thematic review

Thematic review series: Patient-Oriented Research

Recent advances in liver triacylglycerol and fatty acid metabolism using stable isotope labeling techniques

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Abstract **Isotopic measurement of biosynthetic rates of lipids in VLDL particles has long posed difficult technical problems. In this review, key methodologic issues and recent technical advances are discussed. A common problem for all biosynthetic measurements is the requirement to measure isotopic labeling of the true intracellular biosynthetic precursor pool. Two techniques that address this problem for lipid biosynthesis, and that are applicable to humans, have been developed—the combinatorial probability method (or mass isotopomer distribution analysis) and ^2H_2O incorporation. The theoretical basis and practical application of these methods, both of which involve mass spectrometry, are described. Issues relevant to specific lipid components of VLDL, such as differences in the labeling of the various particle lipids (phospholipid, cholesterol, etc.), and the contribution of an intrahepatic cytosolic triacylglycerol (TG) storage pool to VLDL-TG are discussed. In summary, advances in stable isotope-mass spectrometric techniques now permit accurate measurement of liver-TG synthesis and flux. In vivo regulation of the synthesis, assembly, and secretion of VLDL-TG in humans is thereby accessible to direct investigation. Patient-oriented research in conditions such as dyslipidemia and hepatic steatosis is made feasible by these scientific advances.**—Parks, E. J. and M. K. Hellerstein. **Recent advances in liver triacylglycerol and fatty acid metabolism using stable isotope labeling techniques. *J. Lipid Res.* 2006.** 47: **1651–1660.**

Supplementary key words liver metabolism • triglycerides • dietary fat • de novo lipogenesis

The synthesis of lipids and lipoproteins is important to both basic biochemistry and clinical metabolic disorders (1–4). Biosynthetic pathways for triacylglycerol (TG), cholesterol and its ester, and phospholipid are separate, but transcriptionally coregulated (5). Both independent as well as integrated control systems for these hepatic lipids have been identified. Furthermore, the assembly of these lipids, along with apolipoproteins, into VLDL particles is a highly regulated process that fails if the synthesis of any one lipid component is blocked (2, 6, 7). It is therefore important for lipoprotein investigators to be able to measure accurately the biosynthetic rates of any or all of the components of VLDL, depending upon the research question being asked. Over the past decade, significant advances have been made in the measurement of hepatic lipids using stable isotopes. This review will first describe methods for the measurement of fatty acid synthesis and will follow this with a description of methods for quantification of TG secretion and turnover.

All isotopic techniques for measuring synthesis of biological polymers are based on an apparently simple principle. The primary objective of a biosynthetic study is to quantify the proportion of end product molecules that were derived from the biosynthetic pathway during a defined period of time, and from this to calculate the absolute flux rate through the biosynthetic pathway. Toward this end, labeled compounds are administered as tracers that enter the end product of interest through a biosynthetic precursor pool in a tissue (e.g., liver) (8). After a defined period of time, the product pool is sampled and calculation of the contribution of newly-synthesized polymers is based mathematically on the model of the precursor-product relationship. For polymerization biosynthesis, the content of label in the intracellular building blocks determines the content of label in newly-synthesized polymers. If, for example, 5% of tRNA leucines in the hepatic cytosol are labeled during an extended period of time, then as newly-synthesized proteins replace preexisting molecules in the end product pool, the fraction of

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Abbreviations: DNL, de novo lipogenesis; ER, endoplasmic reticulum; FCR, fractional catabolic rate; MIDA, mass isotopomer distribution analysis; TG, triacylglycerols.

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labeled leucines in the protein mixture will approach 5% (the content of label in the intracellular pool). In mathematical terms, the precursor-product relationship simply states that labeling of the end product population approaches that of the precursor pool as unlabeled molecules are replaced by newly-synthesized ones. Because this process typically follows an exponential kinetic, a single time point can be sampled along this exponential curve to calculate the fraction of product derived from endogenous synthesis during any time period. From this, the replacement rate (half-life) of molecules in the end product pool can be determined. The great advantage of this approach is that in principle, an investigator needs only two pieces of information to apply the precursor-product method and measure biosynthesis—the isotope content of the product and the isotope content of the precursor pool (9–11). Measurements at a single time point, or at a small number of time points, are then sufficient to calculate the key biosynthetic parameters.

Unfortunately, the complexity of living systems compromises the apparent simplicity of this isotopic approach. Subcellular organization is characterized by inhomogeneity and complexity: compartmentalization of metabolite pools, membrane-associated microenvironments, metabolite channeling through multienzyme complexes,etc. (12–15). In the case of acetyl-CoA, the building block of fatty acids and cholesterol, there appear to be different pools used in the liver for the synthesis of ketones, fatty acids, and cholesterol, and for citrate in the tricarboxylic acid cycle (12). As a consequence, attempts to use indirect measures of acetyl-CoA labeling (such as secreted ketones) have resulted in "serious errors in the estimation of true precursor labeling and biosynthetic rates," as pointed out by Dietschy and Brown (14) 30 years ago. Because measuring incorporation of label into the polymeric product is usually not difficult, gaining accurate information about the precursor back in the cell has provided the greatest challenge. As described below, the method of mass isotopomer distribution analysis (MIDA) is one means of measuring intracellular precursor enrichment. MIDA allows for the calculation of the precursor enrichment based on the pattern of labeling of the product.

