paper on methodology

Method to measure apolipoprotein B-48 and B-100 secretion rates in an individual mouse: evidence for a very rapid turnover of VLDL and preferential removal of B-48- relative to B-100-containing lipoproteins

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Abstract We have developed a procedure to measure the rates of apolipoprotein (apoB) and triglyceride secretion from the liver of an individual mouse. Using the well-characterized method of Triton WR-1339 injection to block peripheral removal of newly secreted VLDL, the rate of triglyceride accumulation is monitored and at the end of the experimental period, blood is extracted for quantitative VLDL preparation. ApoB species in isolated VLDL are analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the mass of the apoB-48 and apoB-100 species are estimated by Coomassie staining and laser densitometric scanning, using known quantities of LDL-apoB-100 as standards. This methodology was applied to measure the rate of apoB secretion in male and female FVB/N mice and we found that the molar ratio of newly secreted apoB-48 to B-100 is 4.6 in the male, and 3.8 in the female. Measurements of the steady state apoB levels indicate that liver-derived apoB-48 is cleared from the circulation 7.1 times faster than B-100 in the male and 4.7 times faster in the female mouse. VLDL apoB-48 fractional turnover is approximately 1800 pools per day in both the male and female mouse (1814 ± 139 vs. 1831 ± 365 respectively, $P = 0.92$). ApoB-100 fractional turnover rates are much slower and show a statistically significant difference between males and females (255 ± 19 pools per day vs. 386 ± 65 pools per day, respectively, $P = 0.006$). This procedure provides for quantification of secretory rates of these apo proteins in vivo, and may be useful for studying the effects of genetic manipulation on the simultaneous secretion of apoB-48- and apoB-100-containing VLDL, afforded by the panoply of transgenic mouse models now available for study, as well as for effects of diet and drug therapy.—**Li, X., F. Catalina, S. M. Grundy, and S. Patel.** Method to measure apolipoprotein B-48 and B-100 secretion rates in an individual mouse: evidence for a very rapid turnover of VLDL and preferential removal of B-48- relative to B-100-containing lipoproteins. *J. Lipid Res.* 1996. **37:** 210–220.

Supplementary key words laser densitometry • hepatic secretion • mRNA editing • triton • gel electrophoresis

Lipid transport in the circulation is mediated mainly by apolipoprotein B (apoB). Dietary triglycerides are transported in chylomicrons containing apoB-48. Endogenous triglycerides are transported as very low density lipoproteins (VLDL) and contain apoB formed in the liver (1). The human liver produces only apoB-100, whereas several other species edit a variable proportion of their hepatic apoB mRNA, resulting in the secretion of both B-48- and B-100-containing VLDL (2). A limitation in the study of hepatic lipoprotein metabolism has been the difficulty of measuring secretion rates of apoB. Isotopic kinetic methods previously used for estimating lipoprotein secretion rates have significant limitations (3). However, if advances are to be made in our understanding of apoB metabolism, techniques are needed to accurately measure in vivo apoB secretion rates.

There is a growing interest in the use of the mouse model for the study of lipoprotein metabolism. This model is attractive because of the variety of genetic strains available and because of the increasing availability of transgenic and gene knockout mice that contain unique alterations in lipoprotein metabolism. To investigate the quantitative changes in lipoprotein metabolism that are produced by these various models, a method is needed for the measurement of hepatic apoB

Abbreviations: apoB, apolipoprotein B; VLDL, very low density lipoprotein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenymethylsulfonylfluoride; RT-PCR, reverse transcription-polymerase chain reaction.

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secretion rates in such animals. As mouse liver secretes both apoB-48 and B-100-VLDL particles, simultaneous quantitation of both of these species is also important. Therefore, to overcome this deficiency in methodology, we have adapted the existing methodologies of Triton-induced accumulation of VLDL for the simultaneous measurement of secretion rates of triglyceride and apoB in the mouse.

