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APPLICATION NOTE 31

Tracing Lipid Disposition *in vivo* Using Stable Isotope-Labeled Fatty Acids and Mass Spectrometry

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Lipids are ubiquitous molecules which serve a variety of important biological functions, including energy storage (triglycerides), modulation of cellular membrane structure and function (phospholipids and cholesterol), intracellular signaling and hormonal regulation. Dysfunctions of lipid metabolism contribute to a variety of diseases including, among others, atherosclerosis, hypertriglyceridemia and type 2 diabetes. As such, understanding the synthesis, regulation and transport of lipids in the body is important to developing new and improved therapies for these diseases. Stable isotopes have been used to study several aspects of lipid metabolism including the synthesis and disposition of cholesterol,[1,2] phospholipids[3] and VLDL triglycerides.[4] In this application note, we highlight some of the advantages and experimental considerations for using stable isotope-labeled fatty acids as substrates to study lipid metabolism *in vivo* in mice.

Experimental Design

For experiments on lipid synthesis, C57BL6 mice were treated with a vehicle control or a systemic, small-molecule inhibitor of microsomal triglyceride transfer protein (MTP).[5] One hour later, the mice were administered 150 mg/kg of oleic acid, potassium salt ($^{13}C_{18}$, 98%) CP 95% (CLM-8856) mixed with corn oil (Figure 2) or 20% TPGS (Figure 3). Blood samples were withdrawn at serial timepoints following tracer administration and processed to plasma. 10 µL of plasma were mixed with 90 µL of methanol containing heavy internal standards and further diluted with 300 µL of pentanol. The samples were centrifuged briefly to pellet insoluble proteins and 5-10 µL of the supernatant were analyzed by ultraperformance liquid chromatography interfaced with either a triple quadrupole (Waters Xevo TQ) or quadrupole-time of flight (Waters Synapt G2) mass spectrometer. For experiments on fatty acid oxidation, mice were administered a cocktail containing 150 mg/kg of three fatty acids – palmitic acid, stearic acid and oleic acid mixed with intralipid. Experiments were conducted in which one of the fatty acids was perdeuterated palmitic acid (D_{31}, 98%) (DLM-215), stearic acid (D_{35}, 98%) (DLM-379), oleic acid (D_{33}, 98%) (DLM-1891) and the remaining two were unlabeled, or in which all three fatty acids were perdeuterated. 10 µL of plasma were incubated with acetone at basic pH to exchange deuteriums in the plasma water with acetone. The deuterium enrichment in acetone was then measured by headspace gas chromatography interfaced with an isotope ratio mass spectrometer (Thermo Scientific). Further details on the methods employed in these experiments can be found in references.[6-8]

Data Analysis

Total plasma triglycerides were analyzed using a commercially available biochemical kit (Thermo Scientific). All other data were acquired and analyzed using the Waters or Thermo instrument software packages. Multiple-reaction monitoring on a triple-quadrupole mass spectrometer was used to trace the appearance of oleic acid, potassium salt ($^{13}C_{18}$, 98%) CP 95% in selected triglycerides and cholesteryl ester in the blood. The concentration of [$^{13}C_{18}$] oleate-labeled lipids was determined from the peak area ratio of the analyte to its matched, heavy-labeled internal standard. Concentrations were plotted as a function of time, and the area under the curve was determined using the Graphpad Prism software package. Alternatively, data were manually mined from full-scan MSe spectra obtained on the quadrupole time-of-flight instrument. Selected ion chromatograms were extracted, and the relative abundance of each [$^{13}C_{18}$] oleate-labeled lipid was determined based on the peak area ratio to a class-specific internal standard.

Results

In particular, we have studied the microsomal triglyceride transfer protein (MTP), which facilitates the transfer of lipids onto growing lipoprotein particles in the gut and liver prior to their secretion into the systemic circulation (Figure 1). Inhibition of MTP prevents lipidation of these chylomicron and VLDL particles and thereby prevents lipids from entering the blood. The data presented in Figure 2 help to illustrate some of the advantages of using stable isotope tracers to study the effects of MTP inhibition on complex lipid metabolism. Panel (a) shows the effects of a single dose of the MTP inhibitor on total endogenous (i.e. unlabeled) plasma triglycerides following an oral bolus of corn oil. Although the effects of the MTP inhibitor can be quite clearly observed, the basal level of pre-existing, circulating triglycerides (30-50 mg/dL) establishes a lower limit on the window for measuring dynamic changes. Panel (b) shows data from the same experiment but for a specific triglyceride composed of palmitoyl, linoleoyl and [$^{13}C_{18}$] oleoyl fatty acids. Because this species does not exist in the system until after the tracer fatty acid is introduced there is no background level and the time zero sample is truly blank. Since complete inhibition of new triglycerides entering the system can now be feasibly measured, we achieve the theoretical maximum window for measuring dynamic changes in excursion due to the