THE USE OF MIDA FOR MEASURING HEPATIC LIPID SYNTHESIS

Although nonisotopic techniques, such as indirect calorimetry (16–19), are available for the estimation of whole-body de novo lipogenesis (DNL), the focus of the present discussion will be on methods that utilize stable isotopes. The first of these is MIDA. MIDA exploits the mathematics of combinatorial probabilities to infer biosynthetic parameters, after administration of a stable isotope-labeled substrate (10, 11). The precursor-pool enrichment, fractional synthesis, and absolute synthesis of a biological polymer (any molecule that is synthesized by the assembly of repeating subunits) are calculated on the basis of the frequency of multiply-labeled, single-labeled, and unlabeled molecules present after administration of a stable isotope-containing biosynthetic precursor. The abundance of double-labeled relative to single-labeled palmitate molecules, for example, is a calculable function of the probability that acetyl-CoA is labeled in the biosynthetic tissue, after correction for the natural abundance isotope distribution. Knowledge of the true precursor-pool enrichment then allows the precursor-product relationship to be applied in its simplest form to calculate the fraction of newly-synthesized molecules present.

Thus, the distribution of abundances in the product allows the abundance in the precursor to be calculated. It should be emphasized that there can be no legitimate criticism that the "wrong precursor pool" for the polymer was sampled, because the subunits present in the polymer itself were used for the calculation. It is worth considering two potential complications of this model that might arise from metabolic compartmentalization. First, what if more than one metabolic precursor pool of different isotopic abundances mix together and contribute to the true (immediate) precursor pool for a polymer? This might be the case for fatty acid synthesis during an infusion of labeled acetate, for example, if acetyl-CoA derives from both unlabeled glucose and labeled acetate. MIDA is ideally suited for this circumstance, because the isotopic abundance of the true, or mixed, precursor pool is automatically calculated from the measured distributions in the product. A somewhat more complex situation might occur if more than one biosynthetic site is present for a molecule, and these mix in the final pool sampled. The effect on MIDA calculations of different isotope abundances in the precursor pools has been modeled (11). Even if there is a relatively large isotopic gradient, MIDA calculations will reveal the weighted mean abundance of the precursor pools contributing to the product mixture.

In addition, from an operational point of view, one need only perform measurements on the isolated polymer to gain information about both the precursor and the product and thus to calculate biosynthesis. Various calculation algorithms have been developed since the original description of MIDA, including the matrix correction algorithm of Lee et al. (20, 21), the nonlinear least-squares, best-fit approach developed independently by Kelleher and coworkers (22, 23), and the methods of Wolfe and colleagues (24). These approaches differ only in the mathematical strategy used to solve for the precursor enrichments and fractional synthesis rates of the product and give identical results when compared directly (25) (Wolfe, Hellerstein, and Neese, unpublished observations; T. Masterson, personal communication). Some investigators express stable isotope data as isotope enrichments, some apply matrix correction algorithms to account for the natural isotope abundance contributions and express data as percent of labeled species, and some investigators express data as the tracer:tracee ratios. With regard to hepatic lipids, MIDA has been applied to the synthesis of fatty acids (26–28) and cholesterol in plasma and he-

patic stores, and to the synthesis of bile (29, 30). The example of how newly-made fatty acids are used for VLDL-TG synthesis is discussed below.

LABELED-WATER INCORPORATION FOR MEASURING DNL

Because water diffuses freely across cell membranes, compartmentalized pools at different isotope enrichments are unlikely to exist in vivo after administration of labeled water. A certain proportion of the hydrogens that are incorporated from NADPH into newly-synthesized lipids during reductive biosynthetic reactions derive from, or exchange with, the hydrogen in cellular water, in addition to hydrogens that are incorporated directly into lipids from water. Based on these considerations, the technique of $^{3}H_2O$ incorporation for measuring DNL and cholesterogenesis was developed almost 40 years ago by Jungas and others (31). The model, is that hydrogen in H_2O represents the true precursor (by way of cellular H_2O itself or through NADPH) for hydrogen in newly-synthesized lipids, and the H_2O-specific activity can be accurately determined by sampling any body fluid (e.g., plasma, urine, saliva). The biochemistry is more complex than this, however. The proportion of NADPH hydrogens that are derived from water in fact varies according to the predominant metabolic route of NADPH generation in the cell (31, 32). NADPH can be generated via the pentose pathway, malic enzyme, or other enzymatic routes, which exhibit different degrees of hydrogen exchange with cellular water. In practice, an investigator must select from the literature a hydrogen:carbon (H:C) ratio to use for the lipid molecule under analysis in order to convert moles of labeled H_2O incorporated into lipid biosynthesis, because there is no simple means of determining the H:C value actually present during an experiment. This is a potentially important quantitative limitation of the $^{3}H_2O$ method. More recently, a solution to this problem has been developed by use of the stable isotope $^{2}H_2O$ instead of $^{3}H_2O$. Lee and colleagues (20) used an elegant variation of the MIDA technique to calculate directly the number of hydrogen atoms incorporated into lipid polymers synthesized from $^{2}H_2O$ (i.e., the H:C ratio). In this method, $^{2}H_2O$ is administered to rats, and the number of labeled H subunits from $^{2}H_2O$ (n, in probability terminology) is determined, based on the combinatorial ratios measured, using the measured isotope enrichment of the hydrogen in body $^{2}H_2O$ (p). Lee et al. and others have found that the number of subunits labeled with water is highly variable. This approach has since been used in human subjects and experimental animals given heavy water for periods of time up to 3 months to measure DNL and sterol biosynthesis from measured H:C ratios (33, 34). In addition, Jones, Schoeller, and their colleagues have used a lower-dose $^{2}H_2O$ incorporation technique with analysis by isotope ratio-MS (35, 36), and this method has been extended to use GC-MS as well (37). This approach is not able to measure H:C ratios but uses literature values. Important physiologic findings have been reported using this approach (38–40), as have the effects of dietary manipulation (41).