In this paper, we describe a combined approach of Triton injection to measure triglyceride secretion rates (4–19) and quantitative and qualitative analyses of the apoB (20–22) secreted by the liver in an individual mouse. Our methodology allows us to simultaneously quantify any number of protein species in a single sample. Hence, we can apply it to study VLDL secreted by the mouse liver, measuring both B-100 and B-48 secretion rates. In this method, VLDL catabolism is blocked by injection of Triton, and the rate of triglyceride accumulation (i.e., secretion) in the blood is measured. VLDL is subsequently isolated from the blood after a known period of time, and the apolipoproteins are quantitated by SDS-PAGE, Coomassie staining, and laser densitometry. This technique allows for the measurement of both apoB-100 and B-48 secretion rates in a single mouse. Combined with the Triton method for measuring the rate of triglyceride secretion in vivo, this technique is useful for the study of apoB metabolism in various genetic strains and genetically modified mice. Furthermore, it may be useful in the study of the several steps involved in the biogenesis of apoB-containing lipoproteins. Without a quantitative estimation of apoB secretion rates in vivo, the biological significance of various steps in transcription, editing, translation, and lipoprotein assembly cannot be put into perspective.

METHODS

Materials

Tyloxapol (Triton WR-1339), diethyl pyrocarbonate (DEPC), and reagents for triglyceride measurements were obtained from Sigma Chemical Co. (St. Louis, MO), phenylmethylsulfonylfluoride, leupeptin, and reagents for cholesterol determinations were obtained from Boehringer Mannheim (Indianapolis, IN), Trasylol was from Calbiochem (La Jolla, CA), and all other reagents were obtained from local suppliers and were of reagent grade or better. Radiochemicals were purchased from Amersham (Arlington Heights, IL) or DuPont-NEN (Boston, MA). Reverse transcriptase for cDNA synthesis was purchased from Gibco-BRL (Bethesda, MD), and for primer extension from Life Sciences (St. Petersberg, FL).

Animal experimentation

All animal experimental protocols were approved by the local Animal Advisory Board, following AAALAC guidelines. FVB/N, and C57BL mice were animals bred in-house under barrier conditions. The animals were fed a standard rodent diet (Teklad diet #7002, Teklad, Madison, WI) containing 24% protein, 6% fat, and 5% fiber, and maintained on a 12-h light/dark cycle.

Protocol for Triton injection

On the day of experimentation, mouse chow was removed, and the animals were allowed liberal access to water and fat-free food (dry cereal) for 2 h prior to Triton injection. A fasting protocol was not followed because a time course of a total of 7 h is required, and this would represent a period of fasting for a mouse, likely to lead to altered lipid metabolism (23). The supply of fat-free food, which was continued for the period of the experiment, prevented intestinal secretion of apoB-48 and chylomicrons. Blood was drawn from the tail vein for baseline values and a dose of 20 mg tyloxapol was injected in a volume of 100 µl. Tail vein sampling (50 µl) was continued at approximately 30, 90, 180 min, and at 5 h the animals were anesthetized by a ketamine/xylazine mixture, and exanguinated by cardiac puncture to collect blood for VLDL isolation. Blood was collected into tubes containing 10 µl of 0.5 M EDTA, 10,000 IU of Trasylol, and 10 µl of 100 mM PMSF and 50 µg of leupeptin. The liver weights of all animals were recorded, and 100 mg was used for RNA extraction. The rest was flash-frozen in liquid nitrogen and stored at -80°C. Plasma was collected after centrifugation at 12,000 *g* for 15 min at 4°C and an aliquot was stored for triglyceride and cholesterol analysis. The remaining plasma was used for VLDL isolation the same day.

Isolation and analysis of triglyceride and cholesterol

Plasma and VLDL triglyceride analyses were performed using commercial kits (Boehringer Mannheim and Sigma Chemical Co. for triglyceride and cholesterol, respectively) as directed by the manufacturers. In addition, for the analysis of triglyceride in highly hyperlipidemic samples, such as the plasma and VLDL obtained after Triton injection, these samples were analyzed at four dilutions in normal saline to ensure accuracy.