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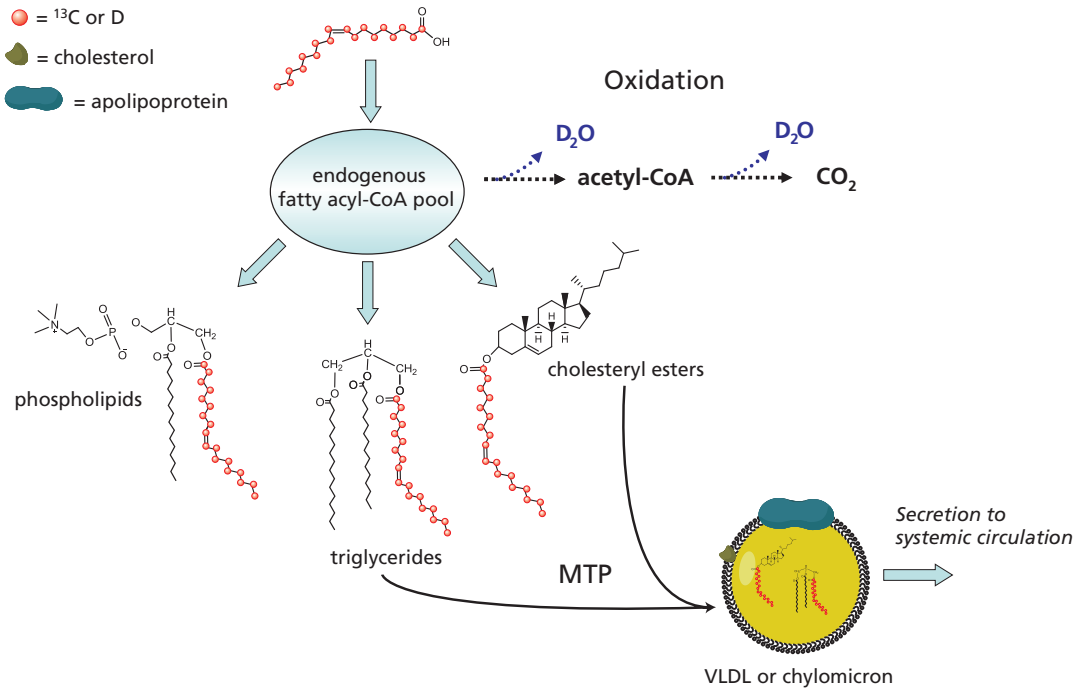

Figure 1. Schematic illustration showing the incorporation of [^{13}C] or [D] oleic acid into larger lipids. The isotope tracer enters the common fatty acid pool and is activated to a fatty acyl-CoA before being synthesized into phospholipid, triglyceride, cholesteryl ester or other lipids. In the liver and intestine, microsomal triglyceride transfer protein (MTP) aids in packaging triglycerides and cholesteryl esters into VLDL or chylomicron lipoprotein particles for secretion to the systemic circulation. Alternatively, [D] oleoyl-CoA can be oxidized to D_2O and CO_2.

pharmacological intervention. Panel (c) compares the effects of the acute dose of the MTP inhibitor on circulating levels of cholesteryl oleate. The turnover of cholesteryl esters *in vivo* is relatively slow compared to that of triglycerides, and the single dose of the MTP compound given has no effect on the endogenous pool of this lipid. In stark contrast, by focusing the analysis on newly synthesized esters (i.e., those tagged with the oleic acid tracer) we are able to observe a highly significant reduction in the amount of new lipid entering the system. Panel (d) illustrates that fatty acid oxidation can also be measured *in vivo* using a very similar experimental approach. In this experiment, we were interested in determining whether the nature of the fatty acid administered (palmitic acid vs. stearic acid vs. oleic acid) would affect the level of oxidation observed in normal mice. As the figure illustrates, comparable data on deuterium enrichment of plasma water was obtained regardless of which fatty acid was used as the tracer. Additionally, when a mixture containing perdeuterated forms of all three fatty acids was administered, the level of enrichment of plasma water was greater than what was observed in experiments when only a single fatty acid was labeled. In principal, experiments can also be designed in which lipid synthesis and fatty acid oxidation are measured simultaneously by simply using a [D]- rather than [^{13}C]-labeled fatty acid to measure synthesis.