HEAVY WATER ($^{2}H_2O$) INCORPORATION FOR MEASURING ALL-SOURCE TG SYNTHESIS AND TURNOVER

The $^{2}H_2O$ labeling method has recently been applied for measuring all-source TG synthesis and turnover, in addition to DNL (33, 34). The cytosolic α-glycerol phosphate used for synthesis of new TG molecules contains ^{2}H incorporated from body water, entering through intermediary metabolic pathways (33, 34). As shown in **Fig. 1**, ^{2}H enters during glycolysis of glucose or during glyceroneogenic synthesis of α-glycerol phosphate (three of the five C-H atoms are exchanged by the former route and five of five by the latter). If the number of exchanged ^{2}H atoms in tissue α-glycerol phosphate is known, the precursor-product relationship can then be applied for calculation of all-source TG synthesis. Accordingly, the glycerol moiety of TG is isolated and the incorporation of deuterium (^{2}H) is measured. Use of MIDA allows calculation of the number of exchanged ^{2}H atoms in tissue α-glycerol phosphate and, thus, the isotopic enrichment of the precursor pool for TG synthesis (33, 34).

Because heavy water can be given safely and with relative ease for long periods of time, this approach has proven very useful for measuring the synthesis and turnover rates of adipose tissue TG. The half-life of subcutaneous adipose tissue TG in humans was confirmed to be about 6 months, on average, but with considerable interindividual variation (33). In rodents, turnover rates of mesenteric adipose tissue TG were shown to be higher than those in femoral tissue TG (34).

This method has been used for measuring VLDL-TG synthesis, as well. In the context of the present discussion, a useful feature of this approach is that the equivalent of a constant infusion protocol can be achieved without requiring intravenous lines or medical monitoring. A bolus injection or oral intake of $^{2}H_2O$ results in a stable body $^{2}H_2O$ enrichment for many hours or days, because the half-life of body water is about 10 days in humans. The precursor-product relationship can therefore be used for measuring the rise in VLDL-TG synthesis to plateau while employing a very simple experimental protocol (34).

Another interesting variation that is possible with $^{2}H_2O$ labeling was described by Chen et al. (42). The contribution from glyceroneogenesis to tissue α-glycerol phosphate can be calculated by MIDA, based on the number of exchanging ^{2}H atoms in TG glycerol. As noted above, glycolysis from glucose results in the exchange of three H atoms, whereas glyceroneogenesis results in the exchange of five H atoms. The actual value in a tissue is between three and five and reflects the relative contributions from these sources (42). The activity and input of glyceroneogenesis is regulated by factors of interest in lipid biology,

