ^{13}C -enriched substrate to the lipogenic acetyl-CoA pool informs its contribution to DNL. By ^{13}C -isotopomer analysis of selected downstream metabolites of acetyl-CoA, the ^{13}C -acetyl-CoA isotopomer distribution may be inferred. Metabolites that retain the acetyl-CoA ^{13}C -isotopomer signature include the fatty acid products of DNL, as well as glutamate, ketone bodies, acetyl carnitine, and acetylated xenobiotics such as sulfomethoxazole [18] and PABA [7]. A comparison of acetyl-CoA isotopomer readouts by different metabolites has revealed a substantial heterogeneity in hepatic acetyl-CoA enrichment from ^{13}C -enriched substrates

[19–22]. At the hepatic tissue level, this may reflect metabolic zonation, where the ^{13}C -enriched substrate concentration, and therefore its contribution to the acetyl-CoA pool, diminishes from periportal to perivenous zones as a function of its arteriovenous concentration gradient [22]. Metabolic zonation exerts more significant effects for substrates that are efficiently extracted by the liver, for example, glycerol or short chain fatty acids, and is less influential for substrates that are less efficiently extracted, such as lactate [23]. At the hepatocyte level, there is evidence that mitochondrial, peroxisomal, and cytosolic acetyl-CoA pools are not homogeneously enriched by ^{13}C -substrates, hence the ^{13}C -enrichment of the cytosolic lipogenic acetyl-CoA pool may be incorrectly inferred by probes or metabolites that sample the enrichment of mitochondrial or peroxisomal acetyl-CoA pools [20]. This is illustrated in Figure 1, where the $[2-^{13}\text{C}]$ acetate tracer is initially transported into the cytosol, where it can be converted to $[2-^{13}\text{C}]$ acetyl-CoA via acetyl-CoA synthetase 1 (ACSS1) thereby directly enriching the cytosolic acetyl-CoA pool. Alternatively, the $[2-^{13}\text{C}]$ acetate tracer may be transported into the mitochondrion *via* the monocarboxylate transporter and converted to $[2-^{13}\text{C}]$ acetyl-CoA by the mitochondrial acetyl-CoA synthetase 2 (ACSS2). This mitochondrial $[2-^{13}\text{C}]$ acetyl-CoA can subsequently be transferred into the cytosolic pool via the citrate shuttle, but it can also be utilized by other mitochondrial pathways. These include the Krebs cycle where it generates $[4-^{13}\text{C}]$ glutamate during its first pass through the cycle. This, and other glutamate $^{13}\text{C}_4$ and C_5 isotopomers derived from $[1-^{13}\text{C}]$ -, $[1,2-^{13}\text{C}_2]$ -, and unenriched acetyl-CoA, can be quantified by ^{13}C NMR spectroscopy thereby reporting the distribution of all four possible mitochondrial acetyl-CoA isotopomers [24–26]. The other potential pathway for mitochondrial acetyl-CoA utilization is ketogenesis, where $[2-^{13}\text{C}]$ acetyl-CoA generates ketone bodies enriched in carbons 2 and 4. Depending on ketone body concentrations and ^{13}C -enrichment levels, this enrichment pattern may or may not be quantifiable by ^{13}C NMR but can be analyzed by GC-MS [20]. Enrichment of the cytosolic and mitochondrial acetyl-CoA pools may not be equivalent since the rate of unlabeled acetyl-CoA synthesis from endogenous sources relative to that of $[2-^{13}\text{C}]$ acetyl-CoA formation from $[2-^{13}\text{C}]$ acetate may not be equivalent for each pool. Consequently, the readout of glutamate and ketone body ^{13}C -isotopomers representing the mitochondrial acetyl-CoA pool may be different from those of *N*-acetyl PABA and product fatty acids representing cytosolic and true lipogenic acetyl-CoA pools, respectively. Even within a single intracellular compartment, acetyl-CoA enrichment may not be uniform. For example, it was shown that the enrichment of acetoacetate and citrate was not equivalent suggesting the existence of more than one mitochondrial acetyl-CoA pool [19]. In another study where several extra-mitochondrial acetylation probes were simultaneously administered to sample acetyl-CoA ^{13}C -enrichment from various ^{13}C -substrates substrates such as $[2-^{13}\text{C}]$ acetate, $[1-^{13}\text{C}]$ octanoate, and $[1,2,3,4-^{13}\text{C}_4]$ docosanoate, they reported different acetyl-CoA ^{13}C -enrichment levels [20]. Thus, for

