patient-oriented research

Delayed secretory pathway contributions to VLDL-triglycerides from plasma NEFA, diet, and de novo lipogenesis in humans

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Abstract **Newly synthesized triglyceride (TG) may exit the liver immediately as VLDL-TG or be stored and secreted after a delay. We quantified the contributions from plasma NEFA, diet, and de novo lipogenesis (DNL) to VLDL-TG via immediate and delayed pathways in five lean, normolipidemic subjects; six obese, hypertriglyceridemic (HPTG) nondiabetics; and six obese, HPTG diabetics. Intravenous [$^{2}H_{31}$]palmitate and [1-$^{13}C_{1}$] acetate and oral [$^{2}H_{35}$]stearate were administered for 30 h preceding an overnight fast. [1,2,3,4-$^{13}C_{4}$]palmitate was infused during the subsequent 12 h fast. Contributions from plasma NEFA via the immediate pathway were 64 ± 15, 33 ± 6, and 58 ± 2% in control, HPTG, and diabetic HPTG, respectively. Delayed pool fractional contributions were as follows: dietary FA, 2.0 ± 0.9, 2.5 ± 1, and 12 ± 2%; DNL, 3 ± 0.3, 14 ± 3, and 13 ± 4%; delayed NEFA, 15 ± 4, 20 ± 4, and 30 ± 3%. VLDL-TG production rates and absolute input rates from the delayed pool were significantly higher in HPTG and diabetic HPTG than in controls. In conclusion, we provide direct kinetic evidence for a hepatic TG storage pool in humans and document its metabolic sources. The turnover time and sources of this pool differ in diabetic HPTG and nondiabetic HPTG, with potential therapeutic implications.—** Vedala, A., W. Wang, R. A. Neese, M. P. Christiansen, and M. K. Hellerstein. **Delayed secretory pathway contributions to VLDL-triglycerides from plasma NEFA, diet, and de novo lipogenesis in humans. *J. Lipid Res.* 2006.** 47: **2562–2574.**

Supplementary key words hypertriglyceridemia • diabetes • obesity • hepatic cytosolic pool • stable isotopes • mass spectrometry • very low density lipoprotein • nonesterified fatty acid

Hypertriglyceridemia (HPTG) is associated with diabetes (1), obesity (2), and coronary artery disease (3). Most studies have evaluated the relationship between fasting plasma triglyceride (TG) concentrations and coronary artery disease (4, 5). Other workers have demonstrated correlations between postprandial TG increases and coronary artery disease (5–7), however, consistent with the proposal (8) that atherogenesis is a postprandial phenomenon. Correlations between fasting plasma TG concentrations and the magnitude and duration of postprandial TG response have also been reported (9, 10). Couillard et al. (9), for example, found that fasting plasma TG, insulin, and HDL levels showed a strong correlation with postprandial triglyceride-rich lipoprotein (TRL) concentrations. High fasting TG and low HDL-cholesterol concentrations have also been associated with increased postprandial TRL levels (10, 11).

Although plasma NEFAs are considered to be the major source of VLDL-TG (12–14), dietary FAs carried in chylomicrons and FAs synthesized by hepatic de novo lipogenesis (DNL) can also affect VLDL-TG metabolism in a number of ways. FAs released from lipolysis of chylomicrons may escape uptake by peripheral tissues and enter the plasma NEFA pool (15–19). This increased substrate supply may then stimulate hepatic TG production. Persistence of TRL particles into the fasting state (20, 21) may continue to provide FAs for fasting VLDL-TG production. Also, remnants of TRL are taken up by a receptor-mediated mechanism in the liver and hydrolyzed in hepatic lysosomes, releasing FAs into the cytosol (22, 23). It is estimated that as much as 50% of dietary fat is taken up as remnant TG by the liver (24). The hepatic cytosolic TG storage pool may then contribute to VLDL-TG released during the fasted state, via a delayed secretory pathway (**Fig. 1**).

Manuscript received 15 July 2005 and in revised form 9 May 2006 and in re-revised form 21 August 2006.

Published, JLR Papers in Press, August 23, 2006.
DOI 10.1194/jlr.M600200-JLR200

Abbreviations: DNL, de novo lipogenesis; FAME, fatty acid methyl ester; GCRC, General Clinical Research Center; HPTG, hypertriglyceridemia, hypertriglyceridemic; MPE, molar percent excess; TG, triglyceride; TRL, triglyceride-rich lipoprotein.

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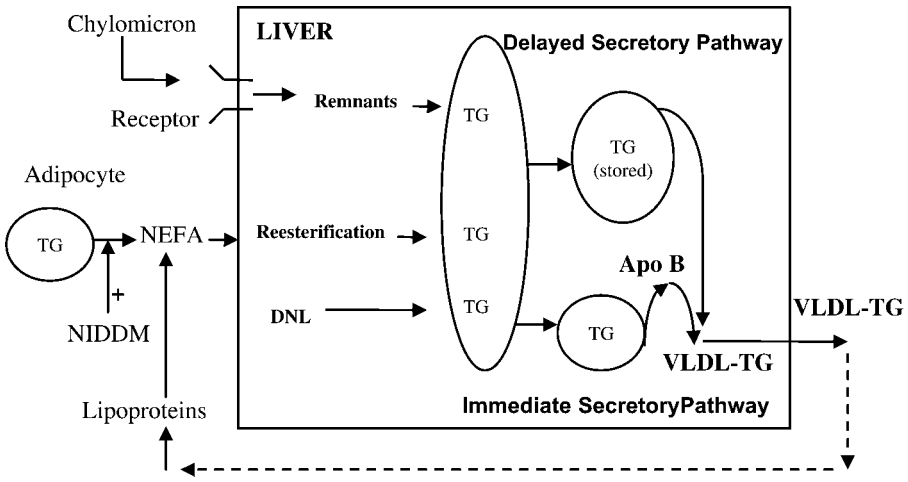

Fig. 1. Metabolic sources contributing FAs to VLDL-triglyceride (TG). The figure shows a simplified model of VLDL-TG synthesis and secretion in the liver. TG may be assembled from FAs derived from plasma NEFAs, chylomicron remnants, or de novo lipogenesis (DNL) pathways and then either secreted into the circulation as VLDL-TG, via the immediate secretory pathway, or stored in the cytosol and secreted after a delay, via the delayed secretory pathway. Dashed arrows indicate recycling of VLDL-TG FAs via the plasma NEFA pool into the liver. Apo B, apolipoprotein B; NIDDM, noninsulin-dependent diabetes mellitus.

Hepatic DNL is also known to contribute newly synthesized FAs to VLDL-TG in both fasting and postprandial states (25, 26). The literature on DNL contribution to VLDL-TG has focused on the "secretion-coupled" or immediate secretory pathway, rather than the delayed secretory pathway. The DNL contribution to fasting VLDL-TG is small (20, 25–27), typically providing at most 5–10% of palmitate to fasting VLDL-TG, with somewhat higher values in the fed state (26).

Conflicting results exist regarding the fate of FAs newly synthesized by DNL in the liver. Duerden and Gibbons (28) found that the TG FAs produced via DNL in cultured rat hepatocytes were partitioned preferentially to VLDL for secretion rather than into the cytosolic storage pool. Evidence for delayed input of DNL-derived FAs in humans comes from Hudgins et al. (29), who found that during a 39 h [1-^{13}C]acetate infusion, the measured DNL contribution was higher on the second morning than on the first. This progressive increase in contribution during a long labeling period suggests the filling of the delayed, or cytosolic, pool with labeled FAs (Fig. 1).

The most widely accepted pathogenic model (14, 30, 31) for fasting HPTG invokes the overproduction of VLDL-TG as a result of increased rates of lipolysis and FFA flux into the liver (32, 33) in the fasting state. This pathogenic model does not provide a mechanism, however, for linking postprandial metabolic events with the overproduction of TG in the fasting state. In this study, we asked whether the hepatic cytosolic TG storage pool could provide such a link between postprandial and fasting TG metabolism.

The existence of a hepatic cytosolic TG pool has been demonstrated in vivo and in vitro (34–36). Obese humans often exhibit higher levels of fat in the liver (37–39), correlating with insulin resistance (38), dyslipidemia (39, 40), and the metabolic syndrome (37). Metabolic studies of FA sources of VLDL-TG in obese and HPTG subjects have reported a substantial percentage of VLDL-TG FAs that are not accounted for by circulating (20, 40) or splanchnic (41–44) NEFAs. Additionally, a multicompartmental model of VLDL-TG transport in humans (45) suggested that VLDL-TG secretion is best explained by fast (immediate) and slow (delayed) pathways. In patients with clinically diagnosed hepatic steatosis, Donnelly et al. (46) measured metabolic sources of hepatic TG and fasting VLDL-TG, but the contributions from delayed versus immediate secretory pathways to fasting VLDL-TG were not assessed.

In this study, we tested the hypothesis that a delayed TG storage pool (34–36) makes an important contribution to fasting VLDL-TG in humans and contributes to fasting HPTG (Fig. 1). Contributions from NEFA, DNL, and dietary FAs to fasting VLDL-TG by the delayed pathway were measured, in addition to contributions from NEFAs by the direct pathway, by combining a prelabeling and a direct labeling stable isotope approach. Nondiabetic HPTG, diabetic HPTG, and normolipidemic subjects were compared.

METHODS

Human subjects

Subjects were recruited by advertisement and gave written informed consent before enrolling in the study. All study protocols received prior approval from the University of California at San Francisco and the University of California at Berkeley human ethical committees.

Subjects were recruited from three groups: *a*) controls (lean, normolipidemic); *b*) HPTG (obese, nondiabetic); and *c*) diabetic HPTG (obese with type 2 diabetes mellitus). The selection criteria were as follows: fasting TG concentration < 100 mg/dl for the control group and 150–300 mg/dl for the HPTG and diabetic HPTG groups; body mass index < 25 kg/m^2 for controls and 25–35 kg/m^2 for the HPTG and diabetic HPTG groups; and body fat $> 30\%$ for the HPTG and diabetic HPTG groups. Diabetic HPTG

subjects had fasting blood glucose concentrations of 140–250 mg/dl and hemoglobin A_1C of 7–10%.

Additional criteria in the control and HPTG groups included having no current or previous diagnosis of diabetes, no family history of diabetes in mother or father, no other significant medical conditions or use of medications, and no abnormalities on screening blood tests. The subjects in the diabetic HPTG group were being managed clinically on either insulin therapy or sulfonylurea hypoglycemic agents but not metformin or thiazolidinediones and were not taking any other medication known to alter lipid or carbohydrate metabolism, such as β-blockers, thiazide diuretics, glucocorticoids, hypolipidemic agents, phenytoin, androgens, or estrogens. Diabetic HPTG subjects had no other significant medical conditions or abnormalities on screening blood tests.