Isolation and analysis of VLDL

A known volume of plasma was adjusted to a volume of 0.5 ml with 0.85% saline when required, added to 1.5 ml of a NaCl/NaBr solution of density 1.019 g/ml (11.422 g NaCl, 16.553 g NaBr per liter of water), and centrifuged at 39,000 rpm in a Beckman 50.3 Ti rotor for 18 h at 12°C. The VLDL was isolated by tube slicing,

and the volume of retovery was measured. The density of the layer beneath the recovered VLDL was routinely around 1.02 g/ml. Aliquots for triglyceride and protein analyses were removed, and the remaining VLDL was frozen at -80°C.

Determination of apolipoprotein B mass

Isolated VLDL was used directly for protein analysis, without performing delipidation. In preliminary experiments, the presence of lipid did not affect the quality of the gels, and delipidation did not improve upon this. An equal amount of VLDL was mixed with 2 × sample buffer, heated, and resolved by 5% SDS-PAGE (24) on 1-mm-thick minigels. As a routine, every gel contained VLDL in duplicate from two preparations and four dilutions of human apoB from LDL as internal standards. The gels were fixed in 45% methanol, 10% acetic acid, stained with 0.25% Coomassie brilliant blue R250 for at least 12 h, destained over 3 h with three changes of solution, dried between two cellophane sheets, and analyzed by laser densitometry. Where possible, the apoB is expressed on a molar basis, assuming B-100 has a molecular weight of 512,772 and B-48 that of 240,855 (25). A typical gel is shown in **Fig. 1A,** and a gel showing the staining pattern of serial dilutions of a VLDL preparation in Fig. 1B. The responsiveness of staining is linear over a range of LDL-apoB protein from 200 ng to 2.5 μg (**Fig. 2**). The data were analyzed by using the linear equation, y = ax + c. As a routine all of the data fit this equation, with a correlation $r^2 \geq 0.98$. The laser densitometer used was from Molecular Dynamics (Sunnyvale, CA) and densitometric quantitation was performed using ImageQuant software. Background correction was

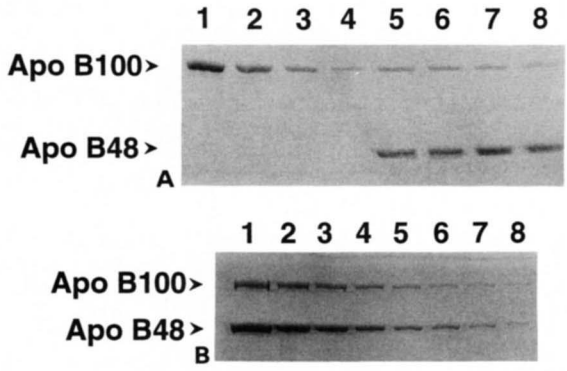

Fig. 1. Analytical SDS-PAGE of mouse lipoproteins. Mouse VLDL samples were analyzed by SDS-PAGE as described in Methods. Fig. 1A shows a typical gel, containing a serial dilution of human LDL apoB-100 standards (2.1 μg, 1.05 μg, 0.525 μg, and 0.168 μg, lanes 1–4, respectively), and duplicate applications of a known amount of mouse VLDL from two VLDL preparations (lanes 5, 6 and 7, 8), resulting in the separation of apoB-100 and apoB-48. Fig. 1B shows the effect of loading decreasing amounts of a VLDL preparation on the staining pattern. Tracks 1–8 contain 15 μl, 10 μl, 7.5 μl, 5 μl, 3.5 μl, 2.5 μl, 1.5 μl, and 0.5 μl of VLDL loaded in a final volume of 30 μl, respectively (see also Fig. 3). The gels are stained, dried, scanned, and quantitated as described in Methods.