FIGURE 1: Sampling the enrichment of mitochondrial and cytosolic [2-¹³C]acetyl-CoA isotopomer from [2-¹³C]acetate by analysis of downstream metabolic intermediates. Mitochondrial acetyl-CoA enrichment may be inferred by analysis of ketone body ¹³C-isotopomers and the ¹³C-isotopomer distribution in glutamate carbons 4 and 5. Cytosolic acetyl-CoA isotopomer enrichment may be sampled by acetylation probes such as *p*-amino benzoic acid (PABA) or by isotopomer analysis of the terminal methyl and methylene ¹³C-isotopomer distributions of the palmitate product. Note that cytosolic and mitochondrial acetyl-CoA pools may be enriched to different levels depending on the relative activities of mitochondrial and cytosolic acetyl-CoA synthase (ACSS1 and ACSS2, resp.).

identifying the contributions of ¹³C-enriched substrates to DNL, it is imperative to verify that the sampled acetyl-CoA pool truly represents the DNL precursors. Of the possible metabolite and acetylation probes for determining lipogenic acetyl-CoA enrichment, only the product fatty acids have a guaranteed provenance. For both mouse and rat models, there is sufficient amount of hepatic lipid for performing high-resolution ¹³C NMR isotopomer analysis *postmortem*. Triglycerides account for the bulk of hepatic lipid and their constituent fatty acids may be analyzed directly, or following hydrolysis to free fatty acids or transesterification to methyl esters. The latter metabolites are preferred because of their higher solubility in chloroform allowing preparation of more concentrated samples that provide higher NMR signal-to-noise ratios. In addition, fatty acid or ester mixture allows the possibility of isolating a specific fatty acid such as palmitate that may yield better resolved NMR signals compared to the mixture of fatty acids within intact triglycerides. In mammalian liver, the principal fatty acids that are labeled via DNL are palmitate, oleate, and stearate. The ¹³C NMR spectrum of each of these fatty acids has well-resolved signals for the initial and terminal acetyl moieties allowing the ¹³C-enrichment of each acetyl position to be measured. Singly enriched acetyl-CoA isotopomers, that is, [1-¹³C]- and [2-¹³C]acetyl-CoA, contribute to the ¹³C singlet signal of the penultimate and terminal ¹³C-resonances, respectively. The [1,2-¹³C₂]acetyl-CoA isotopomer generates a doublet signal due to ¹³C-¹³C coupling that has a characteristic coupling constant. Therefore, ¹³C NMR spectroscopy provides a means of quantifying fatty acid ¹³C-enrichment from [1-¹³C]-, [2-¹³C]-, and [1,2-¹³C₂]acetyl-CoA. In the case of highly enriched fatty acid

molecules where the ¹³C of one acetyl group is bound to the ¹³C of its neighbour, the ¹³C NMR signals are further split by these ¹³C-¹³C coupling interactions. In our experience, these signals are rarely observed with the acetyl-CoA ¹³C-enrichment levels that are typically achieved in rat and mouse studies. Therefore, the ¹³C terminal and penultimate fatty acid signals that are observed in these experiments invariably consist of a singlet and doublet component reflecting the presence of discrete acetyl ¹³C-isotopomers.

