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Review

New methodologies for studying lipid synthesis and turnover: Looking backwards to enable moving forwards☆

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ABSTRACT

Our ability to understand the pathogenesis of problems surrounding lipid accretion requires attention towards quantifying lipid kinetics. In addition, studies of metabolic flux should also help unravel mechanisms that lead to imbalances in inter-organ lipid trafficking which contribute to dyslipidemia and/or peripheral lipid accumulation (e.g. hepatic fat deposits). This review aims to outline the development and use of novel methods for studying lipid kinetics in vivo. Although our focus is directed towards some of the approaches that are currently reported in the literature, we include a discussion of the older literature in order to put "new" methods in better perspective and inform readers of valuable historical research. Presumably, future advances in understanding lipid dynamics will benefit from a careful consideration of the past efforts, where possible we have tried to identify seminal papers or those that provide clear data to emphasize essential points. This article is part of a Special Issue entitled: Modulation of Adipose Tissue in Health and Disease.

1. Introduction

Adipose tissue is broadly divided into two major categories, white adipose provides insulation, cushions vital organs and acts as an endocrine organ whereas brown adipose tissue is generally thought to contribute to thermogenesis. As small molecules, lipids are an excellent fuel source, in addition, they play a critical role in subcellular signal transduction. The accumulation of lipids (or adipose tissue) during growth is comparable to the accumulation of lean body mass, for example, a 3.5 kg newborn baby will accumulate ~12 kg of fat vs ~12 kg of (dry) lean mass as they transition to a healthy 75 kg adult, reminding us that "getting fat" is part of being normal (obviously, too much of a good thing is bad). Note that a majority of weight gain is simply water mass, a relatively small amount of fat-free lean mass reflects true nitrogen accretion whereas fat mass has very little hydration making the accumulation of lipid and muscle comparable.

The aim of this review is to outline methods that are available for quantifying lipid flux, although attention will be directed towards new advances we intend to highlight selected examples from the older literature since this may help to identify gaps for future investigations. The perspective used in discussing these methods is based on our experience studying the biochemical basis of triglyceride accumulation in adipose tissue and the role of adipose dysfunction in the pathogenesis of dyslipidemia and cardiometabolic disorders, comparable methods can be used for both purposes.

Although numerous investigators have studied de novo lipogenesis [3,38,65,155], most experiments do not allow conclusions regarding net lipid balance. We had suspected that in order to develop a comprehensive understanding of lipid accumulation, investigators should consider a tool(s) that yields an estimate of triglyceride synthesis and degradation including the contribution of de novo lipogenesis to the triglyceride-bound fatty acids [20]. Perhaps the most straightforward approach for examining lipid deposition is to determine the change in pool size over time (which equals the synthesis minus the degradation) while quantifying the rate of lipid synthesis via a tracer method, one can solve the equation to estimate the degradation rate [11,12,20].

In our experience, the use of ^{2}H-labeled water offers a number of advantages as compared to other tracers. In addition to the fact that labeled water rapidly and evenly distributes throughout body fluids, it is incorporated into multiple end-products which enables comprehensive studies of metabolic flux while requiring a minimum of resources (Fig. 1). Typical tracer protocols require catheterized subjects, while this is somewhat trivial to setup in a clinical setting the routine catheterization of animal models is labor intensive. Although there are no formal rules regarding how long animals should recover following a surgical manipulation, investigators often allow several days prior to initiating a tracer study in rodents [6]. To expand on this example, assume that one aims to test a hypothesis in a study requiring ~24 animals, how long would it take one FTE to conduct the study and collect

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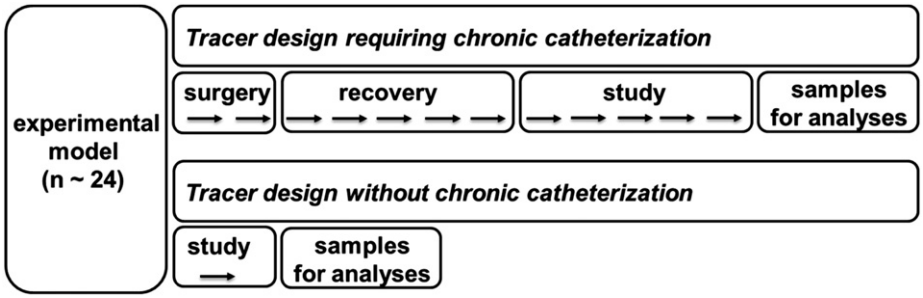

Fig. 1. Design considerations for tracer studies. Assuming a single FTE were available, it would require considerably more time to run a study involving ~24 rodents if catheterization is required; it requires ~2 days to implant catheters, ~4–5 days of post-operative recovery and ~5–6 days to perform tracer infusions vs ~1 day if catheters are not required. Note the each arrow (→) represents the equivalent of one day.

samples? Based on our experience it would take ~2 days to place catheters in rodents, there may be a recovery period of several days followed by several more days of effort to perform the tracer infusions [74,104,119,123,157]. In contrast, using ^2H_2O it is possible to dramatically reduce the time it takes to move from hypothesis generation to sample collection. In addition, if it is necessary to scale up the number of studies, it is possible to run more water-based studies with a minimum of extra effort and in a shorter amount of time as compared to studies that require catheterization. To circumvent the use of chronically catheterized animal models, Kurland and colleagues have used mini-osmotic pumps to deliver tracers whereas Bateman and colleagues have administered a single intraperitoneal bolus of labeled leucine to study protein synthesis [10,159]. While those approaches are somewhat easily implemented in rodent models, it is also possible to administer a more standard intravenous bolus which is suitable for studies in higher species as well [100,101].

We recognize that lipids may mean glycerides to some and sterols to others, the focus here is on triglycerides and cholesterol with a consideration of some related topics. We aim to provide examples of how tracers can and have been used to quantify these flux rates, we hope that our discussion will help guide and expand the use of tracers in future applications [93,113]. Finally, in order to gain a comprehensive understanding of metabolic regulation it is important to attempt linking changes in gene expression with alterations in biochemical flux. Unfortunately there can be disproportionate and/or opposing changes between expression profiles and flux [59,154]; although tracer studies can be used to make statements regarding biochemical flux they cannot necessarily explain points of control just as expression profiles may be useful for identifying potential regulatory sites yet they cannot be used to infer metabolic rates.

2. Why consider lipid flux in white adipose tissue?

A general misconception may be that lipids are stored in adipose tissue to support periods of prolonged fasting/starvation. In fact, the regulation of lipid homeostasis by adipose tissue was rigorously examined nearly 80 years ago [141]. Studies suggested that a large portion of dietary fat passed through adipose tissue each day (presumably, if a subject was in energy balance then equivalent amounts of fat would be stored after a meal and later released during the postabsorptive period). This point seemed so obvious in 1936 that Schoenheimer and Rittenberg [141] wrote:

> "*Instead of comparing the fat tissues to a cellar in which food is stored for times of emergency, it seems more correct to compare them to an ice box in which a part of the food is kept during the short intervals between meals. The fat tissue can therefore be regarded as an energy buffer. During absorption it takes up in the form of fatty acids excess of food material not immediately used for the energy requirements. Conversely, during the postabsorptive period it supplies fatty acids to make up the energy deficit.*"