Body composition was measured by bioelectric impedance analysis (model 1990B; Valhalla Scientific, Inc., San Diego, CA) using the manufacturer's equations. Waist and hip circumferences were measured at the level of the umbilicus and the point of greatest circumference around the hip, respectively.

Study design

For 2 weeks before the metabolic study, subjects received weight-maintaining, controlled diets administered on an outpatient basis from the San Francisco General Hospital's General Clinical Research Center (GCRC) diet kitchen to avoid the effects of recent dietary differences between groups. Diets were calculated to be eucaloric and of identical macronutrient composition, consisting of 50% carbohydrate, 35% fat, and 15% protein. Each subject's daily dietary requirements were estimated by GCRC dieticians, based on the basal metabolic rate of each individual by the Harris-Benedict equation and dietary records. Diet was adjusted by up to 100 kcal/day to maintain constant body weight. Diets were composed of foods normally consumed by the subjects. All meals, beverages, and snacks were prepared by the staff at the metabolic diet kitchen of the GCRC. Each subject reported to the GCRC 3–5 days per week to be interviewed by the study staff, get weighed, and consume either dinner or breakfast. Food to be consumed until the next visit was packed and given to subjects for consumption. Subjects were instructed to consume all of the study food and not to consume any other food during this period. At the end of the 2 week weight maintenance period, subjects were admitted to the GCRC at noon on day 1 of the study. The metabolic infusion study was started at 6:00 PM (**Fig. 2**).

Metabolic infusion protocol

All labeled substrates were >98% enriched and were sterile and pyrogen-free. The metabolic study was started at 6:00 PM on day 1 (Fig. 2). Venous catheters were inserted into the antecubital vein in each arm for separate blood drawing and administration of infusates.

The decision to administer [$^2H_{31}$]palmitate and [1-$^{13}C_1$]acetate for the 30 h period before fasting was based on previous data from our laboratory (29) that demonstrated an increase in the fractional contribution from DNL to VLDL-TG between a 15 h (overnight) and a 39 h (two night) infusion of [1-$^{13}C_1$]acetate but no further increase from 39 to 63 h of [1-$^{13}C_1$]acetate infusion. The 30 h lead-in labeling period used here (Fig. 2) started at 6:00 PM on day 1 and continued to midnight on day 2. An intravenous infusion of [$^2H_{31}$]palmitate (0.6 mg/kg/h) and [1-$^{13}C_1$]acetate (7 mg/kg/h) was administered to label the hepatic TG storage pool and subsequently measure the contribution from NEFAs and DNL, respectively, via the delayed secretory pathway. Subjects were also administered oral [$^2H_{35}$]stearate (0.45 mg/kg/meal) mixed with meals to measure the contribution from chylomicron remnants to fasting VLDL-TG. Hardy-Schmidt and Kien (47) previously demonstrated that oral administration of lipid emulsion containing [^{13}C]palmitate with a meal results in the incorporation of [^{13}C]palmitate label into chylomicrons and VLDL-TG. During the label lead-in period, subjects consumed a total of six meals (four meals and two snacks) that were similar in composition to those consumed during the weight maintenance phase. Each breakfast, lunch, and dinner contained ~28% of total daily calories, and an evening snack contained 16% of total calories. Meal times were as follows: breakfast, 8:00 AM; lunch, 1:00 PM; dinner, 6:00 PM; and snack, 8:00 PM. Each meal was accompanied by a single dose of [$^2H_{35}$]stearate mixed with 25 g of unsweetened apple sauce. [$^2H_{35}$]stearate was mixed to account for 10% of the dietary stearate in each meal. Subjects were required to consume all food given to them during the inpatient period. During the infusion study, subjects in the diabetic HPTG group were managed clinically according to their existing medication regimen. No changes in regimen were required in any of the diabetic HPTG subjects.

After completion of the 30-h lead-in period, the infusion of [$^2H_{31}$]palmitate and [1-$^{13}C_1$]acetate was discontinued and subjects were infused with [1,2,3,4-$^{13}C_4$]palmitate (0.42 mg/kg/h) and [2-$^{13}C_1$]glycerol (18 mg/kg/h) overnight (midnight on day 2 to noon on day 3). The fractional contribution calculated from the infusion of [1,2,3,4-$^{13}C_4$]palmitate represents the direct, or immediate, secretory pathway contribution from NEFAs to VLDL-TG in the fasting state. During this 12 h follow-up period, subjects remained fasted and were only allowed noncaloric, noncaffeine beverages.

Frequent blood samples were drawn during the entire infusion period (Fig. 2). At 8:00–9:00 AM on the morning of day 3, indirect calorimetry was performed using a Deltatrac Metabolic

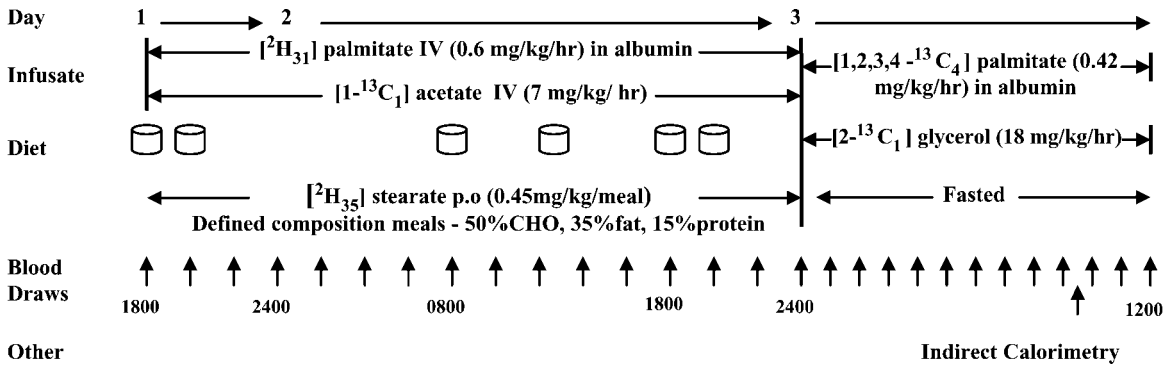

Fig. 2. Metabolic infusion protocol. The 30 h lead-in and the subsequent 12 h labeling periods are shown. CHO, carbohydrate.

Cart (Sensor Medix, Yorba Linda, CA) in the hooded mode. The infusion was stopped at noon on day 3, and subjects were discharged to home.

Metabolite isolation

All blood samples were drawn into iced Vacutainer tubes containing 1 mg/ml EDTA. Plasma was separated by centrifugation (1,500 *g*) at 4°C for 20 min and stored at −20°C until further analysis. For VLDL isolation, plasma samples were ultracentrifuged twice in a 50.3 Beckman rotor at 35,000 rpm for 30 min to separate and remove chylomicrons (1.6×10^6 *g* at 15°C) (20, 48). The remaining sample was used to isolate VLDL (d < 1.006 g/ml) by ultracentrifugation at 40,000 rpm for 17 h (1.3×10^8 *g* at 12°C) (47, 48). VLDL-TG was transesterified, derivatized to fatty acid methyl esters (FAMEs), and reconstituted in heptane for GC-MS analysis (model 5971 mass spectrometer with a model 5890 gas chromatograph and autosampler; Hewlett-Packard Co., Palo Alto, CA). The remaining aqueous fraction, after FAME extraction, containing VLDL-TG glycerol was evaporated to dryness under nitrogen and derivatized with pyridine/acetic anhydride to glycerol triacetate, then reconstituted in ethyl acetate.

Plasma NEFAs were separated (20, 48) from cholesterol and phospholipids by silica gel G thin-layer chromatography (Analtech, Newark, DE) and derivatized to FAMEs (48). Plasma free glycerol was isolated from 350 μl of deproteinized plasma by ion-exchange chromatography. Glycerol triacetate from plasma was derivatized by reaction with acetic anhydride-pyridine (1:1) at 60°C and reconstituted in ethyl acetate (48).

Additionally, TG and FFA in sample meals (made as pairs to the meal consumed) were extracted by Folch solution (chloroform-methanol, 2:1) containing 100 nmol of pentadecanoic acid as an internal standard, at a 1:10 ratio, followed by TLC separation. TG and FFA fractions were then combined and transesterified to FAME for GC-MS analysis to confirm the palmitate enrichment of the food given. Enrichments were within ±0.75 molar percent excess (MPE) of the intended value (10 MPE).

Serum lipids and insulin concentrations were measured by standard methods at the Clinical Laboratories of San Francisco General Hospital. Plasma TG concentrations were analyzed at Unilab (San Jose, CA).

Mass spectrometric analyses

All GC-MS analyses were done in the selected ion monitoring mode. Palmitate and stearate-methyl ester were analyzed by GC-MS using a 25 m DBI fused silica column (48) under electron-impact ionization. Under the major palmitate-methyl ester GC peak, *m/z* 270–274 and *m/z* 301 were monitored to calculate the incorporation of [1,2,3,4-$^{13}C_4$]palmitate and [$^2H_{31}$]palmitate, respectively. The [$^2H_{31}$]palmitate peak exhibited a shift in GC retention time relative to natural abundance palmitate-methyl ester and had to be monitored accordingly. Abundance-matched standard curves of [1,2,3,4-$^{13}C_4$]palmitate and [$^2H_{31}$]palmitate were run concurrently to quantify true isotopic enrichment. Under the major stearate-methyl ester GC peak, *m/z* 298 and 333 were monitored to calculate the incorporation of [$^2H_{35}$]stearate. The shift in GC retention time for [$^2H_{35}$]stearate was taken into account. Abundance-matched standard curves of [$^2H_{35}$]stearate were run concurrently to quantify true isotopic enrichment. Concentrations of FAs in plasma samples were determined simultaneously while measuring isotopic enrichments, using a splitter that diverted a portion of GC effluent to a flame-ionization detector, with comparison to pentadecanoic acid as an internal standard (48). Glycerol triacetate was transesterified to remove NEFA then the aqueous fraction, then derivatized to glycerol triacetate for analysis with a 10 m DB-225 column (chemical ionization mode, using methane). The enrichment was determined by monitoring *m/z* 159 and 160, representing parent mass and mass +1 (M_0 and M_1), with comparison to a concurrently run standard curve of [2-$^{13}C_1$]glycerol (48).