Discussion

One principle of using isotope tracers to study biological processes is that the introduction of the isotope should not perturb the pathways under investigation. Simply put, tracers should be given in small enough amounts so as not to significantly alter the pools of the end products being traced. In order to achieve this, very sensitive analytical methods are required to enable measurement of the tracer enrichments. In the examples explored here, we have used [^{13}C]-labeled fatty acids as precursors for the synthesis of complex lipids (products). By employing ultraperformance liquid chromatography with mass spectrometric detection, we are able to measure very small amounts of [^{13}C]-labeled products in the presence of vastly greater endogenous (i.e. unlabeled) complex lipids.

Figure 3 (a) shows a total ion chromatogram of a mouse plasma lipid profile acquired following analysis by UPLC-qTOF-MS and includes lysophospholipids, phospholipids, diglycerides, ceramides, triglycerides and cholesteryl esters. Figure 3 (b) shows the mass spectrum of triglycerides eluting from the UPLC into the mass spectrometer between 8.5 to 10 minutes. As can be seen, a variety of triglycerides with different masses (based on permutations in fatty acid chain length and unsaturation) are present in this plasma extract. To take one example, the peak

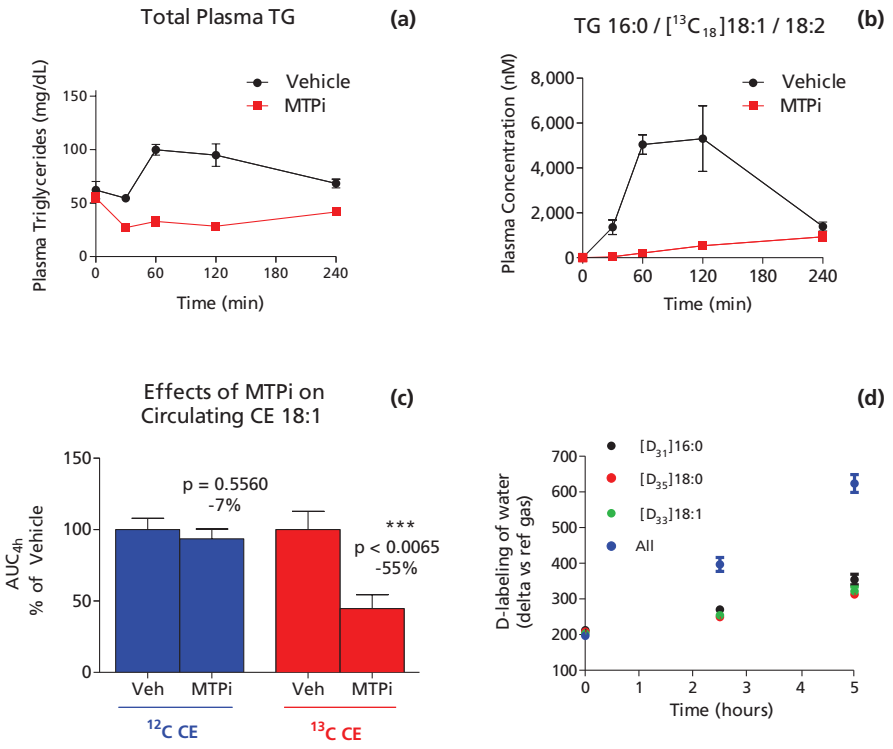

Figure 2. Measurement of lipid synthesis or fatty acid oxidation *in vivo* in mice. Panel (a) shows the influence of an MTP inhibitor on total plasma triglyceride levels in mice given an oral corn oil lipid challenge. Panel (b) shows the improvement in window for measuring the change in triglyceride excursion that can be gained by using a stable isotope-labeled fatty acid to distinguish newly synthesized lipids from endogenous background. Panel (c) illustrates the same advantage as applied to influences on levels of circulating cholesteryl ester over the 4h timecourse of the experiment. Panel (d) shows that oxidation of [D]-labeled fatty acids can be used to study oxidation by measuring the [D] enrichment in plasma water.