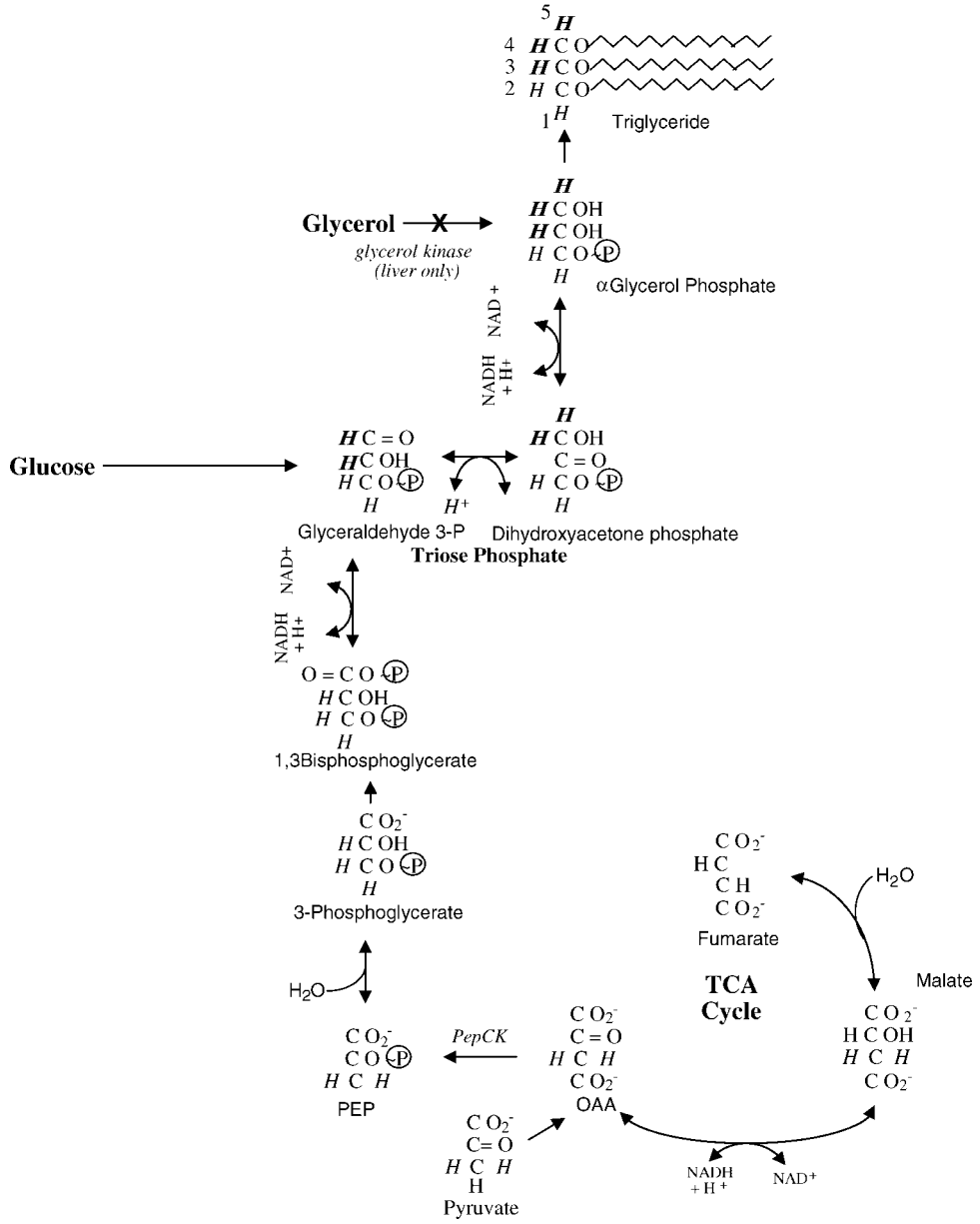

Fig. 1. Biochemistry of ^{2}H incorporation from $^{2}H_2O$ into C-H bonds of the glycerol moiety of acylglycerides. Pathways of hydrogen incorporation during glycolytic and "glyceroneogenic" reactions are shown. The model, described in detail elsewhere (34), demonstrates that the exchange of hydrogen between water and C-H bonds does not occur after α-glycerol phosphate is bound to acyl groups. Accordingly, any acylglyceride containing ^{2}H in its glycerol moiety must have been assembled from α-glycerol phosphate during the labeling period. The pathway of labeled hydrogen incorporation shown here can be traced with pyruvate (glyceroneogenesis; italic *H*, TG hydrogens nos. 1 and 2) or from glucose (glycolysis; boldface H, TG hydrogens nos. 3, 4, and 5).

such as insulin, drugs such as glitazones, and dietary carbohydrate intake (42).

COMPARISON TO ADIPOSE TISSUE LIPID DYNAMICS BY USE OF COMBINED MIDA AND LABELED-WATER INCORPORATION TECHNIQUES

These techniques have been applied to the contribution from DNL to adipose tissue TG, as a comparison to the results of DNL in liver (33, 34). After administration of heavy water to humans (50–80 ml/day) or rodents (4% $^{2}H_2O$ in drinking water), adipose tissue can be sampled and the rate of DNL, as well as all-source TG synthesis, determined concurrently. In human subjects, the average contribution from DNL was 20% of newly-synthesized palmitate in adipose TG (**Fig. 2**). This value, which is somewhat higher than the values observed for hepatic-TG, tended to be stable and characteristic in each individual during serial sampling over 9 weeks. There was consider-

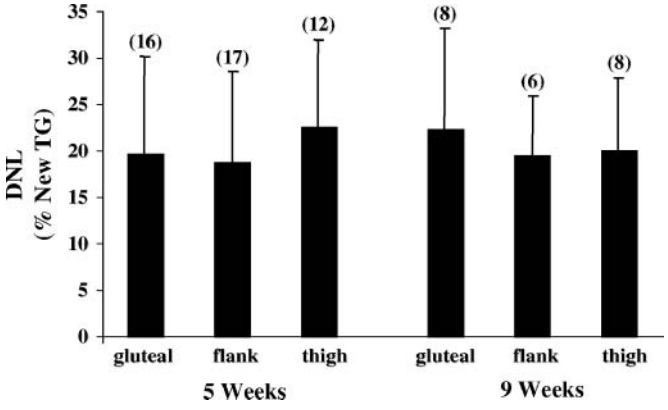

Fig. 2. Calculated half-life ($t_{1/2}$) of subcutaneous adipose-tissue TG in three depots at weeks 5 and 9 of ^2H_2O intake. Healthy subjects consumed heavy water for either 5 or 9 weeks, followed by subcutaneous adipose tissue biopsy. Doses and rate of labeled water administration are described in reference (33). Values in parentheses are the sample size for each measurement. Error bars represent standard error.

able variability among subjects, however. The genetic, dietary, or other factors influencing the DNL contribution in humans will be of interest to study using this approach.