Calculations

The contributions from plasma NEFAs to VLDL-TG were calculated based on the precursor-product relationship. Plasma NEFA contribution via the immediate secretory pathway to VLDL-TG in the fasting state was calculated as (23):

$$\frac{[1,2,3,4{-}^{13}C_4]\text{palmitate enrichment in VLDL-TG (at isotopic plateau)}}{[1,2,3,4{-}^{13}C_4]\text{palmitate in the plasma NEFA pool (average value)}} \quad \textit{(Eq. 1)}$$

The incorporation of [1,2,3,4-$^{13}C_4$]palmitate in VLDL-TG reached or approached a plateau by the 10th h of study. Accordingly, the average enrichment over the last three time points, between 10:00 AM and 12:00 noon on day 3, was used for calculation of the fractional contribution to VLDL-TG.

Plasma and VLDL [$^2H_{31}$]palmitate enrichments were also used to calculate the contribution from plasma NEFAs to fasting VLDL-TG via the delayed secretory pathway. The calculation was as follows:

$$\frac{[^2H_{31}]\text{palmitate enrichment in VLDL-TG (last three time points)}}{[^2H_{31}]\text{palmitate enrichment in plasma (30 h average)}} \quad \textit{(Eq. 2)}$$

A correction for recycling of FAs through the plasma NEFA pool during fasting was included in the calculations. The basis of this correction is as follows. It is possible for some of the labeled FAs administered during the prelabeling period (by intravenous or dietary routes) to enter fasting VLDL-TG via recycling through the plasma NEFA pool during fasting. This could occur by hydrolysis of labeled hepatic TG or VLDL-TG during the fasting period with release of FAs into the plasma and incorporation into VLDL-TG via the immediate secretory pathway (Fig. 1). Indeed, plasma NEFA enrichments of [$^2H_{31}$]palmitate and [$^2H_{35}$]stearate were measurable during the fasting period (see below). Label incorporation by this route is properly classified as immediate pathway input. To correct for recycling through the plasma NEFA pool during fasting, a correction was made to the delayed pathway from [$^2H_{31}$]palmitate, by subtracting the input from fasting plasma NEFA:

$$\frac{\text{VLDL-TG } [^2H_{31}]\text{palmitate enrichment} - \text{VLDL-TG } [^2H_{31}]\text{palmitate enrichment derived from fasting plasma NEFA}}{[^2H_{31}]\text{palmitate enrichment in plasma (30 h average)}} \quad \textit{(Eq. 3)}$$

The VLDL-TG [$^2H_{31}$]palmitate enrichment derived from fasting plasma NEFA in equation 3 was calculated based on the incorporation ratios observed for [1,2,3,4-$^{13}C_4$]palmitate (equation 1). This is illustrated in the following example: assume that VLDL-TG [$^2H_{31}$]palmitate enrichment (last three time points) was 1.13 MPE; residual plasma [$^2H_{31}$]palmitate enrichment (after stopping the 30 h infusion) was 0.55 MPE; average plasma [$^2H_{31}$]palmitate enrichment during the lead-in period was 3.19 MPE; and the fractional contribution from NEFA to VLDL-TG by the immediate secretory pathway was 54% (calculated from

[1,2,3,4-$^{13}C_4$]palmitate incorporation). The calculated contribution of label from the recycling of VLDL-derived [$^2H_{31}$]palmitate through plasma NEFA is then 0.55 MPE × 0.54 = 0.30 MPE, and the corrected delayed pathway contribution = (1.13 − 0.30)/3.19 = 26.1%.

The fractional contribution from dietary FAs to VLDL-TG during the postprandial period via the immediate secretory pathway was calculated by dividing the enrichment of [$^2H_{35}$]stearate in VLDL-TG by the [$^2H_{35}$]stearate enrichment in each meal (10%). [$^2H_{35}$]stearate enrichments at the last three time points of the follow-up study period (10:00 AM to 12:00 noon on day 3) were averaged and calculated (with correction for recycling of FAs through NEFA) as follows:

$$\frac{\text{VLDL-TG [}^2H_{35}\text{]stearate enrichment} - \text{VLDL-TG enrichment derived from recycling through fasting plasma NEFA}}{10\%} \quad \textit{(Eq. 4)}$$

where the correction for VLDL-TG enrichment derived from fasting plasma [$^2H_{35}$]stearate (i.e., immediate pathway input) was calculated from measured fasting plasma [$^2H_{35}$]stearate enrichments and the fractional incorporation from [1,2,3,4-$^{13}C_4$]palmitate, as described above for equation 3.

Mass isotopomer distribution analysis was used to calculate the fractional contribution from DNL to fasting VLDL-TG palmitate and the isotopic enrichment of the true precursor pool, hepatic acetyl-CoA (49, 50). Based on the principle of combinatorial probabilities, the enrichment of acetyl-CoA was inferred from the ratio of excess double-labeled to single-labeled species (EM_2/EM_1). The precursor-product relationship was then applied to calculate the fractional contribution from DNL to VLDL-TG FAs during fasting as well as in postprandial periods. The average of the last three time points on the morning of day 3 was used to calculate the fractional contribution from DNL to fasting VLDL-TG via the delayed secretory pathway.

The VLDL-TG synthetic rate in the fasting state was calculated from the incorporation curves of [1,2,3,4-$^{13}C_4$]palmitate and [2-$^{13}C_1$]glycerol into VLDL-TG. Fractional replacement rates (k_s) were calculated by modeling the increase toward plateau enrichment of [1,2,3,4-$^{13}C_4$]palmitate and [2-$^{13}C_1$]glycerol in VLDL-TG. The data were fit to the equation $y = A_\infty \times [1 - e^{-ks\ (t-c)}]$, where y = VLDL-TG enrichment, A_∞ = the plateau value of VLDL-TG enrichment, t = time in hours, and c = lag period before isotope incorporation into secreted VLDL-TG. The half-life of VLDL in plasma, $t_{1/2}$ (h), was calculated by dividing 0.693 by k_s (51). Absolute VLDL synthesis rate (g/h) was then calculated as:

$$k_s\,(h^{-1}) \times \text{TG concentration (g/dl)} \times \text{estimated plasma volume (dl)} \quad \textit{(Eq. 5)}$$

Plasma volume was estimated to be 5% of the lean body weight.

Absolute contributions (g/h) from NEFAs, diet, and DNL-derived FAs to VLDL-TG were calculated as follows:

$$\text{fractional contribution} \times \text{VLDL-TG synthesis rate (g/h)} \quad \textit{(Eq. 6)}$$

Nonprotein respiratory quotient, energy expenditure, and whole body oxidation of fat and carbohydrate were calculated by indirect calorimetry according to standard equations (52).

Statistical analysis

All data are expressed as means ± SEM. The statistical significance of differences was determined by Kruskal-Wallis one-way ANOVA, and $P < 0.05$ was defined to be statistically significant. If statistically significant, these results were followed up using Bonferroni techniques at a 5% procedure-wise error rate (10% for insulin) in conjunction with the Mann-Whitney *U*-Wilcoxon test. SPSS for Unix, release 6.14 (Sunos), was used for the analysis.

RESULTS

Subject characteristics and lipid profiles

Subject characteristics and metabolic profiles of the three groups are shown in **Table 1**. Control subjects were younger than the HPTG and diabetic HPTG subjects, as we had difficulty recruiting normal control subjects in the 50 year old range. Diabetic HPTG subjects were more obese than the HPTG subjects. Lipid profile was assessed from the samples collected at baseline on day 1 of the metabolic infusion study. TG and VLDL cholesterol concentrations were significantly higher in the HPTG and diabetic HPTG groups compared with the control group. HPTG and diabetic HPTG subjects had significantly higher fasting insulin concentrations compared with the control group ($P < 0.033$). TG concentrations followed a

TABLE 1. Subject characteristics and lipid profiles for the three study groups

Characteristic	Control (means ± SEM)	HPTG (means ± SEM)	DM HPTG (means ± SEM)	*P* (control vs. HPTG)	*P* (HPTG vs. DM HPTG)	*P* (control vs. DM HPTG)
Number (men/women)	6 (4/2)	6 (6/0)	6 (4/2)			
Age (years)	27.2 ± 1.3	49.2 ± 4.2	52.2 ± 5.5	0.0061	0.3272	0.0097
Body weight (kg)	73.9 ± 2.6	93 ± 3.2	102.8 ± 8.7	0.0062	0.3367	0.0176
Fat-free mass (kg)	57.4 ± 3.3	60.4 ± 1.7	70.0 ± 5.7	0.1441	0.1093	0.1003
Fat mass (kg)	16.4 ± 3.3	32.7 ± 2.9	32.8 ± 4.1	0.0062	1	0.0102
Body mass index (kg/m^2)	24.0 ± 1.1	31.1 ± 1.2	35.2 ± 1.8	0.0062	0.0542	0.0061
Waist-to-hip ratio	0.83 ± 0.02	0.96 ± 0.02	0.97 ± 0.02	0.0192	0.5751	0.0119
TG (mg/dl)	61.4 ± 4.1	179.7 ± 16.2	190 ± 22.6	0.0062	1	0.0062
Total cholesterol (mg/dl)	121.2 ± 6.1	147.2 ± 9.5	121.5 ± 12	0.0679	0.1495	1
LDL cholesterol (mg/dl)	72.4 ± 6.0	86.8 ± 7.1	62.2 ± 11.6	0.1745	0.0988	0.3613
VLDL cholesterol (mg/dl)	12.4 ± 0.9	35.2 ± 3.5	38.0 ± 4.5	0.0088	0.8551	0.0061
HDL cholesterol (mg/dl)	36.4 ± 2.8	23.4 ± 1.5	21.3 ± 2.3	0.0117	0.2300	0.0102
HDL/total ratio	0.30 ± 0.03	0.16 ± 0.03 (n = 5)	0.18 ± 0.02	0.0059	0.4184	0.0174
Insulin (μU/ml)	1.83 ± 1.06 (n = 3)	6.15 ± 0.60	20.8 ± 9.07	0.0196	0.0301	0.0201

DM, diabetic; HPTG, hypertriglyceridemic; TG, triglyceride. If data were unavailable for some subjects within a group, n is noted in parentheses.