It is of interest to note that this statement was italicized in the original text, presumably to convey the importance of the observation. This concept was recently revisited by Frayn and colleagues, it is safe to say that the conclusions drawn nearly 80 years ago remain true today despite the marked differences in the methods and models [45,141]. Other studies have reported temporal changes in the activity of lipases in response to changes in glucose and insulin vs fasting suggesting a transient divergence in lipid trafficking away from muscle and towards adipose tissue in humans [42]. A final example regarding the importance of adipose tissue in maintaining daily lipid flux is found in the work of Samuel et al. [134], this indirect evidence serves as an interesting cross-validation. Briefly, they examined how diet-induced fat accumulation affects hepatic signal transduction related to glucose homeostasis. We draw the reader's attention the degree of fatty liver that was observed (see Figure 1, ref [134]), we estimate that the total difference in liver triglyceride is ~0.3 g. Although there were clear differences in the amount of fat in the liver, we believe that that change was trivial in the context of daily lipid flux. We estimate that rats on a high-fat would eat ~15 g of fat per day and that the total lipolysis (i.e. glycerol flux) might be ~5 g of triglyceride per day. Based on these estimates one expects that ~20 g of fat are trafficking through the circulation per day, of which, the slightest imbalance leads to an immediate and profound impairment in normal insulin signaling. In total, these observations further support a critical role for adipose tissue in preventing peripheral lipotoxicity.

The observations noted above raise a final question regarding adipose tissue biology, If a substantial amount of circulating lipids are removed by adipose tissue as part of normal physiological homeostasis are there conditions in which the ability of adipose tissue to clear fat may be limiting? For example, the removal of triglyceride from blood appears to be saturable in vivo [51,128]. Considering the need for adipose tissue to tightly regulate its own lipid flux and its participation in inter-organ lipid trafficking it should come as no surprise that there are a number of approaches for quantifying lipid flux in/out of adipose tissue. One will appreciate that tracer methods per se are helpful in addressing questions but it is often necessary to couple their use with ancillary techniques, e.g. tissue biopsies and/or surgical manipulations [15,18,130].

3. Quantifying triglyceride kinetics in adipose tissue: direct or inferred via plasma measurements

3.1. Dietary interventions

In the classic work performed by Hirsch, subjects were fed modified diets for weeks or months and biopsies of adipose tissue were obtained at various intervals, triglyceride turnover was estimated by

comparing the rate at which the triglyceride composition of adipose tissue remodeled and began to reflect the fatty acid composition of the diet [57]. While intriguing, one obvious consideration with this approach is that feeding a highly modified diet may not be practical and/or may alter the process(es) being studied. It should be noted that this type of design was also utilized nearly 80 years ago in an elegant series of experiments by Kohl [76–78] who fed elaidin to rats and then measured its accumulation and removal from various sites. Both Kohl and Hirsh concluded that once deposited in the adipose tissue, fatty acids generally have a long half-life.

Is it possible to accept the seemingly opposite conclusions reached by Kohl and Hirsh vs Schoenheimer and Rittenberg and Frayn, i.e. lipids in adipose have a long half-life vs substantial amounts of circulating lipids pass through adipose tissue daily? We think "yes" if one considers how the kinetics are described. For example, the early tracer data support the hypothesis that lipids have a long half-life, the fractional turnover of fatty acids in white adipose tissue is quite slow, e.g. a few percent of the total pool is renewed each day. We believe that any misunderstandings in the meaning of these apparently different conclusions come from a consideration of the problem, the flux of fatty acids equals the fractional turnover times the pool size. When we consider how much white adipose tissue is found in normal healthy subjects one recognizes that the absolute amount of lipid which is moving in/out of white adipose tissue in a day approaches that which is typically consumed. Stated another way, since the pool of triglyceride in adipose tissue is relatively large in comparison with dietary intake, stored triglyceride can have a slow turnover (or long half-life) and therein lead to the perception that it is somewhat "inert" yet it is playing a central role in maintaining daily balance and inter-organ lipid trafficking.

A variant on using dietary interventions involves feeding isotopically labeled lipids (e.g. triglyceride tracers) [15,18,130] which is advantageous since dietary modifications are not needed. Also, this allows acute studies lasting a few hours or days. Bragdon and Gordon used this approach to contrast the fate of lipid disposal from triglyceride present in chylomicrons vs albumin-bound free fatty acids in rats [18]. They demonstrated that the nature of the circulating fat (i.e. triglyceride vs free fatty acids) and the nutritional state (i.e. carbohydrate-fed vs fasted) affected the tissue-specific distribution. Havel and Goldfien extended those studies in normal and hepatectomized dogs and observed that by removing the liver there was a minor impact on circulating fatty acids but a nearly complete inhibition of triglyceride production; they concluded that liver plays a minor role in clearing lipids but a central role in converting plasma fatty acids into plasma triglycerides [54]. The work by Havel and Goldfien further emphasizes the importance of coupling tracer methods with surgical techniques in order to understand whole-body integrative physiology.

The use of dietary modifications and/or the administration of labeled lipids suffer from a common assumption. Namely, when using a labeled triglyceride or fatty acid to study triglyceride turnover it is important to recognize that positional specificity of a given lipase may influence the result. Since enzymes may prefer certain substrates [40,47,48,107,108], the chemical structure of the lipid tracer can influence the apparent kinetics, i.e. the tracer may not follow the tracee [15,130]. We previously observed an apparent disconnection between the incorporation of labeled glycerol vs labeled palmitate in epididymal fat pads in growing mice, that discrepancy was resolved when we determined that, in fact, the fatty acid composition of the fat pads had changed over time [20]. For example, based on the incorporation of labeled glycerol we concluded that ~4.4 μmol triglyceride accumulated per day in animals fed a standard rodent diet. We then assumed that ~4 to 5 μmol of palmitate should accumulate (i.e. 3 fatty acids per glycerol, of which, palmitate represents ~30 to 40%). However, we measured the incorporation of labeled palmitate and concluded that slightly less than ~1 μmol accumulated. Direct measurements of the fatty acid composition of adipose tissue then confirmed the tracer data as we found a substantial remodeling had occurred over the course of our study [20].

3.2. Measurements involving arterio-venous balance, microdialysis and microperfusion

In contrast to methods which rely on tissue biopsies it is possible to examine organ-specific uptake and/or production by measuring arterio-venous balances [41,46,149]. Frayn and co-workers have cleverly used this approach to demonstrate a rapid switching of lipase activity in healthy humans during the fasted to fed transition which appears to be altered in obese subjects [33,118]. Such input–output analyses have been used in ex vivo settings as well; the mass-balance concept demonstrated that triglyceride uptake by isolated-perfused fat pads is saturable [128].

For obvious reasons arterio-venous balance measurements can be difficult to make, especially when studying multiple organs or when long term studies are of interest. Although the concepts of the method are relatively easy to understand, i.e. one measures the input and output across an organ, the assumptions may be less obvious. For example, apparent differences in lipid flux between subjects could reflect true differences and/or artifacts related to tissue heterogeneity or perfusion. Namely, one assumes that the relative tissue composition is the same in different subjects and the degree of tissue perfusion is comparable, i.e. any shunting of blood flow should be equal in all conditions.

Microdialysis and microperfusion techniques allow investigators a different type of insight regarding tissue-specific metabolism [5,136,148,151]. One advantage afforded in such studies is the ability to implant multiple probes in the same subject and therein simultaneously study the effect of several perturbations. For example, an investigator can implant three probes in the abdominal fat of humans, the first can be used to deliver vehicle, the second can deliver an activator of lipolysis and the third an inhibitor of lipolysis. The use of microperfusion, as compared to microdialysis, is advantageous in that there are fewer limits in terms of what analytes can be recovered.

