Published in final edited form as:

Metabolism. 2015 May ; 64(5): 580–587. doi:10.1016/j.metabol.2015.01.002.

Triglycerides produced in the livers of fasting rabbits are predominantly stored as opposed to secreted into the plasma

Demidmaa Tuvdendorj^{1,2}, Xiao-jun Zhang^{1,3}, David L. Chinkes^{1,3}, Lijian Wang², Zhanpin Wu^{1,3}, Noe A. Rodriguez^{1,3}, David N. Herndon^{1,3}, and Robert R. Wolfe⁴

¹ Metabolism Unit, Shriners Hospital for Children, University of Texas Medical Branch, Galveston, TX 77550, USA

² Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX 77550, USA

³ Department of Surgery, University of Texas Medical Branch, Galveston, TX 77550, USA

⁴ Department of Geriatrics, Center for Translational Research in Aging & Longevity, University of Arkansas Medical School, Little Rock, AR 72205, USA

Abstract

Objective—The liver plays a central role in regulating fat metabolism; however, it is not clear how the liver distributes the synthesized triglycerides (TGs) to storage and to the plasma.

Materials and Methods—We have measured the relative distribution of TGs produced in the liver to storage and the plasma by means of U-¹³C₁₆-palmitate infusion in anesthetized rabbits after an overnight fast.

Results—The fractional synthesis rates of TGs stored in the liver and secreted into the plasma were not significantly different (Stored vs. Secreted: 31.9 ± 0.8 vs. 27.7 ± 2.6 %•h⁻¹, $p > 0.05$). However, the absolute synthesis rates of hepatic stored and secreted TGs were 543 ± 158 and 27 ± 7 nmol•kg⁻¹•min⁻¹ respectively, indicating that in fasting rabbits the TGs produced in the liver were predominately stored (92±3%) rather than secreted (8±3%) into the plasma. This large difference was mainly due to the larger pool size of the hepatic TGs which was 21±9-fold that of plasma TGs. Plasma free fatty acids (FFAs) contributed 47±1% of the FA precursor for hepatic TG synthesis, and the remaining 53±1% was derived from hepatic lipid breakdown and possibly

© 2015 Elsevier Inc. All rights reserved

Corresponding author: Demidmaa Tuvdendorj, MD, PhD, Assistant Professor, Division of Endocrinology, Department of Internal Medicine, The University of Texas Medical Branch, 8.138 Medical Research Bldg, 301 University Blvd, Galveston, Texas, 77555-1060, Tel: 409 772 1962, Fax: 409 772 8709, detuvden@utmb.edu.

Authors' contribution:

General design of the study: DT, XJZ, DLC, RRW.

Study performance and sample processing and analyses: DT, XJZ, LW, ZW, NAR. Data analyses, interpretation of the results: DT, XJZ, DLC, DNH, RRW.

Manuscript preparation, editing, approval: DT, XJZ, DLC, LW, ZW, NAR, DNH, RRW.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

This manuscript has no potential conflict of interest for all authors.

plasma TGs depending on the activity of hepatic lipase. Plasma palmitate concentration significantly correlated with hepatic palmitoyl-CoA and TG synthesis.

Conclusion—In rabbits, after an overnight fast, the absolute synthesis rate of hepatic stored TGs was significantly higher than that of secreted due to the larger pool size of hepatic TGs. The net synthesis rate of TG was approximately half the absolute rate. Plasma FFA is a major determinant of hepatic TG synthesis, and therefore hepatic TG storage.

Keywords

rabbit liver; stable isotopes; mass spectrometry; very low density lipoprotein; triglyceride synthesis

1. INTRODUCTION

The liver plays a central role in regulating fat metabolism [1-3]. An important function is to take up free fatty acids (FFAs) from the plasma, re-esterify them into triglycerides (TGs), and then secrete them back into the plasma as very low density lipoprotein triglycerides (VLDL-TGs) [4, 5]. The FFAs in VLDL-TGs can be used as an energy source by various tissues, or taken up and stored in adipose tissue [6,7]. Under normal conditions FA release from adipose tissues far exceeds the rate of fat oxidation, but the excess FFAs provide a readily available substrate to enable a rapid increase in fat oxidation when required, e.g., at the onset of exercise [8]. The total amount of FFAs that can be transported in the plasma is limited because FFAs are insoluble and must be transported bound to albumin [2]. Secretion of FFAs as TGs therefore serves as an additional potential energy source in the form of circulating lipids. On the other hand, TGs re-esterified in the liver can also be deposited as TG droplets in hepatocytes. Excess accumulation of hepatic TGs is associated with a variety of pathologies, including insulin resistance [1-3]. The regulatory mechanisms whereby the liver distributes re-esterified TGs to secretion *vs.* storage are unclear. Since hepatic steatosis is associated with insulin resistance and other pathologies [1-3], understanding the differentiation of TGs produced in the liver into storage *vs.* secretion is important.

The production of VLDL-TGs or liver-secreted TGs has been measured in several studies using tracer methodologies [9-11], and shown to be predominately regulated by the provision of plasma FFAs [12-14]. Under most conditions, the re-esterification of plasma FFAs to TGs accounts for the majority of TGs synthesized in liver and the contribution of *de novo* synthesis of FFAs to VLDL-TG production is minor [14]. Thus fairly extensive knowledge regarding regulation of the synthesis of VLDL-TGs secreted into the plasma is available. However, few quantitative data are available regarding the synthesis rate of TGs subsequently stored in the liver. This knowledge gap is largely due to the difficulty in measuring TG synthesis in liver tissue.