This is illustrated in Figure 2(a), which shows ¹³C NMR signals of hepatic lipids that were isolated *postmortem* from a mouse that had previously ingested a mixture of [1-¹³C]glucose and [U-¹³C]fructose in its drinking water during overnight feeding [7]. As described by Carvalho et al., this substrate mixture generated the predicted [2-¹³C]acetyl-CoA and [1,2-¹³C₂]acetyl-CoA isotopomers from conversion of [1-¹³C]glucose and [U-¹³C]fructose to acetyl-CoA via glycolysis and pyruvate oxidation. However, a significant fraction of [1-¹³C]acetyl-CoA was also generated indicating the randomization of the [1-¹³C]glucose label into carbons 2 and 3 of pyruvate via pyruvate recycling [7].

The incorporation of [1-¹³C]- and [2-¹³C]acetyl-CoA into fatty acids contribute singlet resonances to the penultimate and terminal fatty acid carbons, respectively, while [1,2-¹³C₂]acetyl-CoA accounts for the doublet signals. There are small chemical shift differences between palmitic, stearic, and oleic fatty acids that generate three near-isochronous sets of multiplets in each spectral region. Figure 2(b) shows the ¹³C NMR signals of *N*-acetyl PABA derived from the same animal with the corresponding acetyl-CoA assignments. There is a clear correspondence between the multiplet pattern

FIGURE 2: (a), (b) ^{13}C NMR signals of mouse hepatic fatty acids following ingestion of a $[1-^{13}\text{C}]$ glucose and $[U-^{13}\text{C}]$ fructose mixture (a) and urinary *N*-acetyl PABA from the same animal (b). The fatty acid penultimate and terminal ^{13}C signals, centered at 22.7 and 14.1 ppm, respectively, consist of singlet (S) and doublet (D) components reflecting singly and uniformly enriched ^{13}C -acetyl units of the fatty acids. The *N*-acetyl PABA carbon 1 and 2 resonances (C1 and C2) also consist of singlet and doublet components reflecting singly- and uniformly-enriched acetyl-CoA [7].

of *N*-acetyl PABA C1 and C2 and the terminal acetyl moieties of the fatty acids. Notably, the singlet to doublet ratio for *N*-acetyl PABA C2 is higher compared to that of C1 while the singlet to doublet ratio of the fatty acid terminal ^{13}C -signals are also higher compared to those of the penultimate ^{13}C -signals. The systematically higher singlet to doublet signal ratios for the fatty acid compared to the corresponding *N*-acetyl PABA signals is due in large part to a significant contribution of background ^{13}C enrichment to the fatty singlet signals. This is because the large majority of hepatic fatty acids are not derived from DNL but are nevertheless uniformly enriched at the 1.11% natural abundance level. The observed fatty acid singlets therefore represent the sum of contributions from singly enriched acetyl-CoA isotopomers and background ^{13}C -contributions. In contrast, the ^{13}C -isotopomer pattern of *N*-acetyl-PABA reflects a pool of acetyl-CoA that has fully turned over hence the natural abundance contributions are limited to the fraction of acetyl-CoA that was not enriched by ^{13}C -precursors.

From ^1H NMR analysis of the fatty acid sample (data not shown), we estimated a mean fractional enrichment of 3.1% for the methyl carbons with 1.75% accounted for by the singlet and 1.35% by the doublet component. Since the background ^{13}C enrichment level is 1.11% and contributes only to the singlet component, the excess enrichment attributable to $[2-^{13}\text{C}]\text{acetyl CoA}$ was calculated to be 1.75–1.11, or 0.64%. Note that after accounting for the background ^{13}C contribution, the ratio of $[2-^{13}\text{C}]/[1,2-^{13}\text{C}_2]\text{acetyl-CoA}$ for the fatty acid moiety ($0.64/1.35 = 0.47$) is in reasonable agreement with that measured from the *N*-acetyl PABA singlet to doublet ratio (0.43). The precision of the isotopomer analysis can be improved by isolating palmitate, the major product of DNL and the most intense of the fatty acid signals in the ^{13}C -NMR spectrum. Conversion of palmitate to its methyl ester would allow the methyl ester ^{13}C -signal to be used as an intramolecular ^{13}C -enrichment standard for directly calculating positional fatty acid ^{13}C -enrichments from their ^{13}C -signal intensities. This provides a more precise method of

measuring fatty acid excess ^{13}C -enrichment levels compared to ^1H NMR analysis.