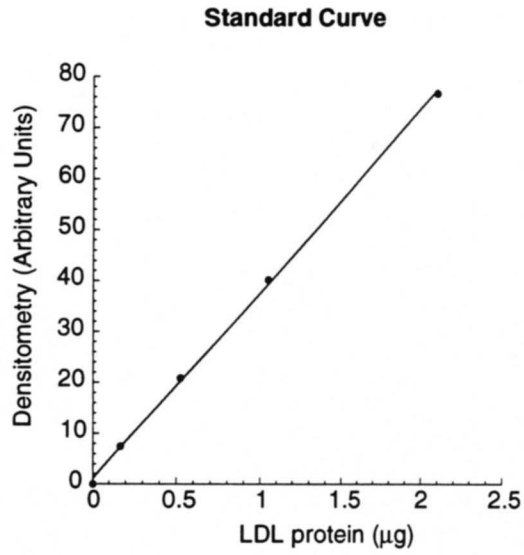

Fig. 2. Linearity of coomassie staining for a range of LDL apoB-100. A series of human apoB LDL proteins (0.2–2.1 μg) were separated by SDS-PAGE, stained, and the intensity of staining was quantitated by laser densitometric scanning as described in Methods. The data shown fit a linear equation, y = 1.1699 + 36.315x (r^2 = 0.99956). As a routine, the measured mouse apoB was in the range 0.5–1.5 μg per VLDL sample.

performed by measuring the background directly above and below the protein bands. Verification of the quantitation was also confirmed by analysis of the images, obtained by laser densitometry, by using NIH Image (26). **Figure 3** shows the linearity of response, and repeated scanning of gels containing a range of VLDL dilutions (such as the one shown in Fig. 1B). Both apoB-48 and apoB-100 show a linear response and, more importantly (Fig. 3B), the ratio of apoB-48 to B-100 does not vary over a broad range. Figure 3B shows VLDL isolated from three different strains of mice after tyloxapol injection; each line represents VLDL from an individual mouse. The mass ratio of apoB-48 to B-100 (fortuitously) varied naturally between these particular animals, and hence afforded an opportunity to examine the effects of loading differing amounts of apoB-48 and B-100 within the same sample. As can be seen, the ratio of B-48 to B-100 remains constant over an approximately 8-fold difference in the proportions of apoB-48 and B-100.

Preparation of the apoB standards

Human LDL apoB was prepared as previously described (27). The protein content was measured by the Lowry assay (28), using bovine serum albumin as a standard, and the quality of apoB was judged by SDS-PAGE.

RNA extraction, cDNA synthesis, and primer extension assay

About 100 mg of fresh liver was homogenized with 1 ml of TriZol (Gibco BRL, Bethesda, MD), and the RNA was isolated as per the manufacturers instructions (29, 30). The RNA was stored at -80°C in 70% ethanol until use. The RNA was treated with DNase I in the presence of RNasin, purified, and used for cDNA synthesis and primer extension assay as previously described (31). Briefly, the cDNA was primed with the reverse oligonucleotide, MB11, and PCR was performed using the forward oligonucleotide, MB10. The PCR products were further purified free of unincorporated nucleotides using the QIAquick PCR purification kit (Qiagen, Chatsworth, CA), though the washing step was increased to three times instead of two, and about a fiftieth of this was used for the primer extension analysis, using ^{32}P-kinased rMouse oligonucleotide. The products of the primer extension assay were resolved by 6% acrylamide-urea gels, dried, and quantitated by phosphorimaging and ImageQuant software (Molecular Dynamics). All RNA samples were also analyzed for the presence of DNA contamination, by parallel processing in the absence of cDNA synthesis as previously described.

Oligonucleotides

The oligonucleotides used in this study, described below, were a kind gift from Dr. Ba-Bie Teng. MB10: 5′ > TCC TCA GCA GAT TCA TCA TTA; MB11: 5′ > CAA GCA TTT TTA ACT TTT CAA T; rMOUSE: 5′ > TTT AAG TCA TGT GGA TCA TAA TTA TCT TTA ATA TAC TG.