All of the methods noted above are useful for probing global aspects on net lipid flux, however, mass-balance approaches do not address questions regarding specific pathways. For example, if one did not observe a difference in glycerol concentration across an adipose depot it is probably incorrect to conclude that there is neglible triglyceride turnover. The use of a glycerol tracer in this scenario could be highly revealing, i.e. finding no difference in concentration but a dilution in the labeling of glycerol would lead one to conclude that there was considerable glycerol uptake with a comparable production [32].

3.3. Glycerol and fatty acid flux

All of the methods noted above have a common limitation, namely, unless every adipose depot is studied one will inevitably make inferences regarding data obtained from one depot to another. The use of glycerol and/or fatty acid tracers allows one to quantify whole-body lipid flux [158]. Before engaging in a detailed discussion it is important to note that whole-body studies are limited since one cannot immediately ascribe the source of any difference between subjects.

It is generally assumed that (i) the release of free glycerol reflects the rate of triglyceride degradation (i.e. since very little glycerokinase is found in adipose tissue glycerol cannot be reincorporated) and (ii) the production of fatty acids can underestimate lipolysis since fatty acids may be reesterified directly in adipose tissue [158]. The logic of quantifying glycerol flux (assuming a metabolic steady-state) is straightforward, one can infuse a glycerol tracer and then measure its dilution in plasma [16,97,158]. Since the infusion rate is known and the dilution in plasma is measured one can calculate the production using the equation:

$$\textit{production rate} = \textit{infusion rate} \times \left[\left(\textit{enrichment}_{\textit{infusate}} / \textit{enrichment}_{\textit{plasma}}\right) - 1\right]$$

where the units for "infusion rate" (e.g. μmol × kg^{-1} × min^{-1}) determine the units for "production rate" [158]. The caveats surrounding

this logic may be obvious and include the assumption that all of the endogenous glycerol mixes with the infused tracer. In addition, the loss of tracer from the plasma is via true utilization and not an isotopic exchange. For example, perfused rat liver will remove [$^2H_5$]glycerol and release other mass isotopomers of glycerol (i.e. [$^2H_4$], [$^2H_3$], etc.) [121]; if this were to occur in vivo, and if unaccounted for, one would overestimate the true rate of glycerol production, i.e. if some of the decrease in the abundance of [$^2H_5$]glycerol were to be caused by conversion of the infused tracer to another form of labeled glycerol. There are other reports which discuss the impact of isotope exchange vs net production [79,83], although those examples surround carbohydrate metabolism the concepts have immediate implications here.

The mode under which tracers are infused and samples are collected will impact conclusions regarding glycerol kinetics. Beylot and colleagues infused postabsorptive and starved rats with [2-^{13}C] glycerol, in some studies the tracer was infused-sampled using the arterial-venous mode whereas in other tracer was infused-sampled via the venous-arterial mode [117]. Regardless of the nutritional state, the rate of glycerol turnover was approximately double in the arterial-venous vs the venous-arterial mode. Since most investigators will presumably choose one mode of tracer infusion-sampling for a given set of studies the effect on the outcome may be minimized nevertheless this may be critical when comparing data across different studies [61]. There are several helpful discussions regarding the importance of infusion-sample modes which review why such striking differences in flux may be observed [70,109].

A subsequent assumption surrounding measurements of glycerol flux is that glycerol production reflects lipase activity of adipose tissue. However, what lipase are we talking about? Namely, the hydrolysis of intracellular triglycerides (e.g. hormone sensitive lipase) will release glycerol just as the hydrolysis of extracellular triglycerides (e.g. lipoprotein lipase). Perhaps differentiating the contribution of intra- and extracellular lipolysis in hyperinsulinemic obese patients could explain how to best approach apparent alterations in lipolytic flux observed in obesity [56,57].

We designed studies to quantify the source of glycerol in humans, [^{14}C]glycerol was used to estimate total glycerol flux and ^2H_2O was used to dissect the contribution of intra- vs extra-cellular lipolysis [60]. The data suggested that ~20% of total glycerol production was not from lipolysis of adipose tissue triglyceride, presumably there was substantial hydrolysis of circulating VLDL triglyceride. Those observations are similar to data obtained by Frayn et al. [46]; using arterio-venous balance measurements they demonstrated that extracellular lipolysis contributes ~20 to 50% to total lipolysis in healthy subjects. Mittendofer et al. considered this problem using a different approach and came to a similar conclusion, i.e. the systemic appearance of fatty acids does not entirely reflect lipolytic flux in adipose tissue [105]. Briefly, they simultaneously compared the production of multiple fatty acids against the fatty acid composition of abdominal and gluteal adipose tissue. Their data suggest that fatty acids are released into the circulation from a tissue source which contains palmitate and stearate in higher concentrations and oleate in lower concentrations than certain adipose tissue depots. Although the site of the unknown lipid depot was not specifically determined, the discrepancies between the fatty acid appearance profile and the distribution of fatty acids in adipose tissue triglyceride were observed during hormone-mediated changes in fatty acid flux therein suggesting that factors which regulate triglyceride turnover in adipose tissue do not necessarily regulate this other depot(s).

4. Quantifying triglyceride production: direct incorporation methods

While the use of the word "triglyceride" is convenient, readers recognize the heterogeneity of this biochemical class. To simplify matters we will initially focus our attention on a higher level overview (Fig. 2). Briefly, triglycerides are made from glycerol-3-phosphate and fatty acids. Analogous to the fact that fatty acids can be derived from multiple

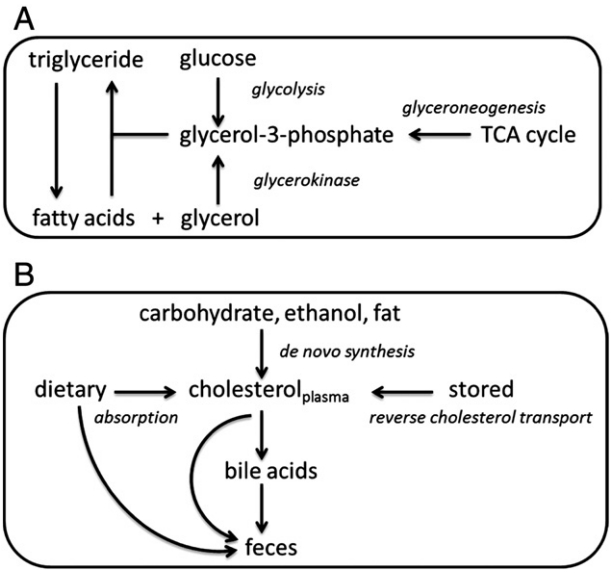

Fig. 2. Overview of lipid flux pathways. Triglyceride synthesis requires a source of glycerol-3-phosphate and fatty acids, the relative contributions of glycolysis, glycerokinase and glyceroneogenesis is expected to vary with tissue-type and nutritional/hormonal status; likewise, fatty acids are derived from variable sources including stored lipids, diet and/or de novo synthesis (not shown) (Panel A). Plasma cholesterol is derived from three sources and is disposed of primarily via two pathways, conversion to bile acids and direct elimination (Panel B). Note that this review does not consider the conversion of cholesterol to other steroids.

sources (e.g. diet or de novo synthesis), glycerol-3-phosphate can be made from glycerol, glucose and "glyceroneogenic" carbon sources (e.g. pyruvate, lactate, alanine). We hope to contrast the use of fatty acid and glycerol tracers against the use of labeled water (both $_2$H and $_{18}$O) as tracers for studying triglyceride production.