For quantification of all possible acetyl-CoA precursor ^{13}C -isotopomers, including acetyl-CoA that is not enriched in either carbons, the fraction of newly synthesized fatty acids via DNL must be known beforehand. This information can be theoretically derived from the fatty acid ^{13}C -enrichment pattern via mass isotopomer distribution analysis (MIDA), which calculates the precursor acetyl-CoA enrichment based the fraction of fatty acid molecules with single and with multiple ^{13}C -enriched acetyl-CoA units [18]. With MS analysis, this approach is optimal for a single acetyl-CoA precursor isotopomer such as $[1-^{13}\text{C}]\text{acetate}$, where the isotopomer signals from singly and multiply enriched fatty acids are well defined. With more complex acetyl-CoA isotopomer distributions, identifying these species is more difficult. As previously mentioned with ^{13}C NMR analysis, fatty acids containing two adjacent acetyl-CoA units can be revealed by ^{13}C - ^{13}C coupling between their neighbouring carbons, but in practice this is limited to conditions of very high precursor acetyl-CoA enrichment levels. Alternatively, the DNL fractional contribution can be assessed independently with $^2\text{H}_2\text{O}$ by MS [27] or by ^2H NMR [9, 28]. Since ^{13}C and ^2H -enrichment distributions within the same lipid sample can be resolved and quantified by sequential ^{13}C and ^2H NMR spectroscopy, the NMR approach allows $^2\text{H}_2\text{O}$ and ^{13}C -substrates to simultaneously administered.

Figure 3 shows a hypothetical analysis of the lipogenic acetyl-CoA precursor enrichment from decomposition of the palmitate methyl ester ^{13}C NMR isotopomer signals in combination with prior knowledge of the newly synthesized DNL fraction. With this approach, the contributions of fatty acid and precursor acetyl-CoA enrichment from all four possible ^{13}C -isotopomers can be resolved from the singlet and doublet components of the terminal and penultimate palmitate ^{13}C NMR signals. This true lipogenic acetyl-CoA ^{13}C -isotopomer pattern can serve a “gold standard” to verify ^{13}C -isotopomer readouts from more accessible and less invasive acetyl-CoA probes such as *N*-acetyl PABA.

FIGURE 3: Schematic depicting determination of the acetyl-CoA precursor ^{13}C -isotopomer pattern from ^{13}C NMR analysis of the terminal and penultimate carbon resonances with prior knowledge of the fraction of newly synthesized palmitate. In this example, 10% of palmitate was newly synthesized. The methyl ester ($-\text{OCH}_3$) signal is an intramolecular natural abundance standard representing 1.1% ^{13}C -enrichment. The ^{13}C palmitate multiplet signals are decomposed into contributions from ^{13}C -enriched acetyl-CoA isotopomers and background ^{13}C . Acetyl-CoA that is not derived from enriched ^{13}C -precursors (shown in light grey) has a 1.1% background ^{13}C -enrichment. Its contribution is calculated as the difference between the newly synthesized fraction (10%) and the sum of the ^{13}C -enriched acetyl-CoA isotopomers (5%). Since 10% of the palmitate was newly synthesized, the precursor acetyl-CoA isotopomer enrichments are 10 times that of the palmitate ^{13}C -isotopomers.