Assumptions of MIDA and labeled-water incorporation techniques

The availability of these two methods for measuring DNL and cholesterol synthesis in humans represents a significant advance in the field over the past several years. Each method is based on assumptions that should be explicitly recognized. For MIDA, one assumes that a polymerization biosynthesis model applies, i.e., that the molecule was built in total from a discrete pool of precursor units. Thus, chain elongation of lipids, which could occur at a different time, in a different cell, or from a different acetyl-CoA precursor pool than synthesis of the 16- or 18-carbon fatty acid core, requires a different calculation algorithm and set of assumptions (10). For the labeled-water incorporation technique, there is the possibility of an isotope effect against ^2H or ^3H in H_2O, in addition to the problem of H:C ratios mentioned above. The combination of heavy water (^2H_2O) labeling with MIDA can solve the H:C problem, as just discussed. It should be noted that isotope discrimination, if it occurs, will be greater for ^3H than for ^2H (relative to ^1H), representing a theoretical advantage of stable isotopes over radiolabeling.

Information about the metabolic source and kinetics of the intracellular acetyl-CoA precursor pool can be learned by MIDA but not by labeled-water incorporation. Oral intake of fructose, for example, results in a marked drop in hepatic acetyl-CoA enrichments during an MIDA labeling study with [^{13}C]acetate that is greater for larger fructose loads [(43) and E. Murphy, C. Beysen, and M. Hellerstein, unpublished observations]. This dilution of intrahepatic acetyl-CoA is metabolically informative, inasmuch as it represents the entry of a triose-phosphate load into the liver and therefore provides information about the substrate load as well as the liver's biosynthetic response. Also, decay curves after pulse labeling can be used very accurately to calculate end product turnover rates and absolute synthesis rates by MIDA (29, 44), but not by ^2H_2O, because enrichment of the body water pool falls too slowly to allow "pulse-chase" applications. Nevertheless, both methods represent valuable alternatives for measuring lipid synthesis in humans. Emerging concepts regarding the regulation of hepatic-TG and VLDL-TG using these methods are discussed below.

Measurement of TG synthesis

The contribution from adipose-derived fatty acids to VLDL-TG production. In the fasting state, the plasma nonesterified fatty acid (NEFA) pool is the primary precursor for VLDL-TG synthesis (45–47). However, previous results with infusions of [^{13}C]palmitate have revealed an interesting complication. The isotope enrichment of palmitate in total VLDL fatty acids typically did not approach a value close to the enrichment of the palmitate in the plasma NEFA pool, even after 12 h of NEFA infusion, contrary to expectations from the precursor-product relationship if plasma NEFA provided most of the fatty acids that entered VLDL-TG. Separation of the TG from other lipids in VLDL revealed that the palmitate used for synthesis of phospholipid and cholesteryl ester was derived from a hepatic fatty acid pool that was labeled less than that used for TG in VLDL (**Fig. 3**). Previous work in isolated hepatocyte systems had suggested that the fatty acids used for cholesteryl ester synthesis are thought to be derived from a specific endoplasmic reticulum (ER)-associated acyl-CoA pool (via the enzyme ACAT) (48). The isotope-labeling results in humans demonstrated that the incorporation of a NEFA label into VLDL can be used to accurately measure liver-TG secretion rates if the TG is separated from these other lipid classes. Furthermore, multiple stable isotope labeling with a combination of fatty acid precursors, fatty acids, glycerol, and leucine can be used to understand VLDL particle assembly (**Fig. 4**). For instance, isolating TG specifically for this analysis, the movement of plasma-derived NEFA into lipoprotein-TG has been shown to occur quite quickly; the label can be detected in large VLDL particles as early as 20–30 min after beginning an intravenous infusion of a labeled NEFA (see Fig. 3, subjects B and C). The rapid rate of appearance in VLDL-TG is surprising, given that in this short time, the fatty acids would have needed to be taken up in the liver, bound to cellular binding proteins, transported to the ER for incorporation into lipoprotein-TG, and secreted into the blood before accumulation was sufficient for detection by GC-MS. Using this methodology, we have found that after a long fast (18 h), the plasma NEFA pool provided nearly 100% of the fatty acids used for VLDL-TG synthesis in healthy normolipidemic subjects [see subjects A and B, Fig. 3 (47)]. For many of the hyperlipidemic subjects studied under the same long fasting conditions, however, the NEFA pool could account for only 70–80% of TG fatty acids (e.g., subject D, Fig. 3). This lack of complete labeling of the VLDL-TG