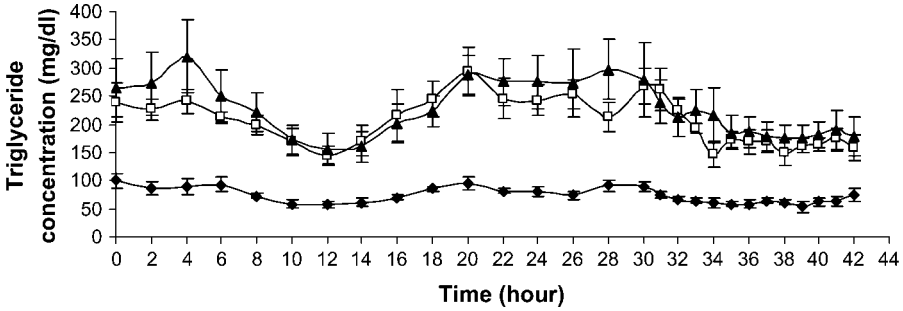

Fig. 3. Plasma TG concentrations during the study period. Black diamonds, control group; white squares, hypertriglyceridemic (HPTG) group; black triangles, diabetic HPTG group. Data shown are means ± SEM.

circadian rhythm in all three groups (**Fig. 3**), with a decrease during fasting followed by an increase during the postprandial period. The TG concentrations were similar in the HPTG and diabetic HPTG groups and were constantly higher than in the control group throughout the study. The TG concentrations at nadir were 58 ± 6, 144 ± 18, and 156 ± 27 mg/dl in the control, HPTG, and diabetic HPTG groups, respectively, whereas the observed peak values were 96 ± 12, 293 ± 44, and 289 ± 34 mg/dl in the control, HPTG, and diabetic HPTG groups, respectively.

VLDL-TG synthesis

The rate of synthesis of VLDL-TG was calculated by kinetic modeling of incorporation curves of [2-$^{13}C_1$]glycerol (**Table 2**). VLDL-TG synthesis rates were 0.38 ± 0.03, 1.28 ± 0.20, and 1.04 ± 0.23 g/h in the control, HPTG, and diabetic HPTG groups, respectively. The rates of synthesis were significantly higher in the HPTG and diabetic HPTG groups compared with the control group ($P < 0.011$). VLDL-TG half-life was nonsignificantly longer in the diabetic HPTG group compared with the control and HPTG groups (Table 2). The rate of appearance of glycerol in the fasting state was nonsignificantly higher in the diabetic HPTG group than in the control and HPTG groups (average values were 1.3 ± 0.2, 1.8 ± 0.1, and 3.0 ± 0.6 μmol/kg/min in the control, HPTG, and diabetic HPTG groups, respectively) ($P > 0.055$).

Immediate pathway contribution from NEFAs to VLDL-TG

The fractional contribution from NEFAs to VLDL-TG via the immediate pathway was calculated from the precursor-product relationship. The [1,2,3,4-$^{13}C_4$]palmitate enrichment in plasma NEFAs reached a plateau by 2 h of infusion and leveled off thereafter. The [1,2,3,4-$^{13}C_4$]palmitate enrichment time course in VLDL-TG is shown in **Fig. 4**. The fractional contribution from NEFAs to fasting VLDL-TG via the immediate secretory pathway was 64 ± 15%, 33 ± 6%, and 58 ± 2% in the control, HPTG, and diabetic HPTG groups, respectively (Table 2). The fractional contribution was significantly lower in the HPTG group compared with the diabetic HPTG group. There was no significant difference between the three groups in absolute contribution from NEFA to VLDL-TG by the immediate secretory pathway (**Table 3**). The absolute contribution rates averaged 0.23 ± 0.10, 0.44 ± 0.12, and 0.61 ± 0.14 g/h in the control, HPTG, and diabetic HPTG groups, respectively.

TABLE 2. Fractional contributions to fasting VLDL-TG from plasma NEFAs, diet, and DNL-derived FAs, and VLDL-TG kinetics

Variable	Control (means ± SEM)	HPTG (means ± SEM)	DM HPTG (means ± SEM)	*P* (control vs. HPTG)	*P* (HPTG vs. DM HPTG)	*P* (control vs. DM HPTG)
VLDL-TG kinetics						
Synthesis (g/day)	9.2 ± 0.8 (n = 4)	30.6 ± 4.8	25.0 ± 5.5	0.0105	0.3367	0.0105
Half-life (h)	3.1 ± 0.2 (n = 4)	3.3 ± 0.7	4.8 ± 0.6	0.0881	0.1495	0.0330
k_s (h^{-1})	0.23 ± 0.02 (n = 4)	0.24 ± 0.04	0.16 ± 0.02	0.0881	0.1495	0.0330
Ra glycerol (μmol/kg/min)	1.3 ± 0.2 (n = 4)	1.8 ± 0.1	3.0 ± 0.6	0.0543	0.0542	0.0550
Fractional contributions (%)						
Immediate						
Plasma NEFA	64 ± 15	33 ± 6	58 ± 2	0.1003	0.0039	0.583
Delayed						
Plasma NEFA	15 ± 4	20 ± 4	30 ± 3	0.3591	0.0367	0.0277
DNL	3 ± 0.3	14 ± 3.0	13 ± 4.0	0.0105	0.631	0.055
Diet	2 ± 0.9	2.5 ± 1.0	12 ± 2.0	0.9256	0.0061	0.0062
Total	83 ± 19	68 ± 11	113 ± 4			
Unaccounted	17 ± 19	32 ± 11	<0	0.5822	0.0047	0.0907

If data were unavailable for some subjects within a group, n is noted in parentheses. VLDL-TG kinetics were calculated from [^{13}C]glycerol incorporation curves. DM, diabetic; DNL, de novo lipogenesis; k_s, fractional replacement rate constant; Ra glycerol, rate of appearance of glycerol.

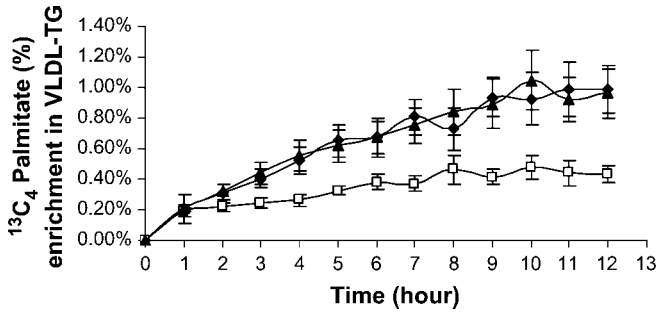

Fig. 4. [1,2,3,4-$^{13}C_4$]palmitate incorporation into VLDL-TG during the 12 h follow-up fasting period. Black diamonds, control group; white squares, HPTG group; black triangles, diabetic HPTG group. Data shown are means ± SEM.

Delayed pathway contribution from NEFAs to VLDL-TG

[$^2H_{31}$]palmitate enrichment in the plasma NEFA pool reached a plateau within 2–3 h of infusion and leveled off during the 30 h lead-in period (**Fig. 5**). Within 1 h of stopping the infusion at midnight on day 2, plasma enrichment decreased to near zero levels in all three groups. [$^2H_{31}$]palmitate enrichment in VLDL-TG increased progressively until 14 h (8:00 AM) on day 2 and stayed at near plateau levels for the rest of the labeling period in the control and HPTG groups (**Fig. 6**). In the diabetic HPTG group, there was a further increase until 24 h (6:00 PM) on day 2. After the infusion was stopped, the VLDL-TG palmitate enrichment decreased gradually in all three groups and reached stable values by the final 2–3 h of the fasting period. It is possible that some of the labeled FAs incorporated into VLDL-TG during the 30 h lead-in period were released into the plasma NEFA pool by lipolysis of circulating TG or hepatic TG stores, then reincorporated into VLDL-TG during the subsequent fasting period via the immediate secretory pathway (Fig. 1). Accordingly, this input was corrected for (see Methods).

At the end of the fasting period, the fractional corrected contribution from NEFAs to VLDL-TG via the delayed secretory pathway was nonsignificantly higher in the diabetic HPTG group compared with the control and HPTG groups, with average values of 15 ± 4%, 20 ± 4%, and 30 ± 3% in the control, HPTG, and diabetic HPTG groups, respectively (Table 2). Absolute contributions from NEFAs to VLDL-TG via the delayed secretory pathway were 1.2 ± 0.5, 6.7 ± 2.5, and 7.0 ± 1.1 g/day in the control, HPTG, and diabetic HPTG groups, respectively (Table 3) ($P <$ 0.05 for the control vs. HPTG and diabetic HPTG groups).

Contribution from dietary FAs to VLDL-TG in the fasting state

[$^2H_{35}$]stearate enrichments in plasma NEFAs and VLDL-TG are shown in **Figs. 7** and **8**, respectively. At all time points, [$^2H_{35}$]stearate enrichments in NEFAs and VLDL-TG were higher in the diabetic HPTG group. Because dietary fat absorption and chylomicron clearance should be long completed by 14–16 h after the last snack at 8:00 PM on day 2, the persistent increase of plasma [$^2H_{35}$]stearate enrichment through noon of the next day, during the follow-up period, implies recycling of FFAs released into the plasma from lipolysis of circulating VLDL-TG or stored hepatic TG (for which a correction was made; see Methods).

The corrected fractional contributions from dietary FAs to fasting VLDL-TG via the delayed secretory pathway were 2.0 ± 0.9%, 2.5 ± 1%, and 12 ± 2% in the control, HPTG, and diabetic HPTG groups, respectively (Table 2). The absolute contribution from dietary FAs to VLDL-TG in the fasting period via the delayed secretory pathway was estimated to be 0.15 ± 0.1, 1 ± 0.6, and 3.5 ± 1.50 g/day, respectively (Table 3). The absolute contribution was higher in the diabetic HPTG group compared with the control group and the HPTG group ($P < 0.01$).

Contribution from dietary FAs to VLDL-TG in the postprandial state

To estimate the contribution from dietary FAs to VLDL-TG in the postprandial state, we averaged the enrichments of [$^2H_{35}$]stearate in VLDL-TG at 2 and 4 h after ingestion of the last meal on days 1 and 2 to represent the postprandial state (Fig. 8). The fractional contributions from dietary FAs to VLDL-TG during the postprandial period were 4, 2, and 7% (day 1) and 4, 4, and 21% (day 2) in the control, HPTG, and diabetic HPTG groups, respectively (with the diabetic HPTG group significantly different from the control and HPTG groups; $P < 0.05$).