Blood volume measurements

Plasma volume estimation in the mouse was made by injection of radioiodinated mouse albumin (a gift from Dr. Stephen Turley). Special care was taken to ensure that the albumin was freshly prepared and had been extensively dialyzed prior to use. The presence of free iodine was checked for by trichloroacetic acid precipitation. Briefly, mice (2 males and 2 females) were anesthetized with ketamine and xylazine, and 100 µl of albumin was injected by tail vein, leaving the needle and syringe in place for 1 min to prevent any significant back flow. After 5 min, blood was drawn by cardiac puncture and collected into a tube containing 10 µl of 0.5 M EDTA. Aliquots of both total blood and plasma were counted in a gamma counter. All potential losses were accounted for, e.g., by measuring the loss of any label remaining within the syringe and needle.

Measurement of liver DNA content

A fresh piece of liver, approximately 100 mg, was blotted gently between two sheets of blotting paper and accurately weighed. It was placed directly into a proteinase K digestion buffer (200 mg/ml proteinase K, 50 mM Tris, pH 7.5, 25 mM EDTA, 1% SDS), containing a

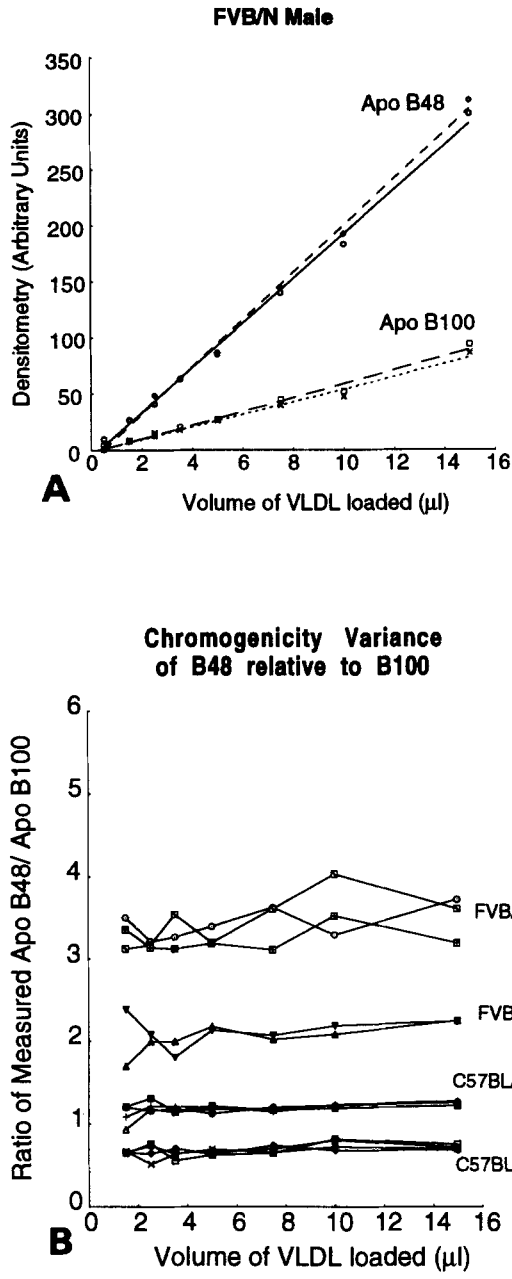

Fig. 3. Chromogenicity of apoB-100 and B-48 in mouse VLDL. Panel A shows the linearity of staining of both apoB-100 and B-48 with increasing amounts of VLDL loaded on the gel. As can be seen, the chromogenicity increases as a linear function ($r^2 > 0.99$). Non-linearity of the chromogenicities between apoB-48 and B-100 were examined by plotting the ratio of B-48/B-100 densitometric units in four VLDL preparations (panel B). Isolated from four mice that showed spontaneously differing proportions of B-48 to B-100. For each animal, multiple measurements were made as shown. Over an eightfold range, the ratio of B-48 to B-100 remains relatively constant, and is reproducible. M and F indicate male and female animals, and the strains are as indicated.

known amount of uniformly labeled [^{3}H]DNA. The liver was digested at 55°C for 10–12 h, nucleic acids were purified by phenol extractions and ethanol precipitation and re-digested with heat-treated RNase A for 3 h at 37°C. The DNA was further purified, dissolved in a known volume of 10 mM Tris/1 mM EDTA pH 8.0 and its purity free from RNA was verified by gel electrophoresis. The DNA content was estimated by UV absorbance, and the loss of DNA was corrected for by normalizing to the [^{3}H]DNA added.