4.1. Fatty acid tracers

There are numerous examples in which labeled fatty acids have been used to estimate triglyceride production [1]. In contrast to the use of labeled glycerol, one can follow the incorporation of labeled fatty acids into cholesterol esters, advanced LC–MS methods greatly facilitate these analyses [100,101,125]. Furthermore, it is possible to simultaneously administer labeled fatty acids orally and intravenously and therein estimate the fate of endogenous vs exogenous lipids [39], depending on the type of labeled fatty acid that is administered it is also possible to estimate lipid oxidation [95].

Wolfe and colleagues recently examined triglyceride synthesis in muscle, the reported observations have immediate implications on measures of triglyceride synthesis in liver and adipose tissue [21,161]. Again, the central problem when following the incorporation of a labeled precursor into a product concerns the dilution of the tracer [21]. Namely, the labeling of the product reflects how much tracer is administered, the turnover of the fatty acid pool or entry of cold fatty acids along the pathway and the rate of triglyceride production.

Various approaches can be used to administer other labeled precursors (e.g. ^{13}C-glucose, ^{13}C-acetate or ^{2}H-water), for example, one can then follow the incorporation of the endogenously synthesized (isotopically labeled) fatty acids into triglycerides and/or other complex lipids. It is possible to estimate the contribution of de novo lipogenesis via the steady-state labeling of palmitate and triglyceride turnover via the time required to reach a steady-state labeling [2,20,30,38,39,87].

A final approach to using "fatty acid tracers" is to measure the disposal of a known amount of Intralipid [133]. Although not necessarily a tracer-method per se, this approach has merit in some instances since one can examine questions regarding the effect of different lipid loads on kinetics, since Intralipid has a somewhat distinct lipid profile

as compared to typical plasma lipids it is possible to simultaneously quantify the removal of several lipid species.

4.2. Glycerol tracers

Glycerol is readily incorporated into liver triglyceride and thus circulating triglyceride, the degree to which adipose tissue will use glycerol vs other substrates is questionable [52,84,91]. One can find several reports in which the temporal change in ^{14}C-labeling of triglyceride–glycerol was measured in humans following the administration of ^{14}C-glycerol, sophisticated mathematical models were used to inform on the rates of different reactions [1]. An important consideration regarding these data centers on the potential for secondary tracers (or isotope recycling). For example, carbon-labeled glycerol can be converted to carbon-labeled glucose which can then generate carbon-labeled lactate, pyruvate, etc. The conversion of those intermediates to triose-phosphates will lead to the (re)incorporation of labeled carbon into triglyceride–glycerol. One potential consequence of this isotopic scrambling is the generation of a "tail" in the apparent labeling curve of triglyceride–glycerol. Although recycling is somewhat difficult to detect when using ^{14}C-tracers, the detection methods used in stable isotope studies permit an immediate assessment of ^{13}C recycling. For example, we previously measured the incorporation of [U-^{13}C]glycerol (M + 3) into glucose [82,121]. Although the predominant shift in glucose labeling occurred in the M + 3 isotopomer, we also observed substantial increases in the abundance of singly and doubly-labeled (M + 1 and M + 2, respectively) glucose. Although our analyses did not aim to definitively measure rates of tracer recycling, the data demonstrate the importance of this concept and suggest that a sizeable amount of labeled glycerol will recycle.

Recent investigations have capitalized on the use of [$^2H_5$]glycerol. As noted above, one might expect the appearance of several mass isotopomers of triglyceride–glycerol following the administration of [$^2H_5$]glycerol. For example, the injected [$^2H_5$]glycerol tracer (i.e. M + 5) is readily phosphorylated and then either immediately incorporated into triglyceride–glycerol (generating an M + 5 product) or equilibrated with the triose-phosphate pool which generates various mass isotopomers of glycerol-3-phosphate depending on the extent of metabolism. Patterson and colleagues clearly demonstrated this in humans (Fig. 3) [114]. Another "proof" of isotopic scrambling centers on the use of ^2H_2O in studies of triglyceride–glycerol flux. For example, the incorporation of ^{2}H, following the administration of ^2H_2O, is possible via a rapid equilibration between the triose phosphates and glycerol-3-phosphate (Fig. 3) [20,153].

It is of interest to discuss the recent use of [$^2H_5$]glycerol in studies of triglyceride production by Qi et al. [124]. Briefly, it appears that triglyceride production was estimated by administering a bolus of [$^2H_5$]glycerol and then measuring the abundance of different ^{2}H-labeled triglycerides. Although Qi et al. [124] concluded that their novel compounds inhibit triglyceride production, we suspect that certain assumptions may complicate the interpretation. For example, it appears that these investigators simply administered [$^2H_5$]glycerol and then measured the concentration of [$^2H_5$]triglyceride$_{52:2}$ in vivo as a marker of triglyceride production. Why is this concerning? The concentration of [$^2H_5$]triglyceride$_{52:2}$ is affected by (i) the dose of [$^2H_5$]glycerol, (ii) the initial dilution of the tracer at the extracellular level which is influenced by differences in glycerol concentration and/or flux between control and treated subjects, (iii) isotope exchange at the level of triose phosphates, (iv) isotope dilution at the level of triose phosphates and (v) the rate of triglyceride production (Fig. 3). Different levels of dilution and/or exchange following the administration of [$^2H_5$]glycerol (i.e. steps *ii*, *iii* and *iv*) make it difficult to definitively interpret the data [124,125].

There are a few additional points to consider regarding the conclusion that the incorporation of ^{2}H-glycerol vs ^{13}C-oleate is specific to a single enzyme (i.e. DGAT2) [124]. First, as eluded to above, the differences between the labeled glycerol and fatty acids could simply be caused by overlooked assumptions. Second, it is not clear whether some form of compensation did not occur, enzymes other than DGAT2 are capable of catalyzing the same reactions. Third, hepatic zonation of metabolic flux is known to affect the interpretation of tracer data. For example, different concentration and/or labeling gradients across the liver (or splanchnic bed) may contribute to the apparent differences in triglyceride labeling that result following the administration of ^{2}H-glycerol vs ^{13}C-oleate.

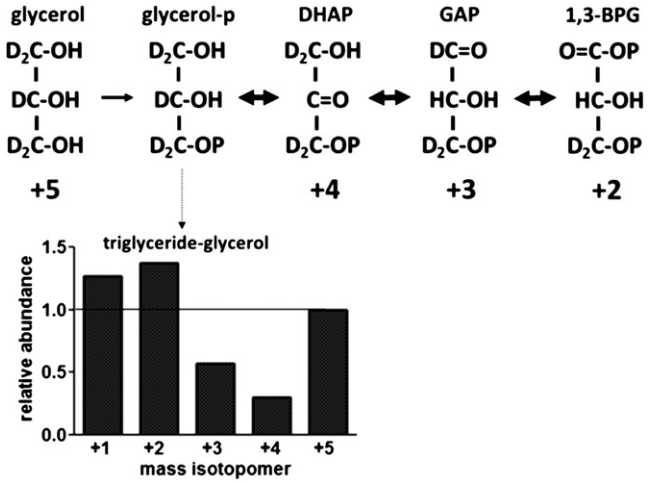

Fig. 3. Isotope exchanges during the incorporation of [$^2H_5$]glycerol into triglyceride. Perdeuterated glycerol is often used in studies of triglyceride synthesis. The bar graph was adapted from Patterson et al. (see Figure 3, ref [114]) and shows the relative abundance of different mass isotopomers of triglyceride–glycerol ~2 h after administration of [$^2H_5$]glycerol. Although the injected glycerol tracer is predominantly M + 5 (set to 1), once activated to glycerol-3-phosphate the tracer will rapidly equilibrate with dihydroxyacetone phosphate, glyceraldehyde phosphate and 1,3-bisphosphoglycerate (DHAP, GAP and 1,3-BPG, respectively). Each interconversion exchanges carbon-bound hydrogens generating lower mass isotopomers. Aldolase (not shown) also participates in these exchange reactions, it can generate other mass isotopomers (e.g. M + 1) depending on the isotopic composition of DHAP and GAP.