Contribution from dietary FAs to the NEFA pool

During the postprandial period (see above), averaged [$^2H_{35}$]stearate enrichments in plasma during the evening of day 1 were 0.67 ± 0.21%, 1.05 ± 0.32%, and 1.69 ± 0.39% in the control, HPTG, and diabetic HPTG groups,

TABLE 3. Absolute contributions to fasting VLDL-TG from plasma NEFAs, diet, and DNL-derived FAs

Variable	Control (means ± SEM)	HPTG (means ± SEM)	DM HPTG (means ± SEM)	*P* (control vs. HPTG)	*P* (HPTG vs. DM HPTG)	*P* (control vs. DM HPTG)
Immediate						
Plasma NEFA	5.5 ± 2.3	10.6 ± 2.9	14.7 ± 3.4			
Delayed						
Plasma NEFA	1.2 ± 0.5	6.7 ± 2.5	7.0 ± 1.1	0.0176	0.4233	0.0062
DNL	0.27 ± 0.03	4.5 ± 1.2	2.8 ± 0.8	0.0105	0.3367	0.0105
Diet	0.15 ± 0.1	1 ± 0.6	3.5 ± 1.5	0.7414	0.053	0.0105
Total measured	7.1 ± 2.8	22.8 ± 6.7	28.0 ± 6.3	0.192	1.000	0.053
Unaccounted	1.5 ± 1.1	7.8 ± 2.4	<0	0.0281	0.0048	0.0907

Absolute contribution values shown are g/day.

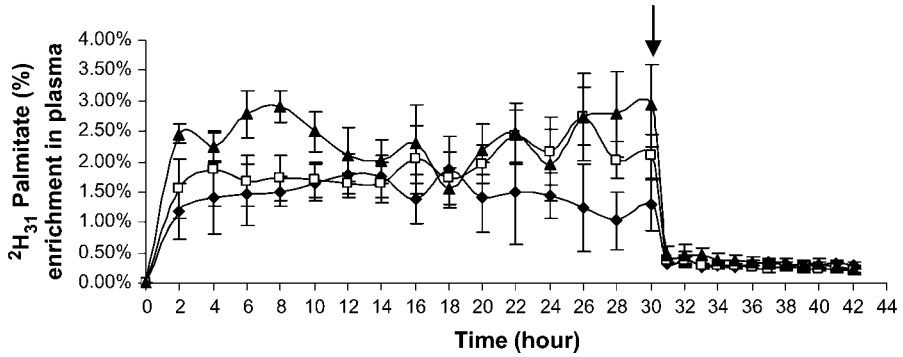

Fig. 5. [$^2H_{31}$]palmitate enrichment (%) in the plasma NEFA pool during the 30 h lead-in and 12 h follow-up periods. The arrow indicates the end of the lead-in period on midnight of day 2. Black diamonds, control group; white squares, HPTG group; black triangles, diabetic HPTG group. Data shown are means ± SEM.

respectively. [$^2H_{35}$]stearate enrichments in plasma increased to 0.93 ± 0.48%, 1.78 ± 0.40%, and 2.12 ± 0.55% in the control, HPTG, and diabetic HPTG groups, respectively, on day 2. These enrichment values represent the entry of a fraction of [$^2H_{35}$]stearate from the diet (10% enrichment) into the NEFA pool. Thus, during the postprandial period, ~7, 10, and 17% on day 1 and 9, 18, and 21% on day 2 of the FAs in plasma NEFAs were derived from recent diet in the control, HPTG, and diabetic HPTG groups, respectively (with the control group significantly different from both the HPTG and diabetic HPTG groups; $P < 0.05$).

Contribution from DNL to VLDL-TG in the fasting state

The percentage of VLDL-TG FAs derived from DNL during the12 h follow-up period is shown in **Fig. 9**. The fractional contributions from DNL to fasting VLDL-TG via the delayed secretory pathway were 3 ± 0.3%, 14 ± 3%, and 13 ± 4% in the control, HPTG, and diabetic HPTG groups, respectively (Table 2). Estimated absolute contributions were 0.3 ± 0.03, 4.5 ± 1.2, and 2.8 ± 0.8 g/day (Table 3). The absolute contribution was significantly higher in the HPTG and diabetic HPTG groups compared with the control group ($P < 0.011$). The fractional contribution from DNL was highly variable in the diabetic HPTG group, although consistent within each individual, and ranged from 3% to 25% (**Fig. 10**). It was not possible to identify any clinical or metabolic variables that correlated with the differences in lipogenesis among the diabetic HPTG subjects.

Contribution from DNL to VLDL-TG in the postprandial state

The percentage of newly synthesized FAs by DNL during the postprandial period is shown in Fig. 9. Midnight on day 1 and 2 represent two consecutive postprandial periods. The peak values of fractional DNL, at midnight on day 1, were 6 ± 2.1%, 9 ± 1.2%, and 8 ± 2.2% in the control, HPTG, and diabetic HPTG groups, respectively. In the control group, after the peak at midnight on day 1, DNL decreased gradually during the fasting period until the consumption of breakfast on day 2. During the daytime, DNL then increased somewhat but stayed at a relatively constant value, only to increase once again during the evening of day 2. In the HPTG group, DNL followed the same pattern as in the control group until after breakfast, followed by a steady increase until midnight on day 2. In the diabetic HPTG group, however, DNL increased progressively from the start of the study to a peak at midnight on day 2. The peak fractional contributions at midnight on day 2 increased to 7 ± 2.9%, 20 ± 1.9%, and 20 ± 4.9% in the control, HPTG, and diabetic HPTG groups,

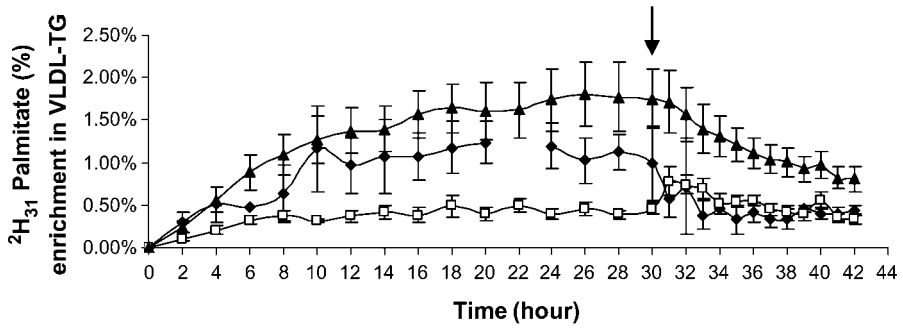

Fig. 6. [$^2H_{31}$]palmitate incorporation into VLDL-TG during the 30 h lead-in and 12 h follow-up fasting periods. The arrow indicates the end of the lead-in period on midnight of day 2. Black diamonds, control group; white squares, HPTG group; black triangles, diabetic HPTG group. Data shown are means ± SEM.

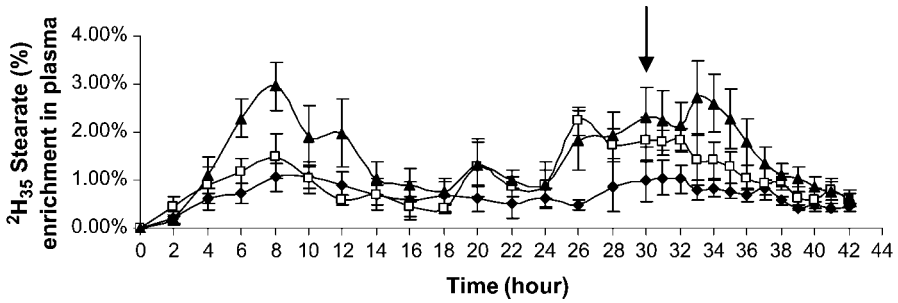

Fig. 7. [$^2H_{35}$]stearate enrichment (%) in the plasma NEFA pool during the 30 h lead-in and 12 h follow-up periods. The arrow indicates the end of the lead-in period at midnight of day 2. Black diamonds, control group; white squares, HPTG group; black triangles, diabetic HPTG group. Data shown are means ± SEM.

respectively. The increase in fractional DNL from midnight on day 1 to day 2 was significant in the HPTG and diabetic HPTG groups ($P < 0.05$). Fractional DNL appeared to have reached a plateau value by midnight on day 2 in all three groups. Fractional DNL was highly variable in the diabetic HPTG group, with the peak values ranging from 3% to 17% and 5% to 37% on days 1 and 2, respectively (Fig. 10).

Energy expenditure

There was nonsignificantly higher resting energy expenditure in the HPTG and diabetic HPTG groups compared with the control group. Resting energy expenditure was 1.31 ± 0.08, 1.34 ± 0.05, and 1.45 ± 0.14 kcal/min in the control, HPTG, and diabetic HPTG groups, respectively. Nonprotein respiratory quotients were 0.80 ± 0.01, 0.82 ± 0.02, and 0.74 ± 0.09, respectively.

Fat and glucose oxidation did not differ significantly in the three groups. Fat oxidation was 0.080 ± 0.01, 0.074 ± 0.01, and 0.13 ± 0.06 g/min, respectively. Glucose oxidation was 0.097 ± 0.01, 0.12 ± 0.02, and 0.10 ± 0.05 g/min, respectively.

DISCUSSION

We present here kinetic evidence confirming a significant contribution from a delayed secretory pathway (or hepatic cytosolic TG storage pool) to fasting VLDG-TG. The relative and absolute inputs from plasma NEFAs, diet, and DNL-derived FAs to fasting VLDL-TG from this delayed pathway were quantified in normolipidemic controls and in HPTG subjects with or without type 2 diabetes.

Earlier studies have provided indirect estimates of the contribution from the hepatic cytosolic pool, ranging from 0% to 50%, based on the difference from the direct NEFA contribution in the fasted state (20, 42, 44). Also, Diraison and Beylot (44) showed a higher rate of VLDL-TG secretion than plasma NEFA esterification in the postabsorptive state, implying that the remaining FAs must have originated from the hepatic TG storage pool. Studies in cultured hepatocytes have revealed storage and secretory pathways for newly formed TG (53–55). Isotopic studies have confirmed TG storage from NEFAs in the livers of human subjects (46, 56). Direct demonstration of a delayed input of labeled NEFAs into fasting VLDL-TG had not previously been available, however.