The present experiments were designed to simultaneously measure both the secretion and storage of TGs synthesized in the liver of rabbits after an overnight fast. We measured the isotopic enrichment of hepatic U-¹³C₁₆-palmitoylCoA (PalCoA) and U-¹³C₁₆-palmitoyl-carnitine (PalCn) to estimate the precursor pool [15,16], and the enrichment of U-¹³C₁₆-palmitate in plasma and hepatic TGs for the product enrichments from the re-esterification

pathway. We have previously shown that in men after an overnight fast there is essentially no *de novo* FA synthesis, meaning that all hepatic TG synthesis is derived from FAs taken up from the plasma [14]. We hypothesized that the same is true for fasting rabbits thus we assumed that the synthesized TGs were derived only from FAs taken up from the plasma. The methodology used in this study also enabled us to further compare the hepatic synthesis of TGs and phospholipids (PLs).

2. MATERIALS AND METHODS

2.1. Animal preparation

Adult male New Zealand White rabbits (Myrtle's Rabbitry; Thompson Station, TN), weighing 4-5 kg, were used. The rabbits were housed in individual cages and fed 150 g•day⁻¹ of unpurified diet (Lab Rabbit Chow 5326, Purina Mills; St. Louis, MO) for weight maintenance. This protocol complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals, and was approved by the Animal Care and Use Committee of The University of Texas Medical Branch at Galveston.

The animals were studied after overnight food deprivation with free access to water. Surgery was performed to insert catheters into the carotid artery and jugular vein under general anesthesia [17]. The arterial line was used for drawing blood and monitoring arterial blood pressure and heart rate; the venous line was used for infusing anesthetics and saline. An additional venous line was installed in a marginal ear vein via a Teflon-top needle (24 G 3/4 in Introcan® Safety™; B. Braun Medical Inc.; Bethlehem, PA), and used exclusively for tracer infusion. Tracheotomy was performed to place a tracheal tube that was connected to a hood filled with oxygen-enriched room air.

2.2. Stable isotope tracer infusion

After surgery, we observed blood pressure, heart rate and rectal temperature for 20-30 min to ensure stable physiological conditions before starting the tracer infusion. U-¹³C₁₆-palmitate (99% enriched; Cambridge Isotope Laboratories), bound to 5% albumin [18] was infused at ~0.10 μmol • kg⁻¹ • min⁻¹ (15 ml/h) after a priming dose of 1.0 μmol • kg⁻¹ (2.5 ml). Arterial blood (1.5-2.0 ml each) was drawn before and during the tracer infusion. At the end of tracer infusion 5 ml of saturated KCl was intravenously injected under general anesthesia, followed immediately by laparotomy. A piece of liver was collected by freeze-clamp technique to avoid any delay in sample processing that could decrease the precursor enrichment [17]. Another piece of liver tissue was then taken using scissors and washed thoroughly in a cup with ~80 ml ice-cold saline. The liver samples were frozen in liquid nitrogen before being transferred into cryogenic tubes and stored at -80 °C. The whole liver was then removed and weighed using a digital scale.

Two groups of rabbits received the above described palmitate tracer infusion. In group 1 (n = 7), the tracer was infused for 3 hours; arterial blood was drawn before the infusion and every 30 min during the infusion. In group 2 (n = 5), the tracer was infused for only 0.5 hour; arterial blood was drawn before the infusion and at 5, 10, 20 and 30 min during the

infusion. Only group 1 was originally planned. However, the high enrichment of hepatic TG-bound palmitate measured from the liver samples taken at the end of the 3-hour infusion suggested possible overestimation of average precursor enrichment if there were tracer recycling. “Tracer recycling” means the release of labeled palmitate from both the plasma and intracellular lipid breakdown to the precursor pool for re-synthesis of lipids. Thus, group 2 was added; we assumed that any increase in precursor enrichment from 0.5 to 3 hours of tracer infusion could be attributed to tracer recycling (*for details see the calculation section below*). Clarification of the recycling allowed us to estimate the average precursor enrichment over the 3-hour tracer infusion, as we could perform liver biopsy only at the end of tracer infusion. Therefore, hepatic lipid synthesis was measured in group 1, which required the results from group 2 to correct for the precursor enrichment.

2.3. Supplemental experiment

The palmitate tracer infusion enabled us to calculate the fractional synthesis rate (FSR) of hepatic stored and secreted TGs (i.e., plasma TGs). However, to convert the FSR to the absolute synthesis rate, we needed to know the pool sizes of stored and secreted TGs, e.g., hepatic and plasma, respectively. The former was calculated by multiplying the liver weight by the TG concentration. The latter pool size was calculated by multiplying the plasma TG concentration by the plasma volume. Because the plasma volume was not available from the above tracer infusion groups, we used additional 2 rabbits for a supplemental experiment to measure plasma volume using indocyanine green (PULSION Medical Inc. East Brunswick, NJ), 0.5 mg/kg was injected intravenously as a bolus at the middle of the 3-hour tracer infusion. Arterial blood was drawn before the injection and every min thereafter for 6 min. The plasma indocyanine green concentration was measured on a spectrophotometer (Genesys 10 Series, Thermo Spectronic) at $\lambda=805$ nm. The decline in plasma concentration of indocyanine green was plotted against time and extrapolated to zero time to calculate the plasma volume, which was used to calculate the pool size of plasma TGs.