Measurement of baseline apolipoprotein B

Preliminary experiments indicated that there was insufficient apoB in the VLDL isolated from an individual mouse to be analyzed in duplicate. Therefore, plasma from 3–4 mice, after a 2-h fat-free food diet, was pooled and the VLDL was isolated as described above. Isolated VLDL was concentrated approximately 20-fold by Centricon 30 (Amicon Corp., Beverely, MA), and analyzed as described above.

Calculation of triglyceride and apoB secretion rates

The rate of triglyceride secretion was determined in every mouse by performing analysis over the linear part of the secretion-rate curve (see Fig. 4 and Results for explanation). This allowed us to negate the effects of the variable lag phase seen in some of our animals. The rates were computed by the division of the increment in triglyceride, between the first time point on the linear phase and the last, by this time interval. Correction for plasma volume was performed from the values derived from plasma volume determinations as described above. For FVB/N males, this was 0.071 ml/g body weight, and for FVB/N females this was 0.09 ml/g body weight.

For apoB secretion rates, the total amount of apoB in VLDL at the end of the experiment was determined as described above. Losses during VLDL isolation were corrected for by assuming that all the plasma triglyceride was present in the VLDL fraction prior to the centrifugation step. Baseline values of apoB were subtracted from these corrected values and corrected for plasma volume for each animal, based on our determinations above. All data were normalized to liver weights for comparison.

Statistical analysis was performed using one-way analysis of variance to compare sex differences.

RESULTS

Table 1 shows the baseline characteristics of the mice used in the experiments. Male and female FVB/N mice differ significantly in their baseline lipid levels, body and liver weights. The cholesterol and triglyceride levels in this strain of mouse are almost twice the levels observed in other strains. These mice were chosen because they are being used in a series of related studies carried out in this laboratory.

Dose response and time course of tyloxapol injection

In preliminary studies, two variables were found to affect the amount of triglyceride accumulation in the plasma. First, at a dose of 500 mg/kg of Triton injected and a conventional time course of 2 h, the response of triglyceride accumulation varied, perhaps because of the difficulty in injecting all of the dose into the tail vein. However, the dose response of Triton injected in a group of male mice showed that increasing the amount of Triton injected, above a threshold level of 400 mg/kg body weight, did not affect the mice unduly, and the triglyceride secretion rates were unaffected by the increased amount of Triton injected (**Fig. 4A**). This resulted in more reliable rate curves. Second, in a fraction of the animals studied, presumably as a result of the Triton not entering the bloodstream rapidly from the site of injection, there was a variable lag phase (Fig. 4A). A prolonged time course revealed that the rate of triglyceride accumulation remained linear for at least 5 h post-injection (Fig. 4B). As a routine, triglyceride accumulation was measured at various time points post-injection (Triton dose of 700–900 mg/kg) for 5 h in every mouse, and all rates were computed over the linear range for each mouse.

Triglyceride secretion rates

Figure 5 shows the triglyceride secretion rates obtained for male and female FVB/N mice. When the data are corrected for blood volume only, there is a significant difference in the triglyceride secretion rates between male and female mice (7.86 ± 1.03 mg/h versus 6.06 ± 0.92 mg/h respectively, $P = 0.0005$). However, as the size of livers in these animals is also significantly different (see Table 1), and when secretion rates were expressed as a function of liver mass, there was no significant difference (6.39 ± 0.58 mg/h • g liver versus 6.30 ± 0.79 mg/h • g liver respectively, $P = 0.78$). Histological examination and measurement of DNA content per wet weight of the livers did not show any significant differences between the sexes (data not shown), suggesting that male and female hepatocytes do not secrete differing amounts of triglycerides. Triglyceride secretion rates, when normalized to body weights, also did not show a difference between male and females (0.289 ± 0.028 mg/h • g versus 0.296 ± 0.034 mg/h • g, $P = 0.60$). However, we have chosen to normalize the data to liver weights, as the amount of apoB secreted is likely to be better reflected by the liver mass than the body mass.