Please note that the points being raised here are not meant to reflect negatively on the work of Qi et al. [124,125], it could be that the concerns outlined above have been evaluated by the authors; these papers are used as examples since experimental details seem to be absent and fundamental assumptions are not discussed, both would help readers better understand the conclusions. Discrepancies between tracer methods and/or other methods are common. For example, the dogma is (or was) that liver and kidney remove glycerol since these organs contain measureable activities of glycerokinase, a critical enzyme involved in glycerol metabolism [91]. However, that concept was challenged by coupling arterio-venous balance measurements of glycerol concentration and isotopic labeling, the data demonstrated substantial extra-hepatic and extra-renal glycerol utilization [84]. This raised the question, How does a tissue use glycerol if glycerokinase is not present? How could a tracer method yield a different result as compared to directly measuring enzyme activity? Could both methods be correct yet they lead to different conclusions? Although it is difficult to answer this question with complete confidence, other pathways of glycerol utilization have been proposed, e.g. glycerol + NADP → glyceraldehyde + NADPH [43,150].

4.3. Application of ^{2}H- or ^{18}O-labeled water

Although readers will likely recognize how ^2H_2O can be used to estimate the contribution of de novo lipogenesis, its use in quantifying triglyceride production may be less clear. We will first briefly outline pivotal discussions in which ^2H_2O was used to study fatty acid synthesis and then expand the review to triglyceride flux.

The seminal studies of Schoenheimer and Rittenberg demonstrated that biosynthetic reactions incorporate protons from body water

[92,142], we now know that the carbon-bound hydrogens in palmitate are derived from water, NADPH and acetyl-CoA [25]; most but not all of the hydrogens become labeled [3,37,65,81,155]. We recently revisited the question of whether the number of hydrogens is constant when different diets are fed to mice, we concluded that it is reasonable to make this assumption when studying palmitate and cholesterol synthesis [122]. Lee and colleagues have carefully considered how to examine questions centered on the elongation of palmitate [3].

Problems of precursor heterogeneity surround studies of fatty acid synthesis that use carbon-labeled acetate (or a related precursor, e.g. carbon-labeled glucose, ethanol) [22,25,35,36,55]. Namely, carbon-labeled tracers may not equally enter all cells which is important for in vivo studies but may be less important in cell-based studies since gradients are expected to be less severe [68,103]; Kelleher and colleagues have demonstrated a mathematical approach that can account for labeling gradients provided that one can measure the abundance of several mass isotopomers [13,14,72,73]. Although ^2H_2O results in a more homogenous precursor labeling, isotope effects may exist therefore one assumes that these would be equal across different subjects. Since ^2H_2O has a relatively long half-life it is somewhat straightforward to maintain a steady-state labeling for a prolonged time, therein allowing investigators to obtain integrative measures of flux [3,20,147]. The long half-life of ^2H_2O has also been considered a problem when studying acute interventions, however, it is possible to use a step-wise dosing routine and therein estimate the flux of some parameters back-to-back [69,122].

In looking back at the classical study of Jungas, in which ^3H_2O was used to quantify fatty acid synthesis, one recognizes that he also measured the incorporation of ^3H into triglyceride–glycerol [65]. ^2H_2O was recently proposed as a tracer for estimating rates of triglyceride production [20,153]. Briefly, ^2H can be incorporated into each of the five carbon-bound hydrogens of triglyceride–glycerol. Since the incorporation of ^2H does not appear to be mediated by non-specific exchange reactions and saponification of isolated triglycerides does not appear to catalyze the back exchange of ^2H, the ^2H-labeling of triglyceride–glycerol should reflect the rate of triglyceride synthesis [12,20,29].

Since triglyceride–glycerol can be derived from glucose, glycerol and glyceroneogenic substrates (e.g. pyruvate) (Fig. 2) [7], our initial question was whether the labeling of the different hydrogens was equivalent [20]. If equivalent, it would be possible to simply measure the total labeling of triglyceride–glycerol and assume a constant number of hydrogens (i.e. n) are incorporated, as is the case when studying the de novo synthesis of palmitate or cholesterol [37,86]. Central to the discussion here is an appreciation for the stereospecific action of enzymes [110,127,131,132]. For example, although glycerol is chemically symmetrical glycerokinase selectively phosphorylates carbon 3 (C3) [23,135], likewise, the labeling of specific hydrogens is affected by distinct enzymes.

Our initial studies relied on direct measurements to determine whether specific carbon-bound hydrogens were differentially labeled [20]. We concluded that quantitation of triglyceride turnover over a short time period (i.e. when one does not model the rate at which triglyceride–glycerol approaches its asymptotic level) required knowledge of the ^2H-labeling on $C1_{triglyceride-glycerol}$. Later studies by our group relied on a simpler analytical strategy for measuring the positional labeling of triglyceride–glycerol [11]. It is important to note the efforts made by Hellerstein and colleagues, although they calculate rates of triglyceride flux their approach does not allow one to determine the labeling in specific positions [153].

Given the observations reported by Patterson et al. [114], we were concerned about the potential to underestimate triglyceride production in plasma if we were to administer ^2H_2O, sample at a single time point and assume a constant for n. To test these concerns, mice were either fed a low-fat or carbohydrate-free diet for ~2 weeks and then given a single bolus of ^2H_2O, samples were collected at various time points and the [^2H]labeling of glyceride–glycerol was determined (Fig. 4). We

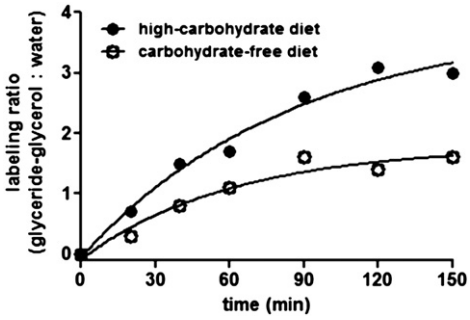

Fig. 4. Incorporation of ^2H_2O into glyceride–glycerol. Mice were fed a high-carbohydrate or a carbohydrate-free diet for ~2 weeks and then given a single intraperitoneal bolus of ^2H_2O, the ^2H-labeling of water and glyceride–glycerol was determined in plasma samples (shown as ave, n = 2 per group per time point). Plasma water labeling reached ~2.2% enrichment within 20 min and remained stable for the duration of the study (not shown). The total enrichment of glyceride–glycerol was determined via GC–MS and is expressed as a ratio relative to the water labeling (y-axis). Each data set was fitted to a single exponential, the asymptotic labeling was expected to reach 3.8 ± 0.4 vs 1.8 ± 0.3 (ave ± se) in animals fed a high-carbohydrate vs a carbohydrate-free diet, respectively.

believe that these observations underscore the importance of collecting temporal data, clearly the asymptotic value of total glyceride–glycerol labeling appears to be influenced by the diet and therefore it would likely be incorrect to assume a constant for n. Presumably it would be more appropriate to estimate triglyceride production by fitting the time-dependent change in labeling. Our observation of a lower n in animals fed a carbohydrate-free diet seems consistent with what might be expected. If we consider that ~90% of the caloric content of the carbohydrate-free diet is lipid and that the carbohydrate-free diet results in substantial triglyceride turnover in white adipose tissue [12], it is likely that the delivery of free glycerol to the liver is increased. The extent to which there is direct conversion of glycerol → glycerol-3-phosphate → triglyceride–glycerol might lead to the observed difference in the asymptotic labeling of glyceride–glycerol being lower in animals fed a carbohydrate-free vs a low-fat diet.