2.4. Sample analysis

Arterial blood was collected in tubes containing EDTA. The plasma FFAs and TGs were isolated and derivatized to produce methyl esters of palmitate, and their enrichments were measured using a gas chromatograph-mass spectrometer (GC-MS, MSD system, Agilent) [17,18]. Total plasma TG was used as an approximation of VLDL-TG in the postabsorptive state [11]. Ions were selectively monitored at mass-to-charge ratios of 270 and 286 for methyl palmitate. Eight FAs in plasma FFAs and TGs were measured using a GC system with flame ionization detection (GC-FID, Model 6890, Agilent, Santa Clara, CA); their individual contributions to the total FAs were expressed as percents. The 8 FAs were myristate (14:0), palmitate (16:0), palmitoleate [16:1(n-1)], stearate (18:0), oleate [C18:1(n-9)], linoleate [18:2(n-6)], linolenate [(18:3(n-3)], and arachidonic acid [(20:4(n-6)].

The liver tissue taken using the freeze-clamp technique was processed for measuring FA-CoA and FA-Cn [15,16]. Frozen liver samples were pulverized into powder with a mortar and pestle pre-chilled in liquid nitrogen. We used dry ice and an ice-water bath to keep the samples cold throughout processing, because these intermediate lipid metabolites are subject

to rapid changes on thawing. FA-CoA and FA-Cn enrichments and contents were measured with a liquid chromatograph-mass spectrometer (1100 series LC, 1956B SL single quadrupole MS; Agilent) [15,16]. Heptadecanoyl-CoA (Sigma Chemical, St. Louis, MO) and d₃-palmitoyl-Cn (CIL, Andover, MA) were added as internal standards for calculating the PalCoA and PalCn contents, respectively. Total FA-CoA and FA-Cn contents were calculated from the percents of PalCoA and PalCn in 7 total FA-CoAs and FA-Cns [18]. The 7 fatty acyls were the derivatives of FAs measured in plasma FFAs and TGs except of arachidonic acid [(20:4(n-6)]. We used the term of content (nmols or μmols/g liver) to express the amounts of hepatic FA-CoAs, FA-Cns, TGs, and PLs per unit of liver weight because they are not dissolved in body fluid.

Liver samples that were washed in ice-cold saline were used to measure the enrichments and contents of TGs and PLs. This washing procedure was important to eliminate blood contamination in the liver samples. Lipids were extracted from 30-50 mg of liver powder in 1:2 (v/v) methanol: chloroform solution containing 0.05 mg/ml butylated hydroxytoluene. Internal standards added for quantification of lipids were triheptadecanoin for TGs and L-α-phosphatidylcholine for PLs. After extraction, the samples were centrifuged, the supernatants dried under nitrogen gas. The pellets were reconstituted with 50 μl chloroform for isolation of TGs and PLs by thin-layer chromatography (TLC, Partisil LK5D, Silica Gel 150 Å, Schleicher & Schuell, Maidstone, England); for TLC, a mixture of hexane: ethyl ether: acetic acid (70:30:1) was used for TG separation, and for PL - chloroform: methanol: water (65:30:5) mixture (20-30 min) and then heptane: ethyl ether: glacial acid (80:20:2) mixture (45 min). The isolated TG and PL fractions were recovered from the TLC plate and reacted with 14 % Boron Trifluoride (Sigma-Aldrich Inc., St. Louis, MO) to produce palmitate methyl esters. Thereafter, the contents of hepatic TGs and PLs were measured on the GC-FID using the same method as was described for plasma FFAs and TGs.

2.5. Calculations

The concentrations (or contents) of palmitate in plasma (or liver) lipids were calculated by the internal standard method (18). Palmitate concentrations (or contents) were divided by % of palmitate in total FAs to obtain total FA concentrations (or contents). FSRs of liver lipids (i.e., TG and PL) were calculated by the tracer incorporation method, which is based on the precursor-product principle [18]. The general equation is

$$FSR = \frac{E_{t2} - E_{t1}}{E_p (t_2 - t_1) \times (t_2 - t_1)} \quad \text{Eq 1}$$

where $(E_{t2} - E_{t1})$ is the increment of product enrichment from t_1 to t_2 and $E_p(t_2 - t_1)$ is the average precursor enrichment from t_1 to t_2 . We used hepatic PalCn enrichment as the surrogate of the precursor enrichment because the enrichment of the true precursor (PalCoA) was subject to tracee dilution from lipid breakdown during sampling and processing.

We assumed that the precursor pool for hepatic lipid synthesis via the re-esterification pathway is derived from two sources: plasma FFAs and lipid breakdown, both plasma or intrahepatic. The enrichment difference between hepatic PalCoA (reflected by PalCn) and plasma free palmitate can be used to calculate the percent contribution of plasma FFAs to

the hepatic precursor pool [17]. The equation to calculate the percent of hepatic lipid synthesis from plasma FFAs is

$$\% \text{ TG synthesis from plasma FFA} = (E_{\text{PalCoA}}/E_{\text{palmitate}}) \times 100\% \quad \text{Eq 2}$$

where E_{PalCoA} is the enrichment of hepatic PalCoA (reflected by PalCn) measured from the liver sample and $E_{\text{palmitate}}$ is the corresponding plasma free palmitate enrichment.

The contribution from lipid breakdown or recycling is thus

$$\% \text{ TG synthesis from recycling of lipids} = 100\% - [(E_{\text{PalCoA}}/E_{\text{palmitate}}) \times 100\%] \quad \text{Eq 3}$$

If the precursor enrichment (i.e., E_{PalCoA}) is constant, Eq 2 is valid at any infusion time point. However, to consider the possible tracer recycling we calculated the enrichment ratio of hepatic PalCN to plasma free palmitate in groups 1 and 2 (e.g., 3 and 0.5-hour time points), and by extrapolating this ratio to time 0, we estimated the precursor enrichment without tracer recycling to obtain the % TG synthesis from plasma FFAs using Eq 2. Meanwhile, we can estimate the average precursor enrichment over the 3-hour infusion period for calculation of FSR using Eq 1.