5. The Effects of Pyruvate Recycling on Hepatic Acetyl-CoA ^{13}C -Isotopomer Generation from Glycolytic ^{13}C -Enriched Substrates

For many substrates such as fatty acids, ketone bodies, and ketogenic amino acids the identity of each carbon is preserved during their catabolism to acetyl-CoA allowing a specific acetyl-CoA isotopomer to be predicted for a given ^{13}C -enriched precursor. For example, catabolism of $[2,4,6,8-^{13}\text{C}_4]$ octanoate yields $[2-^{13}\text{C}]$ acetyl-CoA while that of $[1,3-^{13}\text{C}_2]\beta$ -hydroxybutyrate produces $[1-^{13}\text{C}]$ acetyl CoA. Consequently, quantifying the relative contributions of $[1-^{13}\text{C}]$ - and $[2-^{13}\text{C}]$ acetyl CoA by isotopomer analysis informs the relative contribution of octanoate and β -hydroxybutyrate to acetyl-CoA generation. However, for substrates that are metabolized to acetyl-CoA via pyruvate, which includes glucose and fructose, the ^{13}C -label can participate in pyruvate cycling. In this process, pyruvate is carboxylated to oxaloacetate or converted to malate by malic enzyme and the carbons are returned to pyruvate via PEP-carboxykinase and pyruvate kinase or by reversal of malic enzyme. In the liver,

pyruvate cycling flux is typically several-fold higher than that of pyruvate dehydrogenase [29–31]. This results in extensive randomization of the pyruvate ^{13}C label and, to a lesser degree, enrichment by ^{13}C -isotopomers of Krebs cycle intermediates. As shown in Figure 4, pyruvate cycling has a major influence on acetyl-CoA ^{13}C -isotopomer enrichment from singly enriched pyruvate precursors such as $[2-^{13}\text{C}]$ - and $[3-^{13}\text{C}]$ pyruvate. Precursors that yield $[\text{U}-^{13}\text{C}]$ pyruvate are relatively little affected, since they generate $[1,2-^{13}\text{C}_2]$ acetyl-CoA regardless of whether $[\text{U}-^{13}\text{C}]$ pyruvate participated in pyruvate cycling or not [7]. Thus, when determining the relative contributions of different ^{13}C -substrates that are metabolized to pyruvate, such as, for example, $[1-^{13}\text{C}]$ glucose versus $[\text{U}-^{13}\text{C}]$ fructose, the randomization of the $[1-^{13}\text{C}]$ glucose label into both carbons 1 and 2 of acetyl-CoA via pyruvate recycling needs to be taken into account [7].

In summary, for determining ^{13}C -enriched substrate contributions to the lipogenic acetyl-CoA pool via analysis of acetyl-CoA ^{13}C -isotopomers from product metabolites or acetylated xenobiotics, there are two important considerations. First, the sampled acetyl-CoA must correspond to the true lipogenic acetyl-CoA precursor pool. Second, the effects of hepatic pyruvate cycling must be taken into account for determining acetyl-CoA isotopomer formation from a given ^{13}C -enriched precursor substrate.

6. Lipogenic Acetyl-CoA Fluxes and Sources during High Fructose Feeding

There is currently high interest on the mechanisms by which dietary fructose contributes to excessive levels of hepatic lipid. The conversion of fructose to triose phosphate bypasses several key metabolic control sites of glycolysis, and this is widely cited as an explanation of its higher lipogenic potential compared to glucose. However, fructose also promotes the glycolytic utilization of glucose via the activation of glucokinase [32, 33] hence it can promote the formation of acetyl-CoA from glucose in addition to contributing to acetyl-CoA via its own glycolytic metabolism. High fructose feeding induces increased activities of enzymes that mediate its glycolytic metabolism, including fructokinase and aldolase 13 [34] as well as lipogenic transcription factors such as ChREBP and lipogenic pathway enzymes. There is an increase in hepatic TG levels, postprandial TG secretion, and plasma TG concentrations [35]. The increase in hepatic and systemic TG levels is associated with elevated fractional and absolute rates of DNL in both rodents [36] and humans [37]. The increased DNL flux implies an increase in lipogenic acetyl-CoA generation and current dogma states that the additional acetyl-CoA carbons are contributed by fructose. However, measurements of labeled fructose incorporation into lipid have revealed a paradoxically low contribution. For example, in humans, acute ingestion of various glucose/fructose mixtures resulted in highest DNL rates for the mixture with highest fructose/glucose ratio [37]. Studies on the fate of fructose carbons within 6 hours of ingestion in humans revealed that the bulk of ingested fructose was oxidized or converted to glucose