We aimed to test the suitability of a novel analytical method for measuring the ^2H-labeling of triglyceride. For example, rapidly evolving LC–MS protocols make it possible to analyze the labeling of intact lipids [26,27]. In our experience it seems that one may be able to estimate the rate of triglyceride production by administering a single bolus of ^2H_2O and then measuring the change in total ^2H-labeling of triglyceride over time (Fig. 5, Panel A). Although the labeling curve reflects a composite of triglyceride–glycerol and triglyceride-fatty acids, one should be able to estimate the fractional rate of triglyceride synthesis by fitting the rise to steady-state. While these newer analytical methods allow one to examine distinct analytes it is not always possible to obtain good chromatographic separation and/or clean spectra for all analytes [26,27].

A final point to consider regarding the use of ^2H_2O in studies of triglyceride flux centers on quantifying the specific contributions of glucose, glycerol and glyceroneogenic carbon sources. It is expected that the liver will use any/all of these pathways depending on the nutritional/hormonal status [66,67], however, there is uncertainty regarding the contribution of the various pathways in white adipose tissue in vivo. Presumably, understanding the source(s) of triglyceride–glycerol is important when trying to guide the design of novel therapeutics that are aimed at mediating disease-associated alterations. Although the seminal studies of Hanson and colleagues demonstrated a sizeable glyceroneogenic contribution using an ex vivo model, the contents of the media strongly influenced the contribution of the different pathways [7]. We previously reviewed the merits of the different in vivo data regarding the sources of glycerol-3-phosphate [12], it appears that adipose tissue has a marked flexibility when generating triglyceride–glycerol (consistent with the original report [7]).

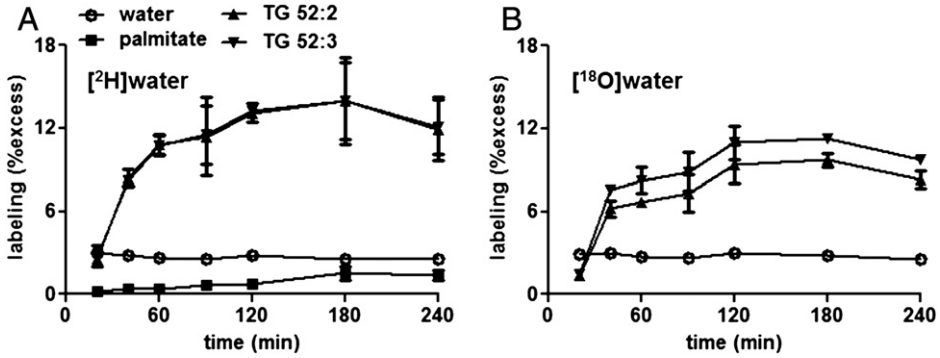

Fig. 5. Incorporation of ^{2}H- or ^{18}O-labeled water into specific triglycerides. Mice were fed a standard diet and then given a single intraperitoneal bolus of either ^{2}H- or ^{18}O-labeled water. The labeling of plasma water and specific triglycerides was determined (data shown as ave ± se, n = 3 per group per time point). The labeling of plasma water was maintained at ~2.5% for the duration of the study and there was substantial incorporation of ^{2}H or ^{18}O from water into the respective triglycerides. Note that in the case where ^{2}H-labeled water was administered, plasma was saponified and the ^{2}H-labeling of total palmitate was also measured, the sizeable difference between the labeling of palmitate and the intact triglycerides suggests that the majority of the increase in triglyceride labeling reflects the incorporation of ^{2}H-glycerol. Regardless of the water tracer, the product labeling reaches several times that of the precursor labeling; this is expected since multiple copies of the precursor are incorporated. Note that triglyceride labeling was determined using LC-Q-TOF analyses on the intact lipid [27].

One can find a few published reports regarding the use of ^{18}O-water in studies of lipid synthesis [80,106,137,138]. In short, since the hydrolysis of complex lipids (e.g. triglyceride and phospholipids) in the presence of ^{18}O-water will lead to the generation of ^{18}O-labeled fatty acids it should be possible to estimate lipid synthesis by measuring the (re)incorporation of those fatty acids into various end-products. Although the previous work relied on rather cumbersome methods for isolating lipids, and caution had to be exercised in regard to sample preparation prior to GC–MS analyses, the data were of value [137]. We were intrigued by the potential of using ^{18}O-water in studies of lipid flux. Briefly, we recently revisited the use of ^{18}O-water for studies of protein flux and thought that the ability to couple measurements of lipid flux would be of interest in certain scenarios [162]. Rodents were given a single bolus of ^{18}O-water and samples were collected at various time points, lipids were extracted from plasma and subjected to LC–MS analyses, the temporal incorporation of ^{18}O-water was determined in select analytes (Fig. 5, Panel B). We found that the asymptotic labeling of $triglyceride_{52:2}$ and $triglyceride_{52:3}$ was several times greater than that of body water which is consistent with the notion of an amplification between the precursor:product labeling ratio of which the final value reflects the number of labeled sites [22]. Despite the marked differences in the pathways which incorporate ^{2}H vs ^{18}O from body water, we observed comparable fractional synthetic rates of the specific triglycerides, i.e. ~2.28 vs ~2.10 pools per hour, respectively (Fig. 5).

Our pilot studies were followed by a study in which we administered ^{18}O-water to mice under various conditions, i.e. control, following intravenous injection of Intralipid or the administration of an inhibitor of microsomal transfer protein [162]. Samples were collected at ~90 min post-tracer administration and relative rates of lipid and apoB flux were estimated. It appears that at lower rates of triglyceride flux there is a fairly direct relationship with apoB flux, this coupling seems to be lost as the rate of triglyceride flux increases (Fig. 6). Although it is beyond the scope of this review to comment on the interpretation of these data, our observation is consistent with an earlier study in humans which relied on the use of separate tracers [102]; the ability to use labeled water allows for a simpler study protocol which may be uniquely suited for animal models (Fig. 1).

5. Ancillary measures when studying lipid kinetics: estimating body composition and total energy expenditure

Studies aimed at understanding lipid flux may consider estimates of body composition and/or energy expenditure. We recognize that various methods can be used to quantify these endpoints and we do not consider the merits of one vs another, our point here is to explain how isotopically labeled water can be used to make those measurements [4,158].

5.1. Body composition

The principle of using isotopically labeled water to estimate fat mass is relatively straightforward. Briefly, a known quantity of either ^{2}H- or ^{18}O-labeled water is administered as a bolus injection. After allowing for isotope equilibration, a blood sample is collected and the ^{2}H- or ^{18}O-enrichment of body water is measured, total body water is calculated from the dilution of the tracer. Once total body water is determined, fat mass can be estimated by assuming that (i) the body is composed of two basic compartments (i.e. fat-free mass and fat mass) and (ii) there is a constant relationship between total body water and fat-free dry mass [98]. Obviously, water-based estimates of body composition do not permit the assessment of tissue-specific fat accumulation, imaging methods and/or direct tissue biopsies would be needed to address questions regarding the localization of fat.