The absolute TG synthesis rate can be calculated from the Eq:

$$\text{Absolute lipid synthesis rate} = \text{FSR} \times \text{pool size} \quad \text{Eq 4}$$

2.6. Statistics

Isotopic enrichments for calculating FSRs are expressed as mole % excess (MPE). Values are expressed as means $\pm$ SE. Differences between the groups were tested by 2-tail unequal Student's *t*-test. Differences in FA profiles between plasma, hepatic TGs and hepatic PLs were evaluated using a one-way ANOVA with post hoc correction using the Holm-Sidak test. The relationships between parameters were examined using linear regression analysis. A *p* value < 0.05 was considered statistically significant.

3. RESULTS

The average body weight for both groups was 4.5 ± 0.1 kg ($p > 0.05$ for a between groups differences). Tracer infusion was completed smoothly in all the rabbits. No abnormal responses to anesthetics or human albumin bound to the palmitate tracer were observed, as the rectal temperature, heart rate and arterial pressure were all stable (data not shown). In group 1, plasma free palmitate enrichment increased rapidly after the start of tracer infusion with $4.09 \pm 0.59\%$ at 0.5 hour, while plasma TG-bound palmitate enrichment increased gradually over time (Fig. 1A). After the 3-hour infusion, plasma TG-bound palmitate enrichment was $55 \pm 5\%$ that of plasma free palmitate enrichment, and the plasma concentrations of free palmitate and TG-bound palmitate were 123 ± 12 and 187 ± 30 nmol•mL⁻¹, respectively. Palmitate accounted for $38 \pm 2\%$ and $43 \pm 4\%$ of total FAs in plasma FFA and TG, respectively. Plasma FFA and TG concentrations, which were

converted from palmitate and % palmitate in total FAs, were 336 ± 45 and 158 ± 31 $\text{nmol} \cdot \text{mL}^{-1}$, respectively.

3.1. Hepatic precursor and plasma FFA contribution

Hepatic PalCoA enrichment was lower ($p < 0.05$) than PalCn enrichment in both groups 1 and 2 (Fig. 1B). Hepatic PalCoA and FA-CoA contents were greater ($p < 0.05$) than PalCn and FA-Cn, respectively, and much less than TG and PL (Table 1). The sum of PalCoA and PalCn (31.5 ± 4.5 $\text{nmol} \cdot \text{g}^{-1}$) was only 0.25% of palmitate bound to liver TG and PL (Table 1).

The percentage contribution to the hepatic precursor pool from plasma FFAs was calculated from the enrichment gradient between plasma free palmitate and hepatic PalCn (representing PalCoA) after 3 (group 1) or 0.5 hours (group 2) of tracer infusion, using Eq 2. In group 1, plasma free palmitate enrichment after 3-hour tracer infusion was $5.31 \pm 0.81\%$ (Fig. 1A), and the hepatic PalCn enrichment was $4.18 \pm 0.45\%$. Thus, $80 \pm 2\%$ of FAs entering the hepatic PalCoA pool came from arterial FFAs. As it was noted this value might have been overestimated if there was significant tracer recycling. In group 2, after 0.5-hour tracer infusion the enrichments of plasma free palmitate and hepatic PalCn were $4.81 \pm 0.23\%$ and $2.59 \pm 0.14\%$, respectively. Accordingly, $54 \pm 2\%$ of FAs in the precursor pool came from plasma FFAs, lower than that in group 1 ($p < 0.001$). We used 54% as the enrichment ratio of PalCn/plasma free palmitate at 0.5 hour in group 1, so the PalCn enrichment at this time point was calculated as $2.86 \pm 0.33\%$. Using Eq 2, the contribution of plasma FFA to hepatic precursor was calculated to be $47 \pm 1\%$. Using Eq 3, the contribution of lipid breakdown to precursor enrichment was $53 \pm 1\%$.

Tracer recycling released labeled palmitate to hepatic precursors so the precursor enrichment increased over time. We calculated the average PalCn enrichment ($3.39 \pm 0.37\%$) from the 0.5- and 3-hour PalCn enrichments.

3.2. Hepatic Triglyceride and Phospholipid synthesis

The enrichments and contents of hepatic TG and PL were measured from the liver samples that were thoroughly washed in ice-cold saline in group 1. The enrichments of TG-bound palmitate in the liver and the plasma after 3 hours were not different ($3.25 \pm 0.39\%$ and $2.69 \pm 0.19\%$; $p > 0.05$) (Table 2 and Fig. 1A). The FSRs of the stored TGs (31.9 ± 0.8 $\% \cdot \text{h}^{-1}$) and secreted TGs (27.7 ± 2.6 $\% \cdot \text{h}^{-1}$) were also not different ($p > 0.05$). The enrichment of hepatic PL-bound palmitate was $0.62 \pm 0.07\%$ after 3-hour tracer infusion (Table 2), resulting in a FSR of 6.9 ± 0.5 $\% \cdot \text{h}^{-1}$, much lower than those of stored and secreted TGs ($p < 0.0001$).