TABLE 1. Baseline characteristics of male and female FVB/N mice

Sex	Age	Body Weight	Liver Weight	Baseline Triglyceride	Baseline Cholesterol
	wk	*g*	*g*	*mg/dl*	*mg/dl*
Male (n = 11	13.6 ± 5.7	27.3 ± 3.3	1.19 ± 0.15	123 ± 14	176 ± 42
Female (n = 11)	11.2 ± 2.9	20.3 ± 1.1	0.98 ± 0.11	145 ± 31	145 ± 23
P value	0.24	0.0002	0.0013	0.041	0.05

All values are expressed as mean ± SD. A statistically significant difference was seen for body weight, liver weight, and for cholesterol and triglyceride at baseline between males and females. Baseline cholesterol and triglyceride values and body weights were measured before tyloxapol injection and liver weights were determined at the end of the experimental period.

Apolipoprotein B secretion rates

The mouse liver edits its apoB mRNA, and as a consequence, synthesizes and secretes both apoB-48 and apoB-100. To measure the apoB secretion rates, VLDL was quantitatively isolated from Triton-injected animals and apoB-48 and B-100 were simultaneously quantitated by SDS-PAGE as described in Methods. Correction for any losses in isolation of VLDL was effected by assuming that all the plasma triglyceride in the final sample was present as VLDL. In view of the mass difference of B-48 and B-100, the data are expressed in molar form. This is also preferable because each VLDL particle contains only one apoB molecule. In the absence of accurate molecular weight data for murine apoBs, we have used the human values, based on the cDNA sequence (25). All rates were normalized to liver weight. Baseline values of apoB-48 and B-100, determined as described in Methods, were 1.97 nM and 3.14 nM in the male and 1.94 nM and 2.44 nM in the female, respectively. Figure 5 shows the rates of apoB-48, B-100, and total apoB secreted by male and female FVB/N mice. Females secreted more apoB-100 than males (77.2 ± 17.4 pmol/h • g liver vs. 56.2 ± 7.3 pmol/h • g liver, $P = 0.035$). However, whilst there is a trend for the female to secrete more apoB-48 (291 ± 70 pmol/h • g liver vs. 251 ± 26 pmol/h • g liver, $P = 0.24$), the difference was not significantly different. The technique allows the measurement of the molar ratios of apoB-48 and B-100 that are secreted simultaneously in an individual mouse. **Figure 6** shows the mean molar ratios of baseline apoB-48 to B-100 in male and female mice that are 0.63 and 0.79, respectively. However, in VLDL, isolated 5 h after the Triton injection, the ratios are 4.50 ± 0.37 and 3.70 ± 0.24, respectively ($P = 0.0098$). Presumably the ratio at 5 h reflects the ratio of apoB-48 to B-100 secreted as nascent particles from the liver.

On the basis of this result, if the ratios of secreted B-48 to B-100 equal the extent of apoB mRNA editing, the extent of editing in the male should be about 80% and 79% in the female. To determine whether these predicted values correspond to actual values, a sensitive RT-PCR primer extension assay was carried out. We analyzed the steady state levels of liver RNA editing in total RNA extracted from six male and six female FVB/N mice (**Fig. 7**). In the male, 60.1 ± 3.4% of RNA was edited, compared to 62.4 ± 1.8% in the female ($P = 0.44$). Assuming that the measured levels of editing represent those present in the mRNA pool that is translated, these data would imply that levels of edited apoB mRNA are similar in both males and females, but B-48 is preferentially recruited for VLDL synthesis and secretion than B-100. Furthermore, this 'preferential recruitment' is greater in the males than the females (see Discussion).