5.2. Total energy expenditure

Total energy expenditure (i.e. basal metabolic rate, thermic effect of food and physical activity) can be determined by administering a bolus of "doubly-labeled" water [89,90]. The difference between the elimination of ^{18}O and ^{2}H from body water equals the fractional rate of CO_2 production, a series of calculations are then performed to estimate energy expenditure [158], the "doubly-labeled" water method assumes that ^{2}H is primarily lost as water and that ^{18}O is primarily lost as water and CO_2.

Lifson and McClintock's pioneering work demonstrated strong agreement between measurements of CO_2 production made using "doubly-labeled" water and indirect calorimetry [89,90], various groups have since expanded the use of "doubly-labeled" [50,62,139,140]. It is important to note that measurements of tracer dilution do not immediately yield an estimate of total energy expenditure. The measurements allow one to calculate the fractional rates of ^{2}H and ^{18}O turnover in body water, a number of equations are then used to calculate the rate of CO_2 production and energy expenditure. It may be difficult to completely verify the application of different eqs. in every experimental setting so some caution should be exercised when interpreting the data [158]. We adapted the "doubly-labeled" water method in order to simultaneously estimate CO_2 production and lipid and protein flux [11]. The aim of those experiments had been to couple concepts of mass-balance

and tracer kinetics, i.e. to link changes in whole-body energy balance to changes in pathway flux that affect adipose vs muscle mass.

6. Cholesterol flux and related processes

The synthesis of cholesterol and related products (e.g. cholesterol esters and bile acids) has received considerable attention. Rates of de novo cholesterol synthesis have been estimated using either carbon-labeled acetate or "heavy" water, however, concerns have been expressed regarding the use of carbon-labeled substrates [25,36]. We aim to provide a simple example of how ^{2}H-labeled water can be used to somewhat easily study the coordinated regulation of pathways surrounding cholesterol homeostasis. Again, the central point to be made here concerns the logic of the methods and not necessarily the biology.

Total cholesterol flux can be estimated by administering a single bolus of labeled cholesterol and then measuring its dilution over time, Ostlund and Matthews demonstrated that stable isotope tracers are well suited for this purpose [111,112]. The ability to differentiate the source(s) of cholesterol requires additional tracers. For example, cholesterol absorption can be studied using dual tracer methods whereas ^{2}H-labeled water can be used to estimate de novo synthesis [17,38,63,64,152,163]. A major pathway for affecting cholesterol elimination is its conversion to bile acids; Stellaard and colleagues have rigorously examined how to approach studies of bile acid kinetics [8,9,143–146]. Briefly, although bile acids are physically separated into plasma, bile and intestinal pools, the rate of mixing between these sites is expected to be much greater than the throughput (Fig. 7). Consequently, since bile acids behave as a single well-mixed pool their kinetics can be studied by administering a bile acid tracer(s) and then determining the initial labeling and the rate of dilution, i.e. the pool size and fractional turnover, respectively.

Important goals in managing dyslipidemia are lowering plasma cholesterol and/or removing cholesterol from peripheral sites (e.g. plaque), apoA1 kinetics may be a marker of the latter. Although one can envision the design of multi-tracer studies to dissect the pathways surrounding cholesterol homeostasis we provide an example of how $^{2}H_2O$ could be used to examine several connections (Fig. 7). Briefly, two groups of mice were fed a standard low-fat, low-cholesterol diet, one group was treated as controls whereas the other group was given cholestyramine (~3% by weight mixed with the food). All animals were given a bolus of $^{2}H_2O$ afterwhich the ^{2}H-labeling of water, cholesterol, cholic acid and apoA1 were measured at various time intervals. Since a bolus of water was administered we expected a slight decrease in the ^{2}H-labeling of body water, however, since the dilution is virtually identical between the two groups it is somewhat straightforward to examine the effect of cholestyramine on this network. Consistent with what one might expect, treatment with cholestyramine induced an increase in cholesterol synthesis, promoted the conversion of newly made cholesterol to cholic acid and increased cholic acid turnover, a somewhat novel finding is the apparent lack of an effect on the fractional rate of apoA1 production (Fig. 7). Again, as mentioned before, the point of this example is to demonstrate the relative ease with which interrelated metabolic flux rates can be studied using a single tracer method, a complete mechanistic understanding of the biochemistry is beyond the scope of this report.

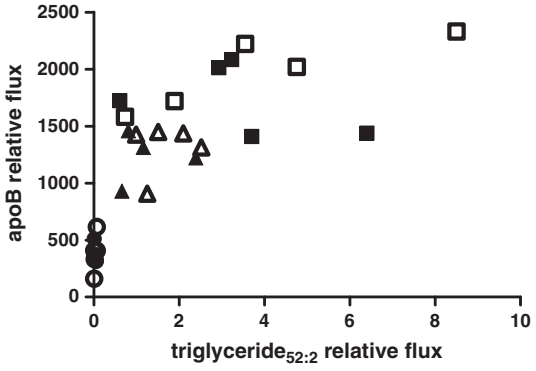

Fig. 6. Coupling measurements of triglyceride and apoB relative flux rates. Mice were studied under conditions that were expected to perturb lipoprotein flux, i.e. control or Intralipid challenge or inhibition of microsomal triglyceride transfer protein (triangles, squares and circles, respectively). The incorporation of ^{2}H- or ^{18}O-labeled water (open and closed symbols, respectively) was used to simultaneously estimate the relative flux of apoB and triglyceride$_{52:2}$, plasma was collected 90 min after the water tracers were given. Lipids and apoB were analyzed as described [27,162].

7. Measuring isotopic labeling by mass spectrometry: is there a best approach?

We suspect that it would be easy to either discourage or offend many readers by choosing a single instrument so we will try to balance our understanding of the pros and cons regarding the use of various mass spectrometry systems. Note that although other analytical platforms can be used to measure the isotopic abundance of different analytes (e.g. NMR), mass spectrometry-based analyses are perhaps the most widely used.

In our experience, a standard GC–MS platform is sufficient for running many of the analyses discussed herein. In addition to the fact that virtually all GC–MS instruments have small footprints as compared to LC–MS instruments, GC–MS are relatively inexpensive to purchase, easy to maintain and they generally have robust performance records. Numerous methods have been reported for analyzing small molecules via GC–MS therefore it is possible to easily get started [16,49,53,115,158]. Some investigators have demonstrated the ability to adapt small molecule analyses for LC–MS configurations [99,129], presumably the utility of LC–MS for lipid analyses centers on methods for lipidomic-type analyses [27,31]. A central question in terms of using LC–MS protocols for tracer studies centers on what type of mass analyzer is better. In our experience, the use of a Q-TOF configuration has proven to be very powerful [26,27,156]. It seems that one can obtain highly reproducible measurements of isotope labeling ratios over a large dynamic range using a non-biased approach. For example, Q-TOF instruments yield highly reproducible measurements of isotopic ratios. QQQ instruments offer comparable reproducibility, however, they usually require pre-determined MRM settings and are therefore more limited to targeted analyses. We have had mixed success using "trap"-based instruments, in our experience the analyses yield reliable information regarding exact mass data but the measurements are typically limited by a lack of precision and/or accuracy when quantifying isotopic ratios, this was most obvious when trying to measure isotope ratios over a wide dynamic range. Recent efforts by our colleagues have demonstrated a novel approach that should now enable routine and reliable measurements of isotopic labeling using "trap"-based mass spectrometers. Briefly, Kasumov and colleagues proposed to acquire data at various resolution settings from which they can construct a calibration curve to account for any systematic bias in the apparent isotopic labeling [58]. A final point to consider here in terms of coupling tracer methods with mass spectrometry-based analyses centers on the emerging use of imaging mass spectrometry to study spatial and temporal flux [75,85].