In the two supplemental rabbits, the measured plasma volumes were 31 and 34 $\text{mL} \cdot \text{kg}^{-1}$; the average plasma volume of 32.5 $\text{mL} \cdot \text{kg}^{-1}$ was used to calculate plasma TG pool size. The average liver weight was 76 ± 2 g, equivalent to 16.9 ± 0.57 g liver $\cdot \text{kg}^{-1}$ body weight. Using the plasma volume and concentrations of plasma TG-bound and free palmitate we calculated the pool sizes of plasma TG-bound and free palmitate as 6.1 ± 1.0 and 4.3 ± 0.5 $\mu\text{mol} \cdot \text{kg}^{-1}$ body weight, respectively. Using the hepatic TG-bound palmitate content and liver weight,

we calculated the pool size of hepatic stored TG-bound palmitate was $106 \pm 33 \mu\text{mol} \cdot \text{kg}^{-1}$ body weight. The pool size of liver stored TG-bound palmitate was 21 ± 9 and 25 ± 5 folds that of plasma TG-bound and free palmitate, respectively. The content of hepatic PL-bound palmitate was $55 \pm 14 \mu\text{mol} \cdot \text{kg}^{-1}$ body weight.

The absolute synthesis rates were calculated using Eq 4. If expressed as nmol of palmitate molecule $\cdot \text{kg}^{-1}$ of body weight per min, the absolute synthesis rate of hepatic total TG was 570 ± 154 , and the absolute synthesis rates of stored and secreted TGs were 543 ± 158 and $27 \pm 7 \text{ nmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, respectively (Fig. 1C). If expressed as nmol of TG $\cdot \text{kg}^{-1}$ of body weight $\cdot \text{min}^{-1}$, the absolute synthesis rate of hepatic total TG was 443 ± 120 , and the absolute synthesis rates of stored and secreted TGs were 422 ± 123 and $21 \pm 6 \text{ nmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, respectively. The conversion of palmitate to TGs was based on the fact that palmitate accounted for 42.3% and 42.9% of FAs in plasma and liver TGs, respectively, and one TG molecule contains three fatty acids. In both cases stored TG synthesis accounted for $92 \pm 3\%$ of total TG synthesis and secretion TG synthesis for only $8 \pm 3\%$. The FSR of hepatic PL was $6.1 \pm 0.4\% \cdot \text{h}^{-1}$, and the absolute PL synthesis rate was 53 ± 12 (expressed as palmitate molecule) or 102 ± 22 (expressed as PL molecules) $\text{nmol} \cdot \text{kg}^{-1}$ body weight $\cdot \text{min}^{-1}$. The conversion of palmitate molecules to PL molecules was based on the fact that palmitate accounted for 26.3% of fatty acids in PL, and there are two fatty acids in each PL molecule. The absolute synthesis rate of total TG was greater than the PL synthesis rate ($p < 0.05$).

Using the body weight ($4.5 \pm 0.1 \text{ kg}$) and liver weight ($76.0 \pm 1.8 \text{ g}$), we can convert the above synthesis rates to show the synthetic capacity of TG and PL in each g of liver. For example, the synthesis rate of hepatic total TG was $25.7 \pm 6.5 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$, reflecting the synthesis rates of stored and secreted TGs of 24.4 ± 6.7 and $1.32 \pm 0.40 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$. The synthesis rate of PL per g of liver was $6.16 \pm 1.44 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$, respectively. Again, the stored TG accounted for 92% of total hepatic TG synthesis, and secreted TG synthesis for only 8%.

There were positive correlations between the plasma palmitate concentration and hepatic PalCoA content ($r^2 = 0.61$; $p < 0.05$), between the plasma palmitate concentration and hepatic TG synthesis ($r^2 = 0.59$; $p < 0.05$), and between the hepatic PalCoA content and hepatic TG synthesis ($r^2 = 0.61$; $p < 0.05$) (Fig. 2). However, there was no correlation between hepatic TG synthesis and PL synthesis ($r^2 = 0.11$; $p > 0.05$, not shown).

3.3. Fatty Acid profile

The fatty acid composition of stored TGs was comparable to that of secreted TGs (i.e. plasma TGs), but different from that of hepatic PLs (Table 3). Both stored and secreted TGs contained more palmitic acid (16:0) but less stearic acid (18:0) than PLs ($p < 0.05$).

4. DISCUSSION

The main purpose of the present study is to distinguish the rates of secretion as opposed to storage of TG produced in the livers of fasting rabbits. We also evaluated the associations between the kinetics and values of liver and plasma lipids (e.g., FFAs, TGs and PLs) and the use of hepatic PalCoA and PalCn as precursors for TG synthesis.

4.1. The majority of TGs are stored in the liver

The FSRs of hepatic storage and secretion TGs were comparable (Table 2); however, the absolute synthesis rate of stored TGs was much greater than that of secreted TG (Fig. 1C). This was because the pool size of hepatic TGs was 21 ± 9 fold that of plasma TG-bound palmitate. Thus, in the postabsorptive state in rabbits the majority of TGs synthesized in liver were deposited there and only a small portion was secreted into the plasma. Given the high synthesis rate of hepatic TGs, these findings may indicate that in the case of acute hyperlipidemia, the liver is able to clear plasma FFA rapidly, which may be reversed when the energy demand is acutely increased, e.g., during exercise [20].

4.2. Liver and plasma TGs, but not PLs, are derived from the same precursor pool

The FSRs and FA compositions of TGs in the plasma and liver were comparable (Table 2, 3). Interestingly, the hepatic PL synthesis rate was 5-fold lower than the TG rate (Table 2), and the FA compositions were also different between these two lipids (Table 3). These data may suggest that while liver and plasma TGs are derived from the same precursor pool (Fig. 2), PLs turnover is regulated differently. PLs are the major components of biological membranes, which may explain these differences in the regulation of PL turnover and its composition.