at m/z 902.8189 corresponds to triolein, a triglyceride containing three equivalents of oleic acid. The mice in this experiment had been given a low, oral dose of oleic acid, potassium salt ($^{13}C_{18}$, 98%) CP 95% (in the absence of any other naturally occurring fatty acids) prior to plasma being collected and processed. Panels (c) – (e) are enlargements of selected spectral features corresponding to various isotopomers of triolein. The peaks at m/z 920.8732, 938.9407 and 957.003 represent distinct molecules of triolein containing one, two or three equivalents of [$^{13}C_{18}$] oleate, respectively. The most intense [$^{13}C_{18}$] oleate-labeled isotopomer occurs at m/z 957.003 and is present at ~ 1/5 the concentration of the unlabeled endogenous triolein. In terms of totals, the sum of all [$^{13}C_{18}$] oleate-labeled triglycerides detected amounted to less than 1% of the concentration of total unlabeled (endogenous) triglyceride, illustrating the feasibility of performing these tracer studies without perturbing the natural biology.

There are a variety of practical benefits to adopting stable isotope tracer approaches for the study of lipid dynamics. One principle advantage is the ability to distinguish acute effects on dynamic lipid synthesis and disposition in the absence of steady-state effects. As illustrated in Figure 2(c), MTP inhibition does have an immediate impact on the appearance of intestinally derived, newly synthesized cholesteryl esters in the blood, though the total levels of the ester did not change over the timeframe of the experiment. These results obtained in mice are similar to what has been observed by others in humans. Although acute doses of this MTP inhibitor in humans was shown to lower VLDL cholesterol content, total and LDL cholesterol were not found to change over a 24-hour period.[5] Prolonged treatment, however, did result in changes in total and LDL cholesterol after three days of dosing. By using stable isotope tracers to distinguish newly synthesized from pre-existing cholesteryl esters, it is possible to get an indication of the effects of this compound in a much shorter period of time. Another related benefit of the tracer approach is the improvement in the window for measuring a dynamic change in lipid synthesis. By examining Figure 2(a) and (b), we can see that an approximately two-fold window is available for measuring changes in total plasma triglycerides. If this is the maximum change that can be observed, and with some knowledge of the inter-subject variability in these measurements, statistical power calculations can be carried out to determine the number of subjects that would be required to observe a specific % change in plasma TG. In contrast, the tracer data provides an approximately seven-fold window for measuring this change. Based on this data set, power calculations suggest that the tracer methodology has the power to detect changes in new triglyceride synthesis using five-six times fewer subjects than traditional methods based on measuring changes in total triglycerides.