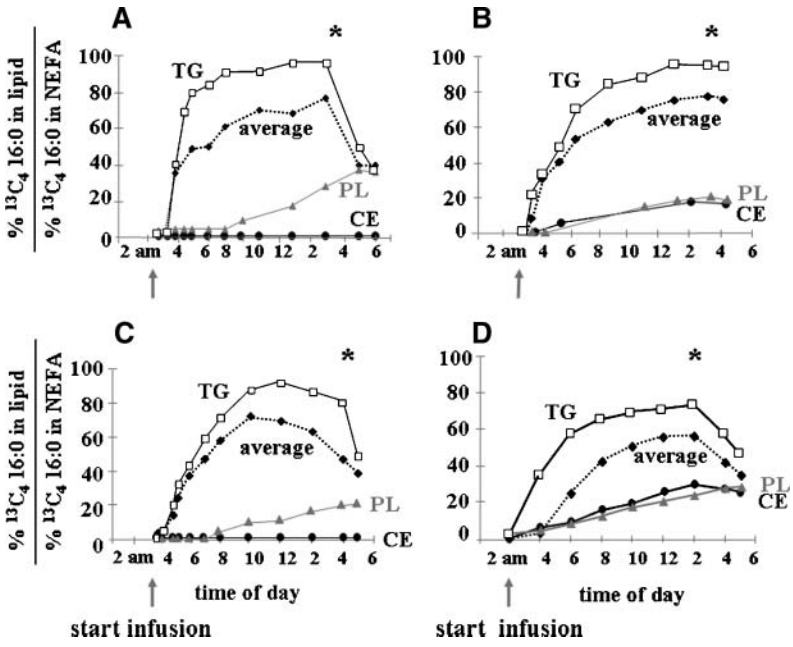

Fig. 3. Pattern of labeling of VLDL lipids with a stable isotope of palmitate infused into the plasma nonesterified fatty acid (NEFA) pool. [$^{13}C_4$]palmitate (16:0) was infused intravenously for 12 h in four healthy, fasting subjects (A–D). Cessation of the infusion is denoted with an asterisk. VLDL particles were isolated by ultracentrifugation and the lipids (TG, triacyglycerols; PL, phospholipids; and CE, cholesteryl esters) separated by thin-layer chromatography (TLC). Fatty acids in each of these lipid classes were transesterified and analyzed by GC-MS as described previously (47). Each of the curves represents the enrichment of the label in these lipids as they appeared over time in VLDL. In addition, an aliquot of VLDL was extracted, and fatty acids from all lipids combined were transesterified before analysis (i.e., no TLC was performed). The mean change in enrichment of total VLDL palmitate over time is represented by the curve denoted "average."

pool, even after an 18 h fast, suggested that other sources of lipids contributed significantly to hepatic-TG synthesis. As will be described in the following sections, these sources can include hepatic-TG stores derived from dietary fatty acids, or fatty acids made via the process of DNL.

Dietary fatty acids. Recently, our laboratory (49–53), and Vedala and Hellerstein (unpublished observations) and others (54) have investigated the flux of dietary fatty acids into liver-TG pools. The time needed for dietary fatty acids to enter the liver and be used for subsequent VLDL-TG synthesis is longer than that for the direct plasma NEFA pool and depends on at least three factors: the rate at which meal fats appear in the chylomicron-TG pool, the rate of lipolysis of intravascular TG-rich lipoproteins, and the rate of chylomicron remnant uptake by the liver. Chylomicrons secreted shortly after the onset of fat consumption contain a majority of fatty acids derived from the