4.3. Fatty acid availability determines liver TG synthesis

Plasma FFAs accounted for ~50% of total hepatic lipid synthesis, and the other 50% was derived from lipid breakdown. Therefore, the net TG synthesis rate was ~50% that of the absolute rate measured via the traditional isotopic technique. If calculated using plasma palmitate as the precursor, the FSR of VLDL-TG may be underestimated because the plasma palmitate tracer is diluted by unlabeled palmitate in the precursor pool within the liver. At the same time, our results emphasize the importance of plasma FFAs in liver TG synthesis. The correlations between the plasma palmitate concentration with the hepatic PalCoA content (Fig. 2A), and hepatic TG synthesis (Fig. 2B), and between hepatic PalCoA content and hepatic TG synthesis (Fig. 2C) indicate that FA availability is a determinant of hepatic TG synthesis, supporting the previously reported data [12-14].

4.4. Precursors for TG synthesis

The technical challenge of measuring hepatic lipid synthesis lies in the difficulty of measuring hepatic precursor enrichment. We used hepatic PalCn enrichment as a surrogate of precursor enrichment. The enrichment of PalCoA, which is the true precursor, was consistently lower than that of PalCn (Fig. 1B). This is the same observation as in the measurement of precursor enrichment for intramuscular lipid synthesis (17). One possible explanation for the lower enrichment of PalCoA is its dilution by the unlabeled PalCoA due to the intra-mitochondrial beta oxidation that converts stearate (C:18) into palmitate (C:16). However, hepatic stearyl-CoA and palmitoyl-CoA accounted for $14.4 \pm 1.7\%$ and $24.2 \pm 1.1\%$ of total FA-CoAs (unpublished data), suggesting that the contribution of unlabeled stearate to PalCoA was negligible (20). Another issue in measuring hepatic precursor enrichment is tracer recycling (*see method section*). In future experiments, we may consider infusing two palmitate tracers, one starting at the beginning and the second 0.5 hour before

the end of the infusion period, which could enable completing the experiment without a second group, but with simultaneous estimation of the recycling and the true precursor enrichment during the study period.

4.5. Study limitations

4.5.1. We measured the synthesis rate of plasma TGs to reflect hepatic TG secretion via the formation of VLDL. In the postabsorptive state when no exogenous fat enters the circulation, plasma TG is mainly carried in the hydrophobic core of VLDL [11]. In this experiment the unpurified diet for rabbits contained less than 10% fat calories, and the experiment was performed after 16 hours of fasting, so the experimental design ensured negligible exogenous fat uptake. Additionally, the use of the enrichment of total plasma TGs rather than VLDL-TGs may have resulted in underestimating the FSR and production rate of VLDL-TGs; however, the use of total plasma TGs may have resulted in overestimation of the VLDL-TG pool size, so these may have cancelled each other, and the absolute hepatic synthesis rate should be valid.

4.5.2. The hepatic TG and PL enrichments were measured at a single 3-h time point and a linear rate of tracer incorporation was assumed. Based on the PalCn enrichment data from 0.5- and 3-hr time points we extrapolated hepatic PalCn enrichment at 1, 1.5, 2 and 2.5 hrs using both linear and exponential models (data not shown). The averages of these estimates of PalCn enrichment were 3.384 and 3.334 % by the linear and exponential models, respectively. If we use these values and calculate the storage TG synthesis, the difference between these values was 0.44%/hr (e.g., storage TG synthesis was 32.01 vs. 32.45 %/hr, via the linear vs. exponential models). Thus, we speculate that, at least during the time frame of the study (3 hrs), the model of tracer incorporation may not affect the FSR value significantly.

4.5.3. Our study did not clarify either hepatic ketogenesis or fat oxidation. Havel et al., [21] demonstrated that in fasting humans about 60% of FFA up taken by the liver is converted into ketone bodies and CO₂. The current study quantified the fate of the non-oxidized plasma FAs, which might be < 50% of total FFA flux in the fasted state. Thus, our measurement of the ratio of hepatic stored TGs to secreted TGs should not be affected by the extent of oxidation of FAs. Interestingly, both our study and that of Havel et al., [21] demonstrated that the fractional contributions of plasma FFAs were comparable between the hepatic stored TGs and secreted TGs. However, different conditions, e.g., fed state, obesity or hyperinsulinemia may significantly alter these contributions [22-25]. For example, it has been shown previously that although hyperinsulinemia acutely suppressed VLDL-TG secretion in both healthy and type 2 diabetic individuals, the postabsorptive VLDL-TG secretion was higher in type 2 diabetic individuals [22,25]. However, the mechanisms underlying these responses are not known, and neither is the storage of TGs in the liver.

In summary, our current study is one step toward filling the gap in our knowledge regarding the *in vivo* mechanisms of hepatic storage as compared to secretion. It would be speculation to extrapolate our results to circumstances significantly different than the experimental conditions of our study. Thus future studies evaluating TG synthesis and hepatic fat oxidation along with ketogenesis under different physiological and pathological conditions

are of interest. Also, our results support a single TG pool in the liver, which uses plasma FFAs and FAs from both the plasma and intrahepatic lipid breakdown for reesterification of TGs. However, the main result of this study suggests that the majority of TGs synthesized in the liver are stored there, and only a small portion is secreted into plasma. Thus these data may suggest that factors controlling the VLDL secretion rate are at least to some extent independent of the rate of hepatic TG synthesis, and that the rate of VLDL secretion, rather than the rate of TG synthesis, is the key determinant of the extent to which TGs accumulate in the liver. Future studies evaluating these mechanisms are warranted and of a significant interest and translational value in evaluating the mechanisms of non-alcoholic hepatic steatosis.