The contribution from the delayed pathway (TG storage pool) to fasting VLDL-TG was measured here by prelabeling the TG storage pool for 30 h. Overall, plasma NEFAs contributed 15–20% of VLDL-TG FAs in the control and nondiabetic HPTG groups via the delayed pathway, whereas the fractional contribution was higher in the diabetic HPTG group. In contrast, the fractional contribution from NEFAs by the immediate secretory pathway was 58–64% in the diabetic HPTG and control groups, respectively,

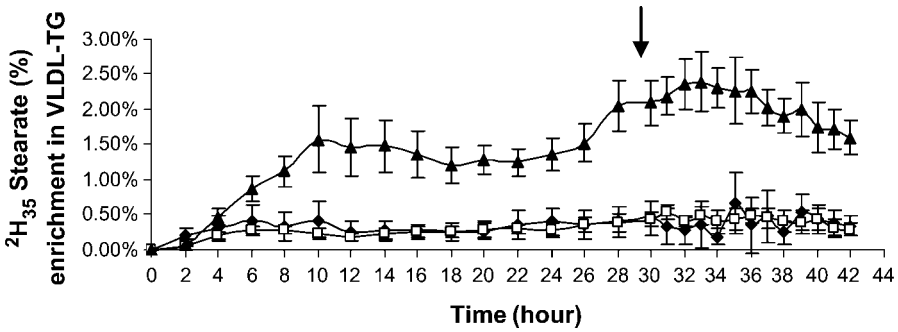

Fig. 8. [$^2H_{35}$]stearate incorporation into VLDL-TG during the 30 h lead-in and 12 h follow-up fasting periods. The arrow indicates the end of the lead-in period at midnight of day 2. Black diamonds, control group; white squares, HPTG group; black triangles, diabetic HPTG group. Data shown are means ± SEM.

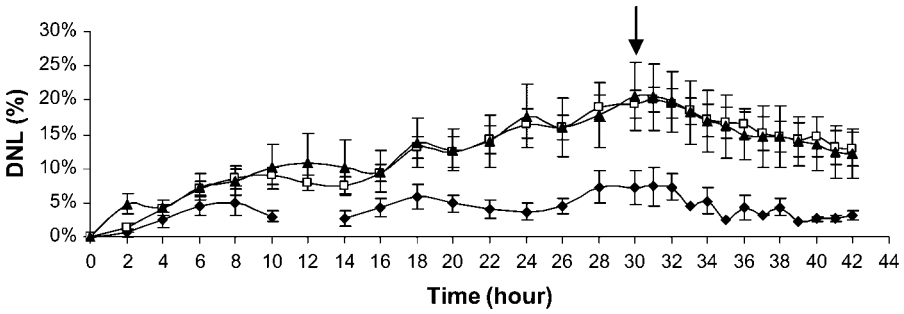

Fig. 9. Fractional DNL during the 30 h lead-in and 12 h follow-up fasting periods. The arrow indicates the end of the lead-in period at midnight of day 2. Black diamonds, control group; white squares, HPTG group; black triangles, diabetic HPTG group. Data shown are means ± SEM.

higher than the contribution in the nondiabetic HPTG group (33%). These results demonstrate differences in the pathways by which plasma NEFAs enter VLDL-TG not only between HPTG and normolipidemic subjects but between diabetic and nondiabetic HPTG subjects as well.

In humans, acute increases of plasma NEFAs stimulate VLDL production (14). In our study, NEFAs contributed 15–30% of the VLDL-TG FAs via the delayed secretory pathway and 33–64% via the immediate secretory pathway. Accordingly, plasma NEFA was a major contributor of FAs to fasting VLDL-TG, particularly when the delayed pathway input was included, in all of the groups that we studied. As it is possible that there is dilution of labeled NEFAs by FAs in the portal circulation, our estimation of the NEFA contribution is, if anything, an underestimation of the true contribution to VLDL-TG.

The measured contribution from NEFAs to fasting VLDL-TG via the immediate secretory pathway was lower in the nondiabetic HPTG group than in the control and diabetic HPTG groups. It has previously been shown that the contribution from plasma NEFAs to VLDL-TG is lower in HPTG than in normotriglyceridemic subjects (20). Barter and Nestel (42) also reported that a substantial percentage of VLDL-TG was not derived from plasma NEFAs in obese subjects and that increased body mass index correlated with a lower contribution from NEFAs to VLDL-TG. Visceral (portal) input of NEFAs is diluting and is not measured as a systemic plasma NEFA contribution but appears as an "unaccounted" input in our protocol. Accordingly, these observations are consistent with a larger contribution from either the delayed pathway (the TG storage pool) or visceral fat-derived (portal vein) NEFAs, or both, in obese HPTG subjects.

The diabetic HPTG group exhibited the highest fractional contribution from NEFAs to VLDL-TG via the delayed pathway, and the contribution via the immediate pathway was also higher than in the nondiabetic HPTG group. The explanation for significantly different fractional contributions through both the immediate and delayed secretory pathways from NEFAs between the HPTG and diabetic HPTG groups (Table 2) is not certain. Whole body oxidation rates of fats were not significantly different, so they cannot account for the differences in the fractional NEFA contribution to stored TG. The most likely unifying explanation is a difference in the turnover time of the delayed (cytosolic) TG pool, with more rapid release of stored TG from the liver in diabetic HPTG subjects. There was no unaccounted FA in the diabetic HPTG group, whereas 32% of FAs in fasting VLDL-TG remained unlabeled from all sources (i.e., were unaccounted for) in the HPTG group. Interestingly, Donnelly et al. (46) reported that >60% of hepatic TG remained unlabeled from all sources even after 4 days of labeling in nondiabetic HPTG subjects with fatty liver. Although we did not characterize

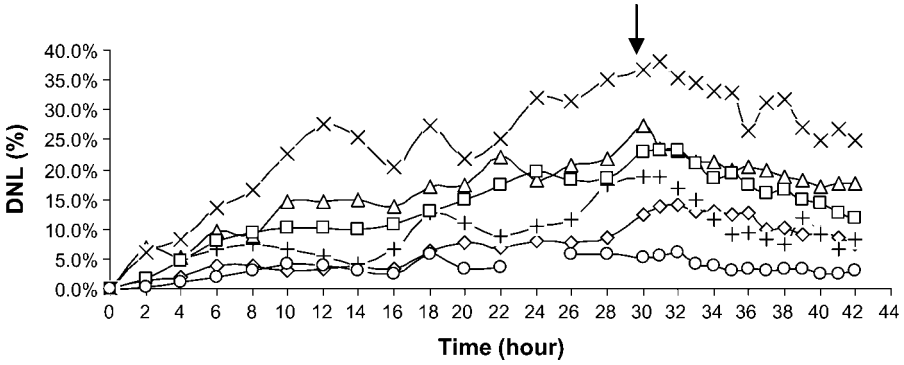

Fig. 10. Fractional DNL in six study subjects in the diabetic HPTG group during the 30 h lead-in and 12 h follow-up fasting periods. The arrow indicates the end of the lead-in period at midnight of day 2.

whether steatosis was present in our HPTG subjects, these results are all consistent with the presence of a slow-turnover, persistently unlabeled TG pool in the liver of nondiabetic HPTG subjects that is evidently not present in diabetic HPTG subjects.

Our results also demonstrate that dietary fat is a source of FAs in VLDL-TG in both the fasting and postprandial states. Dietary fat contribution via the delayed pool accounted for 2–12% of fasting VLDL-TG in the three groups, so this route was not a major contributor of FAs to VLDL-TG during fasting. The fractional contribution was highest in the diabetic HPTG group (12%). As dietary energy and fat intakes were similar in the HPTG and diabetic HPTG groups, the higher fractional contribution observed in the diabetic HPTG group is probably not attributable to the influence of recent diet.

Another novel finding here was that diet-derived NEFAs can persist in the circulation for a long time after a meal. We demonstrated the entry of dietary FAs into the NEFA pool soon after ingestion of a meal, as shown by the appearance of [$^2H_{35}$]stearate in plasma FFAs. On day 1, 2–4 h after the last meal, dietary stearate constituted 7–17% of NEFAs, and on day 2, dietary fats accounted for 9–21% of the plasma NEFA pool. These results are compatible with recent reports (17, 18, 57, 58). Roust and Jensen (17) showed that meal FAs appear rapidly in plasma NEFAs, and up to 33% of meal TG FAs entered the plasma NEFA pool. Frayn et al. (18) have also shown that FAs generated by the action of LPL on circulating TG are released directly into venous plasma. The persistence of [$^2H_{35}$]stearate in plasma well into the fasted state in our study (Fig. 7) also indicates that dietary FAs are extensively recycled.

DNL peaked twice during the lead-in period, first at midnight on day 1, then at midnight on day 2. The peak value at midnight on day 2 appeared to represent a plateau or near plateau in all three groups. The results observed here with [$^2H_{31}$]palmitate prelabeling were similar, with apparent plateau values not approached until at least 30 h of infusion. In a previous study, Hudgins et al. (29) observed that DNL in plasma VLDL-TG after the second evening was higher than after the first evening but that no further increases in DNL occurred after the third evening (63 h of [$^{13}C_1$]acetate infusion). Donnelly et al. (46) also reported that plateau values were reached for DNL in VLDL-TG after 1–2 days. Continued "unaccounted" input may still exist after that time, however (Table 2), even after an apparent plateau is reached. This may reflect persistent diluting sources (e.g., visceral NEFA input) or lower turnover of a subfraction of the hepatic TG pool (e.g., phospholipid FA or a slow-turnover TG subcompartment). It is also possible that a subpopulation of plasma VLDL-TG that does not turn over is present as a source of label dilution, although turnover of VLDL-TG was nearly complete during fasting. Taking these findings together, one can speculate that plateau labeling of the hepatic TG pool requires an infusion of at least 24–30 h, which should include two evening feeding periods, but that unlabeled TG may still be present.

Higher fractional DNL values in the HPTG and diabetic HPTG groups in this study are compatible with previous reports in which DNL was increased in obesity and in insulin-resistant states (25, 59, 60). Higher DNL in the HPTG and diabetic HPTG groups may be explained by high levels of insulin (61). Fractional DNL, however, was highly variable within the diabetic HPTG and nondiabetic HPTG groups. Highly variable fractional DNL in the setting of carbohydrate-induced HPTG was also found by Hudgins et al. (29). The metabolic or genetic basis for this interindividual variability will be worth exploring in future studies.

These findings provide an integrated accounting of the metabolic sources of FAs in the liver that contribute to fasting VLDL-TG. The summed contributions from plasma NEFAs, diet, and DNL to fasting VLDL-TG FAs were 83 ± 19%, 68 ± 11%, and 113 ± 4%, leaving 17 ± 19%, 32 ± 11%, and <0% of FAs unaccounted for, in the control, HPTG, and diabetic HPTG groups, respectively. These findings may be explicable based on the known actions of insulin on lipid metabolism. Insulin is well established to inhibit fasting VLDL production by the liver acutely in humans (12, 14, 62, 63). This effect appears to be independent of NEFA suppression induced by hyperinsulinemia (14). In experimental systems, insulin promotes the synthesis of TG by the liver but prevents its secretion by preferentially partitioning newly synthesized TG into the intracellular storage pool (61). FAs released by hepatic lysosomal lipolysis of cytosolic TG (64) appear to contribute to VLDL-TG (35). It is possible that lipolysis in the hepatocyte is inhibited by insulin, leading to the suppression of TG secretion (64). Insulin also impairs the association of apolipoprotein B with TG (65) and causes a reduction in the translational activity of apolipoprotein B mRNA in HepG2 cells (66) while stimulating the degradation of newly synthesized apolipoprotein B (67).