FIGURE 4: The effect of pyruvate cycling flux on the enrichment of acetyl-CoA from $[1-^{13}\text{C}]$ glucose. For clarity, the unlabeled pyruvate that is also generated is not shown. Pyruvate carboxylase converts $[3-^{13}\text{C}]$ pyruvate to $[3-^{13}\text{C}]$ oxaloacetate which is in rapid exchange with malate and fumarate. This results in a randomization of the ^{13}C label between carbons 2 and 3 of these intermediates and the subsequent formation of $[2-^{13}\text{C}]$ pyruvate and $[1-^{13}\text{C}]$ acetyl-CoA isotopomers, shown in red.

and lactate, with less than 1% incorporated into triglyceride synthesis. [38]. While fructose contributed more than glucose to the appearance of circulating VLDL, the fraction of VLDL fatty acid carbons derived from the fructose load was minor compared to the total fraction of newly synthesized lipid [39]. This suggests that the fractional contribution of fructose carbons to lipogenic acetyl-CoA was also small. Thus, the increased production of lipogenic acetyl-CoA must involve other substrates which hitherto have not been identified. In our recent study of mice fed on normal chow, we sought to measure the contributions of supplemented fructose and glucose to the lipogenic acetyl-CoA pool to determine the extent of their utilization by DNL. Fructose and glucose were provided at concentrations of 5.0%/5.0% and 17.5%/17.5% w/v in the drinking water. The latter formulation is equivalent to 35% sucrose—a widely used concentration for inducing fatty liver in rodents. However, even at these high fructose/glucose levels, the fractional contribution of fructose to the acetyl-CoA pool was only ~10% [7]. Moreover, this was not substantially higher than that of glucose, although the latter could not be precisely measured because of isotopic exchange via pyruvate cycling. The identities of the substrate(s) that contributed the remaining ~80% of acetyl-CoA were not identified.

7. Conclusions and Outlook

Hepatic DNL normally contributes a minor fraction of both liver and circulating triglyceride fatty acyls (the majority are derived from reesterification of circulating NEFA originally derived from adipose tissue TG hydrolysis). However, increased consumption of so-called lipogenic substrates such as fructose or ethanol can result in a several-fold rise of DNL such that it may become a significant contributor to elevated hepatic lipid and plasma TG levels. Since DNL fluxes are intrinsically low compared to other intermediary metabolic fluxes, such as glycolysis, lactate production, and gluconeogenesis, the absolute fraction of carbon flow into DNL is a minor share of the total hepatic substrate disposal. Under conditions of overnutrition, it remains unclear to what extent DNL elevation is contributed by “top-down” mechanisms, where a concerted increased expression of DNL and NADPH-generating enzymes such as acetyl-CoA carboxylase, fatty acid synthase, and G6P-dehydrogenase act to pull in more acetyl-CoA units for fatty acid synthesis, or a “bottom up” process where elevated levels of lipogenic acetyl-CoA precursors serve to push carbons into the DNL pathway. In the case of fructose overconsumption, the evidence strongly leans towards “top-down” actions in stimulating DNL given that it contributes relatively little to the lipogenic acetyl-CoA pool. This leaves a key question as to the identities of the acetyl-CoA sources that supply the majority of DNL carbons. Possible candidates include short-chain fatty acids (SCFA) such as acetate and butyrate that are generated by intestinal fermentation. These are cleared into the hepatic portal vein and efficiently extracted by the liver. While butyrate is initially oxidized to acetyl-CoA by mitochondrial β -oxidation and is therefore dependent on the citrate transport system for incorporation into the lipogenic acetyl-CoA pool, acetate can be directly converted to lipogenic acetyl-CoA in the cytosol. Given that intestinal SCFA production may be influenced by diet [40] and that intestinal SCFA profiles in turn may modify host metabolic fluxes including *de novo* cholesterol synthesis [41], their possible role as major suppliers of lipogenic acetyl-CoA deems further investigation.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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