Acknowledgements

The authors are grateful to Yun-xia Lin, Guy Jones, Guarang Jariwala, Cindy Locklin, Christopher Danesi, and Dr. Chenyue Meng for technical assistance. We also thank the Animal Resource Center of University of Texas Medical Branch for professional care of rabbits, and Dr. David Konkel for critically editing the manuscript. This study was supported by Shriners grant 86500 and 84090, National Institute of Health (NIH) grants RO1 DK 034817 and P30AG028718.

All authors have approved the final version of the manuscript.

Abbreviations

FSR	fractional synthesis rate
FFA	free fatty acids
FA-Cn	fatty acyl carnitine
FA-CoA	fatty acyl coenzyme A
GC-FID	gas chromatography flame ionization detector
GC-MS	gas chromatography mass spectrometry
LPL	lipoprotein lipase
MPE	mole percent excess
palCn	palmitoyl-carnitine
PalCoA	palmitoyl-Coenzyme A
PLs	phospholipids
TLC	thin layer chromatography
TGs	triglycerides
VLDL-TG	very-low density lipoprotein triglyceride

REFERENCES

1. Lavoie JM, Gauthier MS. Regulation of fat metabolism in the liver: link to non-alcoholic hepatic steatosis and impact of physical exercise. *Cell Mol. Life Sci.* 2006; 63:1393–409. [PubMed: 16649140]

2. Yki-Järvinen H. Liver fat in the pathogenesis of insulin resistance and type 2 diabetes. *Dig Dis.* 2010; 28:203–209. [PubMed: 20460912]
3. Reddy JK, Rao MS. Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. *Am. J. physiol. Gastrointest. Liver Physiol.* 2006; 290:G852–858. [PubMed: 16603729]
4. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J. Clin. Invest.* 2005; 115:1343–1351. [PubMed: 15864352]
5. Postic C, Girard J. Contribution of *de novo* fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J. Clin. Invest.* 2008; 118:829–838. [PubMed: 18317565]
6. Jackson, RL. Lipoprotein lipase and hepatic lipase. In: Boyer, PD., editor. *The enzymes*. Academic Press; New York: 1983. p. 141–181.
7. Wolfe, RR. The role of triglyceride-fatty acid cycling and glucose cycling in thermogenesis and amplification of net substrate flux in human subjects. In: Muller, MJ.; Danforth, E., Jr.; Burger, AG.; Siedentopp, U., editors. *Hormones and Nutrition in Obesity and Cachexia*. Springer-Verlag; N.Y.: 1989. p. 59–65.
8. Romijn JA, Coyle EF, Sidossis LS, Zhang XJ, Wolfe RR. Relationship between fatty acid delivery and fatty acid oxidation during strenuous exercise. *J. Applied Physiol.* 1995; 79:1939–1945. [PubMed: 8847257]
9. Smith GI, Magkos F, Reeds DN, Okunade AL, Patterson BW, Mittendorfer B. One day of mixed meal overfeeding reduces hepatic insulin sensitivity and increases VLDL particle but not VLDL-triglyceride secretion in overweight and obese men. *J Clin Endocrinol Metab.* 2013; 98(8):3454–62. [PubMed: 23750033]
10. Magkos F, Fabbri E, Patterson BW, Eagon JC, Klein S. Portal vein and systemic adiponectin concentrations are closely linked with hepatic glucose and lipoprotein kinetics in extremely obese subjects. *Metabolism.* 2011; 60(11):1641–8. [PubMed: 21632078]
11. Magkos F, Sidossis LS. Measuring very low density lipoprotein-triglyceride kinetics in man in vivo: how different the various methods really are. *Curr. Opin. Clin. Nutr. Metab. Care.* 2004; 7:547–555. [PubMed: 15295275]
12. Rigazio S, Lehto HR, Tuunanen H, Nagren K, Kankaanpää M, Simi C, Borra R, Naum AC, Parkkola R, Knuuti J, Nuutila P, Iozzo P. The lowering of hepatic fatty acid uptake improves liver function and insulin sensitivity without affecting hepatic fat content in humans. *Am. J. Physiol.* 2008; 295:E413–419.
13. Vedala A, Wang W, Neese RA, Christiansen MP, Hellerstein MK. Delayed secretory pathway contributions to VLDL-triglycerides from plasma NEFA, diet, and *de novo* lipogenesis in humans. *J Lipid Res.* 2006; 47(11):2562–74. [PubMed: 16929033]
14. Aarsland A, Chinkes DL, Wolfe RR. Contributions of *de novo* synthesis of fatty acids to total VLDL-triglyceride secretion during prolonged hyperglycemia/hyperinsulinemia in normal man. *J. Clin. Invest.* 1996; 98:2008–2017. [PubMed: 8903319]
15. Sun D, Cree MG, Wolfe RR. Quantification of the concentration and ^{13}C tracer enrichment of long-chain fatty acyl-coenzyme A in muscle by liquid chromatography/mass spectrometry. *Analytic Biochem.* 2006; 349:87–95.
16. Sun D, Cree MG, Zhang XJ, Boersheim E, Wolfe RR. Measurement of stable isotopic enrichment and concentration of long-chain fatty acyl-carnitines in tissue by HPLC-MS. *J. Lipid Res.* 2006; 47:431–439. [PubMed: 16301738]
17. Zhang XJ, Rodriguez NA, Wang L, Tuvdendorj D, Wu Z, Tan A, Herndon DN, Wolfe RR. Measurement of precursor enrichment for calculating intramuscular triglyceride fractional synthetic rate. *J Lipid Res.* 2012; 53(1):119–125. [PubMed: 21934122]
18. Wray-Cahen D, Caperna TJ, Steele NC. Methyl- β -cyclodextrin: an alternative carrier for intravenous infusion of palmitate during tracer studies in swine (*Sus scrofa domestica*). *Comp Biochem Physiol A Mol Integr Physiol.* Aug; 2001 2001 130(1):55–65. [PubMed: 11672683]
19. Wolfe, RR.; Chinkes, DL. *Isotopic Tracers in Metabolic Research: Principle and practice of kinetic analysis*. 2nd. Wiley-Liss; New Jersey: 2004.