Severely insulin-resistant states such as type 2 diabetes may be characterized by resistance to the acute inhibitory effect of insulin on VLDL production (12, 62). One might speculate that, as human subjects undergo transition from obesity-associated HPTG states to frank diabetes with HPTG, there is a progressive loss of sensitivity to this action of insulin on hepatocytes and a reduced hepatic TG pool size.

The higher insulin concentrations in the diabetic HPTG group may have been contributed in part by their drug therapy. Whether or not higher insulin levels are attributable to pharmacotherapy should not change the physiologic actions or impact, however. We considered discontinuing antihyperglycemic therapy in the diabetic subjects but concluded that this would introduce greater uncertainty if cessation was for less than several weeks (because of the possible nonsteady state, as glycemia and lipids deteriorated) and would certainly not be in the patients' interest if continued for many weeks. We specifically excluded metformin and thiazolidinedione treatments, because these agents may have direct actions independent of insulin and might thereby have introduced confounding effects. Moreover, untreated dyslipidemic diabetes is not as relevant to clinical diabetic

dyslipidemia, for which the most relevant clinical context is when it persists in the face of standard antihyperglycemic therapy.

The explanation for a total contribution somewhat >100% in diabetic HPTG is also uncertain. Perhaps the fact that dietary stearate input was added to endogenous palmitate inputs could have contributed to this result (i.e., if their relative oxidation vs. incorporation rates are not identical), or it may reflect small systematic errors.

The possible impact of potential complicating factors on our model can be estimated. Measurement of circulating NEFA enrichments corrects for recycling of label via adipose tissue or lipoprotein TG. Admixture of portal and systemic blood flow makes the true precursor pool enrichment for hepatic uptake of the plasma NEFA complex, but dilution by portal/visceral FAs appears in the model as an unaccounted input. For the other input sources, the true precursor pool enrichment is known (DNL by mass isotopomer distribution analysis and diet directly). Finally, the differential oxidation of stearate and palmitate could influence the additivity of dietary input with other sources, but the dietary contribution was relatively small. Thus, the impact of these factors is not known, but it is unlikely to be quantitatively significant.

In summary, this study represents a first survey of many pathways in three populations of subjects. The analytic intensity of this protocol (monitoring up to four tracers in plasma and VLDL every 2 h for 42 h) limited the number of subjects who could be studied here. Even with six subjects per group, however, statistically significant results were observed. Future investigations can focus on specific pathways or subject groups, building on the results reported here. Differences between diabetic and nondiabetic HPTG for contributions from diet and NEFA or for turnover time required to fully label the delayed pool, for example, may be of particular interest.

The authors thank the nursing staff of the GCRC at San Francisco General Hospital for their help, Doris Dare and Laurie Herraiz for diet planning, and Dr. Mark Hudes for statistical analysis. This work was funded by Research Grant 010754 from the American Diabetes Association (to M.K.H.). A National Institutes of Health grant from the Division of Research Resources (MOI-RR00083) supported the General Clinical Research Center.

REFERENCES

1. Reaven, G. M., Y. D. Chen, J. Jeppesen, and P. Maheux. 1993. Insulin resistance and hyperinsulinemia in individuals with small, dense low density lipoprotein particles. *J. Clin. Invest.* **92:** 141–146.
2. Brown, C. D., M. Higgins, K. A. Donato, F. C. Rohde, R. Garrison, E. Obarzanek, N. D. Ernst, and M. Horan. 2000. Body mass index and the prevalence of hypertension and dyslipidemia. *Obes. Res.* **8:** 605–619.
3. Austin, M. A., J. E. Hokanson, and K. L. Edwards. 1998. Hypertriglyceridemia as a cardiovascular risk factor. *Am. J. Cardiol.* **81:** 7B–12B.
4. Ebenbichler, C. F., R. Kirchmair, C. Egger, and J. R. Patsch. 1995. Postprandial state and atherosclerosis. *Curr. Opin. Lipidol.* **6:** 286–290.
5. Patsch, J. R., G. Miesenbock, T. Hopferwieser, V. Muhlberger, E. Knapp, J. K. Dunn, A. M. Gotto, Jr. W. Patsch. 1992. Relation of triglyceride metabolism and coronary artery disease. Studies in the postprandial state. *Arterioscler. Thromb.* **12:** 1336–1345.
6. Dallongeville, J., and J. C. Fruchart. 1998. Postprandial dyslipidemia: a risk factor for coronary heart disease. *Ann. Nutr. Metab.* **42:** 1–11.
7. Karpe, F., G. Steiner, K. Uffelman, T. Olivecrona, and A. Hamsten. 1994. Postprandial lipoproteins and progression of coronary atherosclerosis. *Atherosclerosis.* **106:** 83–97.
8. Zilversmit, D. B. 1979. Atherogenesis: a postprandial phenomenon. *Circulation.* **60:** 473–485.
9. Couillard, C., N. Bergeron, D. Prud'homme, J. Bergeron, A. Tremblay, C. Bouchard, P. Mauriege, and J. P. Despres. 1998. Postprandial triglyceride response in visceral obesity in men. *Diabetes.* **47:** 953–960.
10. Lewis, G. F., N. M. O'Meara, P. A. Soltys, J. D. Blackman, P. H. Iverius, W. L. Pugh, G. S. Getz, K. S. Polonsky. 1991. Fasting hypertriglyceridemia in noninsulin-dependent diabetes mellitus is an important predictor of postprandial lipid and lipoprotein abnormalities. *J. Clin. Endocrinol. Metab.* **72:** 934–944.
11. O'Meara, N. M., G. F. Lewis, V. G. Cabana, P.H. Iverius, G. S. Getz, and K. S. Polonsky. 1992. Role of basal triglyceride and high density lipoprotein in determination of postprandial lipid and lipoprotein responses. *J. Clin. Endocrinol. Metab.* **75:** 465–471.
12. Malmstrom, R., C. J. Packard, M. Caslake, D. Bedford, P. Stewart, H. Yki-Jarvinen, J. Shepherd, and M. R. Taskinen. 1997. Defective regulation of triglyceride metabolism by insulin in the liver in NIDDM. *Diabetologia.* **40:** 454–462.
13. Kissebah, A. H., S. Alfarsi, P. W. Adams, V. Wynn. 1976. Role of insulin resistance in adipose tissue and liver in the pathogenesis of endogenous hypertriglyceridaemia in man. *Diabetologia.* **12:** 563–571.
14. Lewis, G. F., K. D. Uffelman, L. W. Szeto, B. Weller, and G. Steiner. 1995. Interaction between free fatty acids and insulin in the acute control of very low density lipoprotein production in humans. *J. Clin. Invest.* **95:** 158–166.
15. Fielding, B. A., J. Callow, R. M. Owen, J. S. Samra, D. R. Matthews, and K. N. Frayn. 1996. Postprandial lipemia: the origin of an early peak studied by specific dietary fatty acid intake during sequential meals. *Am. J. Clin. Nutr.* **63:** 36–41.
16. Griffiths, A. J., S. M. Humphreys, M. L. Clark, B. A. Fielding, and K. N. Frayn. 1994. Immediate metabolic availability of dietary fat in combination with carbohydrate. *Am. J. Clin. Nutr.* **59:** 53–59.
17. Roust, L. R., and M. D. Jensen. 1993. Postprandial free fatty acid kinetics are abnormal in upper body obesity. *Diabetes.* **42:** 1567–1573.
18. Frayn, K. N., S. W. Coppack, B. A. Fielding, and S. M. Humphreys. 1995. Coordinated regulation of hormone-sensitive lipase and lipoprotein lipase in human adipose tissue in vivo: implications for the control of fat storage and fat mobilization. *Adv. Enzyme Regul.* **35:** 163–178.
19. Frayn, K. N., S. Shadid, R. Hamlani, S. M. Humphreys, M. L. Clark, B. A. Fielding, O. Boland, and S. W. Coppack. 1994. Regulation of fatty acid movement in human adipose tissue in the postabsorptive-to-postprandial transition. *Am. J. Physiol.* **266:** E308–E317.
20. Parks, E. J., R. M. Krauss, M. P. Christiansen, R. A. Neese, and M. K. Hellerstein. 1999. Effects of a low-fat, high-carbohydrate diet on VLDL-triglyceride assembly, production, and clearance. *J. Clin. Invest.* **104:** 1087–1096.
21. Schneeman, B. O., L. Kotite, K. M. Todd, and R. J. Havel. 1993. Relationships between the responses of triglyceride-rich lipoproteins in blood plasma containing apolipoproteins B-48 and B-100 to a fat-containing meal in normolipidemic humans. *Proc. Natl. Acad. Sci. USA.* **90:** 2069–2073.
22. Wu, X., N. Sakata, J. Dixon, and H. N. Ginsberg. 1994. Exogenous VLDL stimulates apolipoprotein B secretion from HepG2 cells by both pre- and post-translational mechanisms. *J. Lipid Res.* **35:** 1200–1210.
23. Craig, W. Y., and A. D. Cooper. 1988. Effects of chylomicron remnants and beta-VLDL on the class and composition of newly secreted lipoproteins by HepG2 cells. *J. Lipid Res.* **29:** 299–308.
24. Hultin, M., R. Savonen, and T. Olivecrona. 1996. Chylomicron metabolism in rats: lipolysis, recirculation of triglyceride-derived fatty acids in plasma FFA, and fate of core lipids as analyzed by compartmental modelling. *J. Lipid Res.* **37:** 1022–1036.
25. Faix, D., R. Neese, C. Kleteke, S. Wolden, D. Cesar, M. Coutlangus,