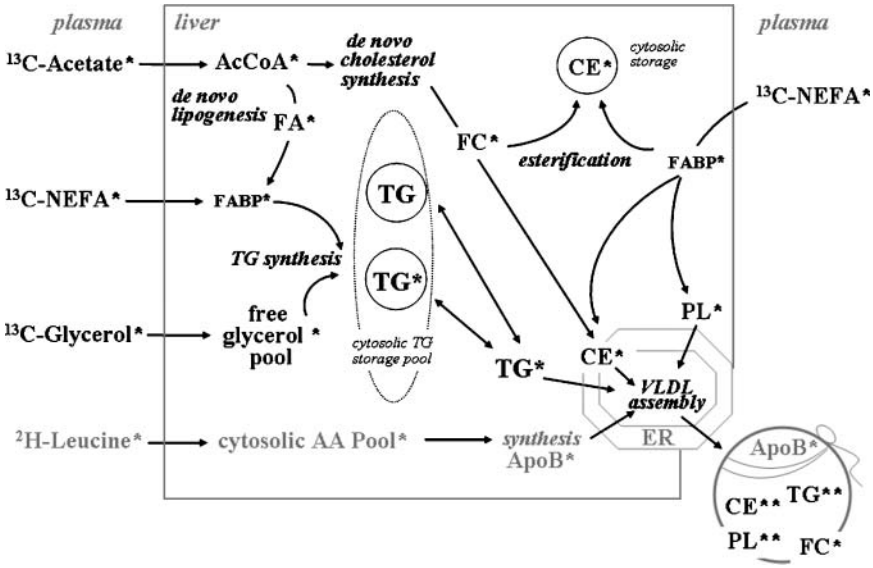

Fig. 4. Simultaneous labeling of VLDL particle components. Metabolic labeling in human subjects using multiple stable isotopes administered intravenously can produce concurrent enrichment of lipoprotein components. In this example, [^{13}C]acetate can be used to measure the synthesis of fatty acids and cholesterol in either free (FC) or ester (CE) form. [^{13}C]glycerol will label the backbone of both triacylglycerol (TG) and phospholipid (PL). A NEFA label can be detected in triacylglycerol (TG), phospholipid (PL), and cholesteryl ester (CE) fatty acids. Finally, labeled leucine is routinely used to label VLDL apolipoprotein B-100 (ApoB) for the measurement of particle turnover rates in the plasma compartment. FABP, fatty acid binding protein; ER, endoplasmic reticulum.

previous meal that had accumulated in a putative intestinal storage pool (55, 56). As the time after meal fat consumption lengthens, more and more of the chylomicron-TG is derived from the meal itself (57). After entering the body through chylomicron synthesis, dietary fatty acids enter the liver in at least two ways. First, through the action of lipoprotein lipase, the liberation of fatty acids from chylomicron-TG occurs at a rate that exceeds tissue uptake (58). As a result, a portion of these fatty acids spill over into the plasma NEFA pool where they can be cleared to the liver in a manner similar to fatty acids derived from adipose tissue. Diet-derived NEFAs appear in VLDL-TG within an hour of meal consumption (50). However, later in the postprandial phase, the dietary label in VLDL-TG is larger than can be accounted for by spillover of dietary fatty acids into the plasma NEFA pool. This additional input was thought to come from chylomicron remnant uptake by the liver (50). The timing of this occurrence (4.5 h post meal and later) is consistent with the delay that would be due to chylomicron disassembly within the hepatocyte and transfer of these fatty acids to the ER. At peaks in postprandial lipemia, 35–50% of VLDL-TG palmitate can be derived from hepatic recycling of dietary palmitate, with fatty acid entry from the spillover pathway and chylomicron remnant uptake providing roughly equal contributions (50, 57). It is striking that these data were derived from research subjects consuming a standardized meal in which only 30% of the energy was derived from fat (49–53). Dietary fat entry into the hepatocyte might be expected to be even greater in individuals consuming meals with higher fat content, although other factors might complicate this relationship. Very similar results were observed by sampling of plasma VLDL-TG after oral administration of labeled stearate in the diet for 2 days (Vedala and Hellerstein, unpublished observations). The contribution from dietary fatty acids to TG assembled in the liver and secreted in VLDL was greater in hypertriglyceridemic subjects than in healthy controls. The ability of dietary fat to contribute to hepatic-TG stores is supported by the data of Musso et al. (59), who found that patients with fatty livers consumed significantly more grams per day of saturated fats ($P < 0.001$) compared with control subjects matched for age, sex, and body mass index (BMI). Similarly, Tiikkainen et al. (60) found that of all dietary and physical parameters assessed (e.g., BMI, body fat, waist circumference), total and saturated fat intake (as a percentage of total energy) were the only variables significantly correlated with a wide range of liver fat contents in overweight women ($r = 0.44$, $P < 0.05$). We have analyzed the sources of hepatic fat in liver biopsy samples from patients with fatty liver who, for the preceding four days, had been infused with stable isotopes and had consumed a 30% fat diet that had also been labeled with a stable isotope (51). Liver biopsies were taken in the fasting state. Of the TG fatty acid sources identified, 59% were derived from the plasma NEFA pool, 15% from dietary fatty acids, and 26% from DNL. Thus, as confirmed by direct measurement, dietary fat can contribute significantly to liver-TG storage pools.

DNL. The other remaining source of fatty acids that can be used for VLDL-TG synthesis is the hepatic DNL pathway. The 3–4 h delay in appearance of newly-made fatty acids in VLDL-TG after the onset of the first meal of the day (53) suggests that de novo-synthesized fatty acids are directed first toward an intracellular liver-TG pool. This concept is supported by a number of observations. First, the timing of stable isotope labeling influences the fractional appearance of de novo-synthesized fatty acids in fasting VLDL. If an infusion of [1-^{13}C]acetate is begun at midnight, the average lipogenesis in healthy, fasted subjects 7–8 h later is typically <5% (47). However, higher values are observed if the infusion is begun at 6 PM the night before the measurement and if the infusion occurs during the consumption of the evening meal [a total of 13 h of labeling, (53, 61)]. A further elevation of 1.5 times in the fasting lipogenesis rate is observed if the infusion continues for an additional 24 h. The explanation for the increase in DNL from the first to the second morning is that some of the fatty acids made during the postprandial state enter the hepatic cytosolic pool and contribute to fasting VLDL-TG the next morning, and that food intake stimulates the movement of fatty acids through this storage pool. Not only are newly-made fatty acids stored, but the process of DNL appears to be strongly influenced by insulin resistance. Patients with insulin resistance alone (62) or with insulin resistance and fatty liver (51) exhibit significantly elevated rates of fasting DNL. Furthermore, in these subjects, fractional lipogenesis failed to increase significantly with meal consumption (51), suggesting that DNL is contributing significantly to hepatic-TG stores and that the process may have reached a maximum value in insulin-resistant subjects.