20. Morio B, Holmback U, Gore D, Wolfe RR. Increased VLDL-TAG turnover during and after acute moderate-intensity exercise. *Med Sci Sports Exerc.* 2004; 39(5):801–806. [PubMed: 15126713]
20. Brunengraber H. What is the proper precursor-to-product labeling relationship for calculating the fractional synthetic rate of muscle triglyceride? *J Lipid Res.* 2012; 53(1):1–3. [PubMed: 22075970]
21. Havel RJ, Kane JP, Balasse EO, Segel N, Basso LV. Splanchnic metabolism of free fatty acids and production of triglycerides of very low density lipoproteins in normotriglyceridemic and hypertriglyceridemic humans. *J Clin Invest.* 1970; 49(11):2017–35. [PubMed: 5475985]
22. Sorensen LP, Andersen IR, Sondergaard E, Gormsen LC, Schmitz O, Christiansen JS, Nielsen S. Basal and Insulin Mediated VLDL-Triglyceride Kinetics in Type 2 Diabetic Men. *Diabetes.* 2011; 60(1):88–96. [PubMed: 20858686]
23. Sondergaard E, Sorensen LP, Rahbek I, Gormsen CL, Christiansen JS, Nielsen S. Postprandial VLDL-triacylglycerol secretion is not suppressed in obese type 2 diabetic men. *Diabetologia.* 2012; 55:2733–2740. [PubMed: 22752024]
24. Barrows BR, Timlin MT, Parks EJ. Spillover of dietary fatty acids and use of serum nonesterified fatty acids for the synthesis of VLDL-triacylglycerol under two different feeding regimens. *Diabetes.* 2005; 54(9):2668–73. [PubMed: 16123356]
25. Malmstrom R, Packard CJ, Caslake M, Bedford D, Stewart P, Yki-Jarvinen H, Shephard J, Taskinen MR. Defective regulation of triglyceride metabolism by insulin in the liver in NIDDM. *Diabetologia.* 1997; 40:454–462. [PubMed: 9112023]

Figure 1.

Plasma free and TG-bound palmitate enrichments during a 3-hour tracer infusion in Group 1 (A) and the enrichments of hepatic precursors – palmitoyl coenzyme A (PalCoA) and palmitoyl-carnitine (PalCn) in groups 1 and 2 (B), the data are presented in Mole % excess. (C), the absolute synthesis rate of stored and secreted triglycerides presented in $\text{nmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. Data are presented as means $\pm$ SE.

Figure 2.

Correlation analyses between (A) plasma concentration of free palmitate and hepatic palmitoyl-coenzyme A (PalCoA); (B) plasma concentration of free palmitate and the total TG synthesis rate; and (C), Hepatic PalCoA content and the total TG synthesis rate.

Table 1

Hepatic lipid contents in group 1.

Fatty acyl-CoA			Fatty acyl-carnitine			TG			PL		
fatty acyl-CoA (nmol/g)	PalCoA (nmol/g)	PalCoA (%)	fatty acyl-carnitine (nmol/g)	PalCn (nmol/g)	PalCn (%)	TG (μmol/g)	TG-Pal (μmol/g)	TG-Palmitate (%)	PL (μmol/g)	PL-Palmitate (μmol/g)	PL Palmitate (%)
95.3±18.8	23.4±4.9	24.2±1.1	33.7±12.5	8.0±2.7	25.5±1.0	6.5±2.1	6.1±1.8	42.9±4.5	6.5±1.9	3.3±0.9	26.3±1.1

Values are presented as Means ± SE, N = 7.

Table 2

FSRs of hepatic lipids in group 1

Average PalCn MPE over 3 hours infusion (%)	Liver storage TG- palmitate MPE at 3 hour (%)	Plasma TG- palmitate (MPE) at 3 hour (%)	Liver PL- palmitate (MPE) at 3 hour (%)	Liver PL FSR (%/h)	Liver storage TG FSR (%/h)	Plasma TG FSR (%/h)
3.39 ± 0.36	3.25 ± 0.39	2.69 ± 0.19	0.62 ± 0.07	6.1 ± 0.4	31.9 ± 0.8	27.7 ± 2.6

Values are means ± SE from 7 rabbits.

Table 3

Profile of plasma and liver Triglycerides (TG) and liver Phospholipds (PL).

Fatty Acids	Plasma TG (%)	Liver TG (%)	Liver PL (%)
C14:0	1.03±0.06 [*]	1.41±0.08 ^{*,#}	0.23±0.08
C16:0	41.24±4.48 [*]	46.17±5.15 [*]	25.98±0.86
C16:1	1.86±0.17	1.82±0.37	0.90±0.28
C18:0	6.14±0.86 [*]	6.55±1.55 [*]	39.65±2.90
C18:1	22.34±1.77	18.43±1.77	18.37±3.88
C18:2	23.64±2.74	22.93±4.06	14.08±5.39
C18:3	2.32±0.41	2.69±0.65 [*]	0.80±0.17
C20:4	0.42±0.09	0.73±0.18	2.51±0.75

Data are presented as means±SE. Statistical analyses were performed using One Way ANOVA with Holm-Sidak post-hoc correction.

^{*} statistically significantly different between the TG and PL groups;

[#] statistically significantly different within the TG groups, $p < 0.05$.