C.H. Shackleton, and M. K. Hellerstein. 1993. Quantification of menstrual and diurnal periodicities in rates of cholesterol and fat synthesis in humans. *J. Lipid Res.* **34:** 2063–2075.
26. Hellerstein, M. K., M. Christiansen, S. Kaempfer, C. Kletke, K. Wu, J. S. Reid, K. Mulligan, N. S. Hellerstein, and C. H. Shackleton. 1991. Measurement of de novo hepatic lipogenesis in humans using stable isotopes. *J. Clin. Invest.* **87:** 1841–1852.
27. Jones, P. J., G. L. Namchuk, and R. A. Pederson. 1995. Meal frequency influences circulating hormone levels but not lipogenesis rates in humans. *Metabolism.* **44:** 218–223.
28. Duerden, J. M., and G. F. Gibbons. 1988. Secretion and storage of newly synthesized hepatic triacylglycerol fatty acids in vivo in different nutritional states and in diabetes. *Biochem. J.* **255:** 929–935.
29. Hudgins, L. C., M. K. Hellerstein, C. E. Seidman, R. A. Neese, J. D. Tremaroli, and J. Hirsch. 2000. Relationship between carbohydrate-induced hypertriglyceridemia and fatty acid synthesis in lean and obese subjects. *J. Lipid Res.* **41:** 595–604.
30. Aarsland, A., D. Chinkes, and R. R. Wolfe. 1996. Contributions of de novo synthesis of fatty acids to total VLDL-triglyceride secretion during prolonged hyperglycemia/hyperinsulinemia in normal man. *J. Clin. Invest.* **98:** 2008–2017.
31. Kissebah, A. H., S. Alfarsi, D. J. Evans, and P. W. Adams. 1982. Integrated regulation of very low density lipoprotein triglyceride and apolipoprotein-B kinetics in non-insulin-dependent diabetes mellitus. *Diabetes.* **31:** 217–225.
32. Egusa, G., W. F. Beltz, S. M. Grundy, and B. V. Howard. 1985. Influence of obesity on the metabolism of apolipoprotein B in humans. *J. Clin. Invest.* **76:** 596–603.
33. Howard, B. V., W. G. Abbott, G. Egusa, and M. R. Taskinen. 1987. Coordination of very low-density lipoprotein triglyceride and apolipoprotein B metabolism in humans: effects of obesity and non-insulin-dependent diabetes mellitus. *Am. Heart J.* **113:** 522–526.
34. Gibbons, G. F., and D. Wiggins. 1995. Intracellular triacylglycerol lipase: its role in the assembly of hepatic very-low-density lipoprotein (VLDL). *Adv. Enzyme Regul.* **35:** 179–198.
35. Francone, O. L., A. D. Kalopissis, and G. Griffaton. 1989. Contribution of cytoplasmic storage triacylglycerol to VLDL-triacylglycerol in isolated rat hepatocytes. *Biochim. Biophys. Acta.* **1002:** 28–36.
36. Gibbons, G. F., and F. J. Burnham. 1991. Effect of nutritional state on the utilization of fatty acids for hepatitic triacylglycerol synthesis and secretion as very-low-density lipoprotein. *Biochem. J.* **275:** 87–92.
37. Marceau, P., S. Biron, F. S. Hould, S. Marceau, S. Simard, S. N. Thung, and J. G. Kral. 1999. Liver pathology and the metabolic syndrome X in severe obesity. *J. Clin. Endocrinol. Metab.* **84:** 1513–1517.
38. Kelley, D. E., T. M. McKolanis, R. A. Hegazi, L. H. Kuller, and S. C. Kalhan. 2003. Fatty liver in type 2 diabetes mellitus: relation to regional adiposity, fatty acids, and insulin resistance. *Am. J. Physiol. Endocrinol. Metab.* **285:** E906–E916.
39. Nguyen-Duy, T. B., M. Z. Nichaman, T. S. Church, S. N. Blair, and R. Ross. 2003. Visceral fat and liver fat are independent predictors of metabolic risk factors in men. *Am. J. Physiol. Endocrinol. Metab.* **284:** E1065–E1071.
40. Barter, P. J., and P. J. Nestel. 1973. Precursors of plasma triglyceride fatty acids in obesity. *Metabolism.* **22:** 779–783.
41. Havel, R. J., J. P. Kane, E. O. Balasse, N. Segel, and L. V. Basso. 1970. Splanchnic metabolism of free fatty acids and production of triglycerides of very low density lipoproteins in normotriglyceridemic and hypertriglyceridemic humans. *J. Clin. Invest.* **49:** 2017–2035.
42. Karpe, F., G. Steiner, T. Olivecrona, L. A. Carlson, and A. Hamsten. 1993. Metabolism of triglyceride-rich lipoproteins during alimentary lipemia. *J. Clin. Invest.* **91:** 748–758.
43. Boberg, J., L. A. Carlson, and U. Freyschuss. 1972. Determination of splanchnic secretion rate of plasma triglycerides and of total and splanchnic turnover of plasma free fatty acids in man. *Eur. J. Clin. Invest.* **2:** 123–132.
44. Diraison, F., and M. Beylot. 1998. Role of human liver lipogenesis and reesterification in triglycerides secretion and in FFA reesterification. *Am. J. Physiol.* **274:** E321–E327.
45. Zech, L. A., S. M. Grundy, D. Steinberg, and M. Berman. 1979. Kinetic model for production and metabolism of very low density lipoprotein triglycerides. Evidence for a slow production pathway and results for normolipidemic subjects. *J. Clin. Invest.* **63:** 1262–1273.
46. Donnelly, K. L., C. I. Smith, S. J. Schwarzenberg, J. Jessurun, M. D. Boldt, and E. J. Parks. 2005. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J. Clin. Invest.* **115:** 1343–1351.
47. Schmidt D. E., J. B. Allred, and C. L. Kien. 1999. Fractional oxidation of chylomicron-derived oleate is greater than that of palmitate in healthy adults fed frequent small meals. *J. Lipid Res.* **40:** 2322–32.
48. Hellerstein, M. K., N. L. Benowitz, R. A. Neese, J. M. Schwartz, R. Hoh, P. Jacob, 3rd, J. Hsieh, and D. Faix. 1994. Effects of cigarette smoking and its cessation on lipid metabolism and energy expenditure in heavy smokers. *J. Clin. Invest.* **93:** 265–272.
49. Neese, R. A., J. M. Schwarz, D. Faix, S. Turner, A. Letscher, D. Vu, and M. K. Hellerstein. 1995. Gluconeogenesis and intrahepatic triose phosphate flux in response to fasting or substrate loads. Application of the mass isotopomer distribution analysis technique with testing of assumptions and potential problems. *J. Biol. Chem.* **270:** 14452–14466.
50. Hellerstein, M. K. 1995. Methods for measurement of fatty acid and cholesterol metabolism. *Curr. Opin. Lipidol.* **6:** 172–181.
51. Wolfe, R. 1992. Radioactive and Stable Isotope Tracers in Biomedicine: Principles and Practice of Kinetic Analysis. Wiley-Liss, New York. 119–144.
52. Ferrannini, E. 1988. The theoretical bases of indirect calorimetry: a review. *Metabolism.* **37:** 287–301.
53. Gibbons, G. F., S. M. Bartlett, C. E. Sparks, and J. D. Sparks. 1992. Extracellular fatty acids are not utilized directly for the synthesis of very-low-density lipoprotein in primary cultures of rat hepatocytes. *Biochem. J.* **287:** 749–753.
54. Wiggins, D., and G. F. Gibbons. 1992. The lipolysis/esterification cycle of hepatic triacylglycerol. Its role in the secretion of very-low-density lipoprotein and its response to hormones and sulphonylureas. *Biochem. J.* **284:** 457–462.
55. Yang, L. Y., A. Kuksis, J. J. Myher, and G. Steiner. 1995. Origin of triacylglycerol moiety of plasma very low density lipoproteins in the rat: structural studies. *J. Lipid Res.* **36:** 125–136.
56. Sidossis, L. S., B. Mittendorfer, E. Walser, D. Chinkes, and R. R. Wolfe. 1998. Hyperglycemia-induced inhibition of splanchnic fatty acid oxidation increases hepatic triacylglycerol secretion. *Am. J. Physiol.* **275:** E798–E805.
57. Heath, R. B., F. Karpe, R. W. Milne, G. C. Burdge, S. A. Wootton, and K. N. Frayn. 2003. Selective partitioning of dietary fatty acids into the VLDL TG pool in the early postprandial period. *J. Lipid Res.* **44:** 2065–2072.
58. Summers, L. K., S. C. Barnes, B. A. Fielding, C. Beysen, V. Ilic, S. M. Humphreys, and K. N. Frayn. 2000. Uptake of individual fatty acids into adipose tissue in relation to their presence in the diet. *Am. J. Clin. Nutr.* **71:** 1470–1477.
59. Schwarz, J. M., R. Neese, C. H. L. Shackleton, and M. K. Hellerstein. 1993. De novo lipogenesis (DNL) during fasting and oral fructose in lean and obese hyperinsulinemic subjects (Abstract). *Diabetes.* **42 (Suppl. 1):** 39A.
60. Schwarz, J. M., P. Linfoot, D. Dare, and K. Aghajanian. 2003. Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets. *Am. J. Clin. Nutr.* **77:** 43–50.
61. Sparks, J. D., and C. E. Sparks. 1994. Insulin regulation of triacylglycerol-rich lipoprotein synthesis and secretion. *Biochim. Biophys. Acta.* **1215:** 9–32.
62. Lewis, G. F., K. D. Uffelman, L. W. Szeto, and G. Steiner. 1993. Effects of acute hyperinsulinemia on VLDL triglyceride and VLDL apoB production in normal weight and obese individuals. *Diabetes.* **42:** 833–842.
63. Lewis, G. F., B. Zinman, K.D. Uffelman, L. Szeto, B. Weller, and G. Steiner. 1994. VLDL production is decreased to a similar extent by acute portal vs. peripheral venous insulin. *Am. J. Physiol.* **267:** E566–E572.
64. Debeer, L. J., A. C. Beynen, G. P. Mannaerts, and M. J. Geelen. 1982. Lipolysis of hepatic triacylglycerol stores. *FEBS Lett.* **140:** 159–164.
65. Patsch, W., S. Franz, and G. Schonfeld. 1983. Role of insulin in lipoprotein secretion by cultured rat hepatocytes. *J. Clin. Invest.* **71:** 1161–1174.
66. Adeli, K., and A. Theriault. 1992. Insulin modulation of human apolipoprotein B mRNA translation: studies in an in vitro cell-free system from HepG2 cells. *Biochem. Cell Biol.* **70:** 1301–1312.
67. Sparks, J. D., and C. E. Sparks. 1990. Insulin modulation of hepatic synthesis and secretion of apolipoprotein B by rat hepatocytes. *J. Biol. Chem.* **265:** 8854–8862.