Quantitation of lipoprotein-TG secretion rate

Many investigators have administered labeled glycerol—most have used radioactively labeled glycerol (63)—to estimate VLDL-TG production and secretion rates. Continuous infusion of the stable isotope [2-^{13}C]glycerol and analysis of the rise to plateau in the VLDL-TG pool has been used (47, 64), as has a bolus injection of $^{2}H_5$ glycerol, followed by the appearance of the label in the backbone of lipoprotein-TG and analysis of the subsequent decay in VLDL-TG label to quantitate TG turnover (63). During the decay phase, persistent incorporation of ^{2}H-labeled glycerol that remains in the cell or reincorporation of label released from previously-labeled TG can occur and can result in an underestimation of TG turnover. Patterson et al. (63) have compared VLDL-TG fractional catabolic rate (FCR) using bolus injection of glycerol and/or palmitate, and bolus glycerol administered with continuous infusion of palmitate. These authors provide a comprehensive comparison of results obtained from healthy subjects using a variety of methods for VLDL-TG kinetics. Compartmental modeling of lipid kinetics, which accounted for hepatic recycling of label from either bolus glycerol or palmitate, provided identical VLDL FCRs. One benefit of the bolus administration is that it may require less material and therefore is less expensive than contin-

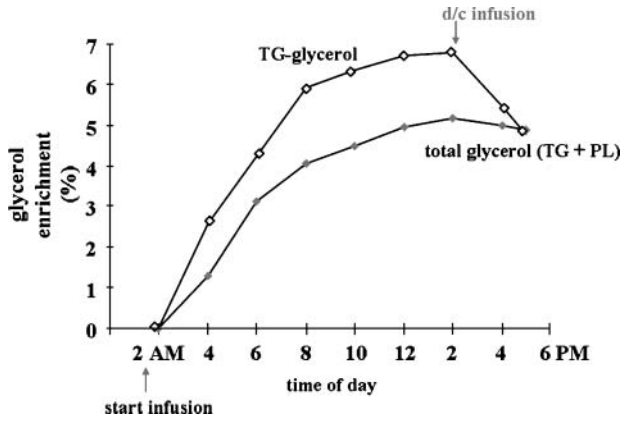

Fig. 5. Change in enrichment of labeled glycerol in the backbone of VLDL-TG and in glycerol isolated from total VLDL lipids. [^{13}C]glycerol was infused intravenously for 12 h in a healthy, fasted subject. Cessation of the infusion is denoted with an asterisk. VLDL particles were isolated as described in Fig. 3, and lipid glycerol was liberated during the transesterification of fatty acids as described previously (47).

uous infusion of label. However, continuous infusion of isotope allows for simultaneous measurement of adipose tissue fatty acid release, a variable that is frequently also of interest in studies of VLDL-TG kinetics. As shown in **Fig. 5**, care must be taken to isolate VLDL-TG from particle phospholipid (PL), because it is likely that the glycerol backbones of TG and PL are derived from different cellular glycerol pools. As pointed out by Patterson et al. (63), the use of deuterated glycerol to label VLDL turnover is complicated by fast and extensive loss of deuterium from the glycerol, presumably due to hepatic metabolism of glycerol through glycolysis. The bolus methods, when combined with compartmental modeling, gave VLDL FCRs that were faster (~0.92–1.02 pools/h) compared with continuous infusion of label into VLDL-TG (~0.28–0.53 pools/h). The exact reason for this discrepancy is unknown. Beyond the choice of tracer used, the rate of VLDL-TG turnover measured using labeled glycerol depends most on characteristics of the study population, such as the subjects' gender and obesity [elevated body weight was associated with greater VLDL-TG secretion in men, but not in women (65)], NEFA flux [greater flux was associated with a higher VLDL-TG production rate (66, 67)], lipoprotein lipase activity [lower lipase activity leads to slower VLDL-TG turnover (68)], and the presence or absence of type 2 diabetes [diabetes is associated with a greater VLDL-TG synthetic rate (69)].

SUMMARY

In view of the increasing prevalence and public health consequences of dyslipidemia and hepatic steatosis, understanding the regulation and pathology of hepatic lipid assembly is a high research priority. Tools for measuring the various aspects of the synthesis, assembly, and secretion of hepatic lipids have advanced considerably in recent years. In particular, stable isotope-mass spectrometric techniques for measuring DNL, the synthesis of TG, and the assembly of VLDL particles in humans are now available. Stable isotope tracers are safe for use in humans and provide increasingly specific information about the pathways involved in hepatic lipid metabolism, ranging from contributions from the bloodstream to intracellular substrate fluxes to intrahepatic assembly. The emergence of these approaches makes human investigations feasible and detailed, patient-oriented research an increasingly practical goal.

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