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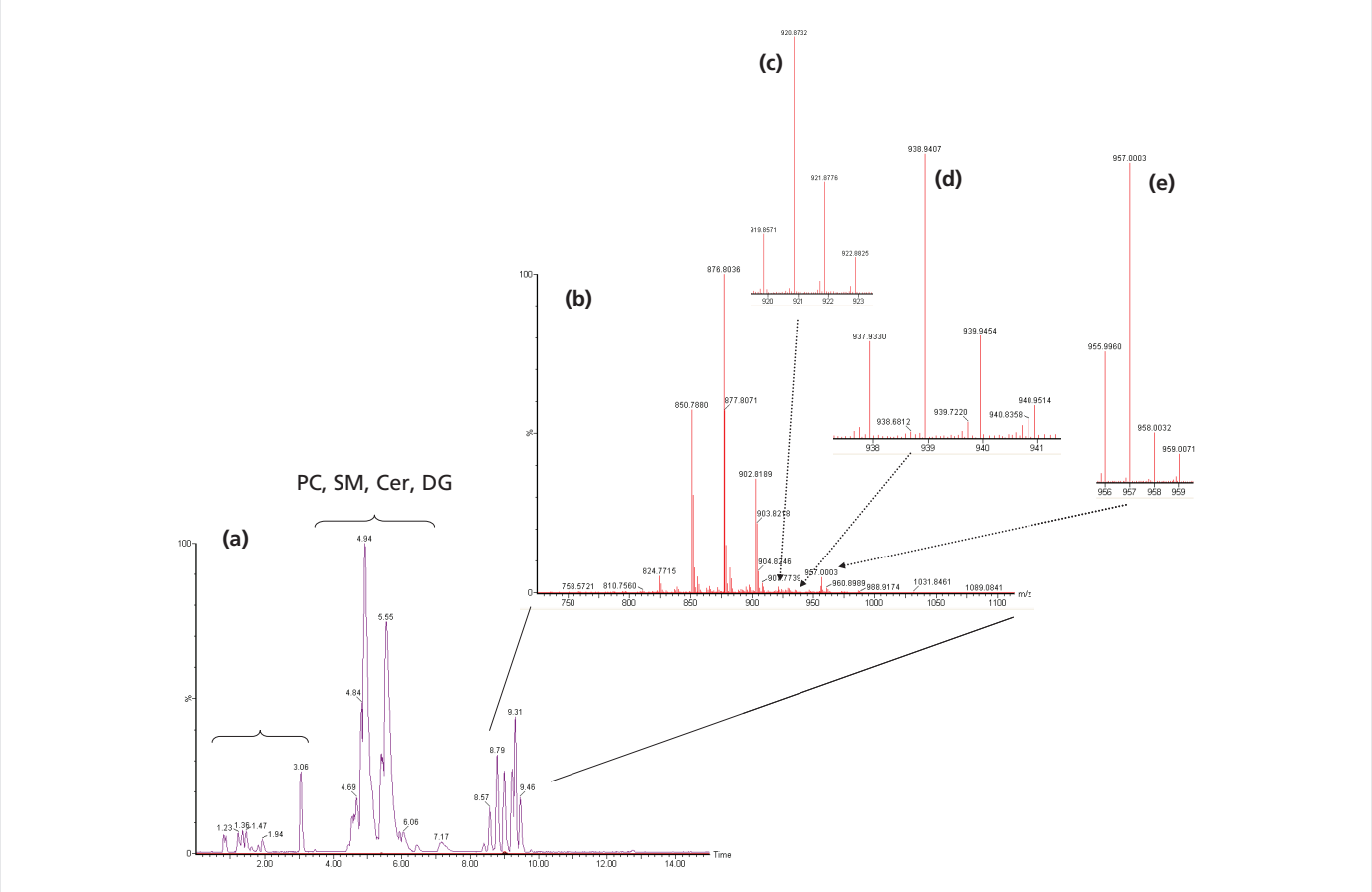

Figure 3. Untargeted analysis for the incorporation of oleic acid, potassium salt ($^{13}C_{18}$, 98%) CP 95% into lipids in mouse plasma using ultra-performance liquid chromatography coupled to a quadrupole-time of flight mass spectrometer. Panel (a) shows the total ion chromatogram for the full lipid profile; panel (b) shows a portion of the mass spectrum from 8.5 to 10 minutes (where triglycerides and cholesteryl esters are known to elute); panels (c), (d) and (e) show expanded spectra for the incorporation of 1, 2 and 3 equivalents of oleic acid, potassium salt ($^{13}C_{18}$, 98%) CP 95% into triolein, respectively. This figure highlights the detection of low levels of stable isotope-labeled fatty acids in the presence of much larger amounts of endogenous lipid.

These examples help to highlight how the use of stable isotope tracers can have both practical and ethical benefits when conducting pharmaceutical research. By using stable isotope tracers to evaluate the effects of novel drug candidates in preclinical species, we can reduce the number of animals required to complete a given study. An extension of this is that we can also complete individual studies in less time, thereby making informed decisions more quickly. These approaches thus have the potential to reduce both the cost and time for development of new drugs, hopefully allowing novel treatments for diseases to be discovered and effective therapies to be advanced more rapidly for the patients that need them.

Related Products

Catalog No.	Description
CLM-8856	Oleic acid, potassium salt ($^{13}C_{18}$, 98%) CP 95%
DLM-1891	Oleic acid (D_{33}, 98%)
DLM-215	Palmitic acid (D_{31}, 98%)
DLM-379	Stearic acid (D_{35}, 98%)