Our discussion to this point has avoided the topic of how to obtain lipids for any analyses [31]. In general, unless some level of purification is used one must interpret the data with caution. In our experience, white adipose tissue contains an overwhelming amount of triglyceride as compared to other glyceride species so that it may be possible to simply assume that any glycerol released after saponification is from triglyceride [20]. When studying plasma-derived lipids, investigators typically use ultracentrifugation to separate lipoprotein fractions before subjecting the samples for further preparation and mass spectrometry-based analyses [88,114]. We recently capitalized on the use of a gel-based separation technique which seems reasonable for studies of lipid flux [101].

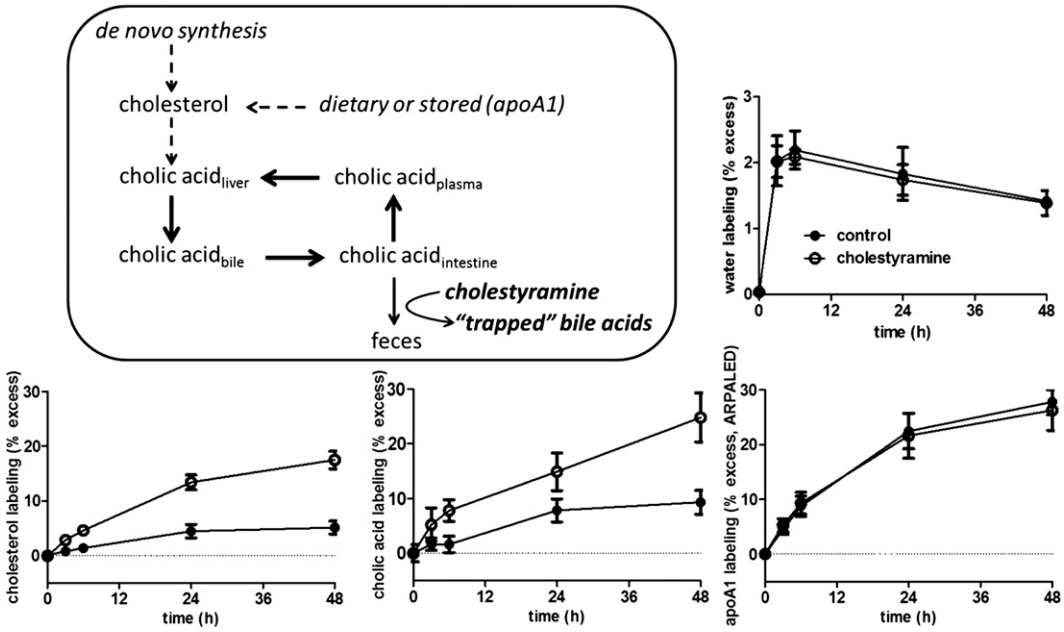

Fig. 7. Simultaneous assessment of multiple indicators of cholesterol homeostasis. Mice were fed a standard low-cholesterol diet ± cholestyramine for 5 days and then given a single intraperitoneal bolus of ^2H_2O. The ^2H-labeling of water, total cholesterol, cholic acid and apoA1 were determined using plasma samples were collected over 48 h (data are shown as ave ± se, n = 3 per time point per group). Cholestyramine-feeding did not influence the ^2H-labeling of body water but there was a sizeable increase in the contribution of newly made cholesterol and conversion to cholic acid, in contrast, there was no apparent effect on the fractional rate of apoA1 synthesis. The ^2H-labeling of water and cholesterol was determined by GC–MS [59] whereas LC–MS was used to determine the ^2H-labeling of cholic acid and apoA1 [162].

8. Mathematical modeling

Central to metabolic flux studies is an alignment between the physiological and the mathematical models. We previously reviewed several concepts regarding compartmentation of triglycerides in adipose tissue, presumably that discussion has implications in regard to lipid flux in other locations [20]. Our efforts towards studying lipid accretion relied on a straightforward logic, by measuring the change in the pool size over time and the rate of synthesis via ^2H_2O we could calculate the rate of degradation [11,12,20]. Obviously, in a metabolic steady-state the production is equal to the degradation. Berman, Grundy and Howard published a highly useful collection of reports regarding early efforts aimed at modeling plasma lipoprotein kinetics [1]. Other valuable resources, in regard to general concepts, are the papers by Foster et al. [44] and Ramakrishnan [126]. One important area that has perhaps seen little attention surrounds reproducibility and/or propagation of error [11,122,160]; Magkos, Patterson and Mittendorfer carefully considered this matter using a range of tracers surrounding lipoprotein kinetics [94] whereas Matthews et al. [96] rigorously examined this for studies of leucine flux.

9. Summary

It should be clear that there are a number of methods for studying lipid kinetics. Obviously, the questions will drive the utilization of the method(s) and/or influence the development of new tools. We hope to have outlined several of the approaches that can be considered when examining the biochemical basis of lipid flux, presumably the methods discussed herein can be used to address questions surrounding the pathogenesis and management of obesity, (diabetic) dyslipidemia, cardiovascular disease and/or lipotrophic disorders.

Although our attention was mainly focused on questions related to the dynamics of circulating lipids as well as white adipose tissue homeostasis, the logic outlined here should allow investigators to apply the methods in novel areas (e.g. neuroscience or cancer) and/or shed light on gaps in the field. In particular, we believe that attention to data processing methods will be of great value in the future. For example, there are numerous examples in the literature which demonstrate the power of lipidomics [31]. As we recently observed, it is possible to extract information regarding kinetics by coupling stable isotope tracer methods with LC–MS/MS analyses [26,27]. In our experience, the hardware outperforms the ability of the software to readily extract the labeling data.

Lastly, we have found that ^2H_2O can be used to measure most of the major reactions that affect triglyceride accumulation (i.e. the rates of triglyceride synthesis and breakdown, the contribution of de novo lipogenesis to the pool of triglyceride-bound fatty acids and the rate of remodeling of adipose tissue triglycerides). As well, ^2H_2O can be used to study coupled processes (e.g. lipid and protein flux), including a dissection of carbohydrate flux and DNA synthesis [19,24,28,38,120,147,162]. We expect that advances in instrumentation will continue to improve the limits of detection and reduce the tracer requirements. This will have obvious advantages in terms of cost and presumably affect safety concerns. Aside from occasional/transient nausea and vertigo, we have not observed any overt signs of toxicity when administering ^2H_2O. We generally enrich body water to ~2.5% in rodents, side effects and toxicity have been demonstrated at higher doses [34,71,116]. Studies performed in non-human primates typically use doses of ^2H_2O that are closer to what is reported in humans (e.g. ~0.5% enrichment), as in human subjects, the required dose of tracer is given in small volumes over an extended time period without any apparent adverse reactions [56,122].

Acknowledgements

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of (often unstated) assumptions and potential limitations of different approaches.

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