The measurement of the relative proportions of the apoB species secreted, compared to the baseline values, allows us to gain a measure of the turnover of the two species for the mouse. **Figure 8** shows the relative turnover of apoB-48 and B-100 in the female and male FVB/N mouse. ApoB-48 had a very rapid turnover in the male and female mouse (1814 ± 139 vs. 1831 ± 365 pools per day, $P = 0.92$), whereas B-100 was less rapidly cleared (255 ± 19 vs. 386 ± 66 pools per day, $P = 0.0055$). Note that there is a significant difference between the clearance of the apoB-100 between the sexes, but not so for apoB-48. Expressed another way, apoB-48 was cleared about 7 times faster in the male and about 5 times faster in the female. Very high turnover rates for both forms of VLDL account for the low steady state levels of apoB levels that are characteristic of the mouse.

DISCUSSION

We report a methodology that allows the simultaneous measurement of triglyceride and apolipoprotein B (apoB) secretion rates from the liver in an intact mouse. The methodology relies on the previously well characterized method of Triton injection to block all peripheral removal of newly secreted triglyceride (as VLDL) from the blood (4–19). Although similar approaches have been described previously (32), we have combined this with a laser densitometric measurement of the apoB isolated from these animals (20–22), allowing us to simultane-

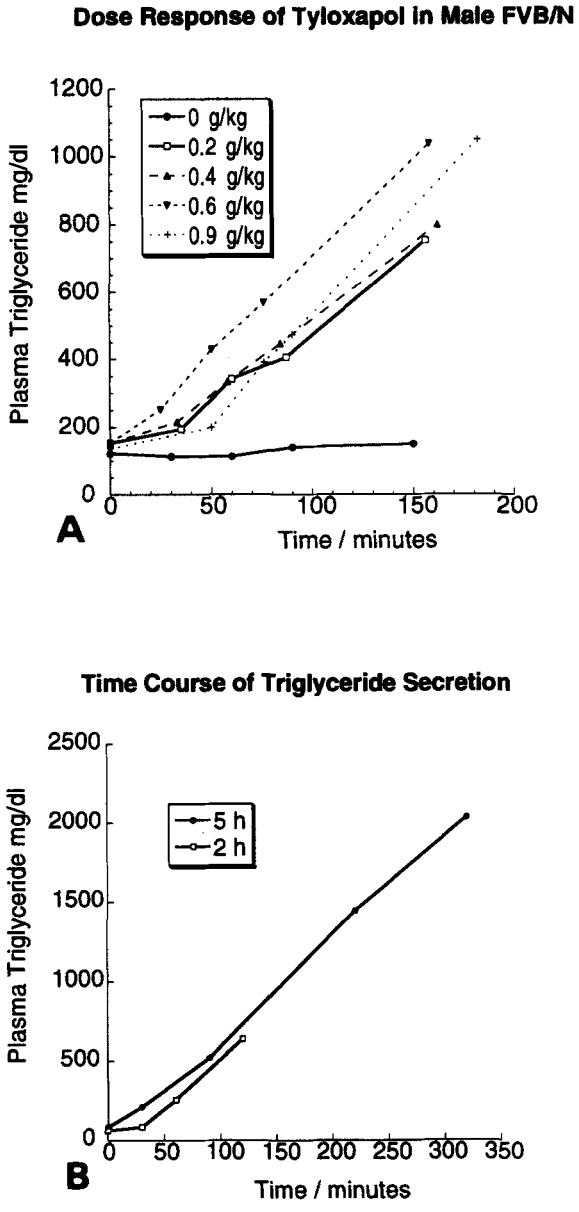

Fig. 4. Dose response and time course of tyloxapol injection. Panel A shows the dose response of male FVB/N mice to increasing concentrations of tyloxapol injection, as indicated in the inset. The data have not been corrected for differing body weights. The rates of triglyceride secretion were not significantly different at doses greater than 0.4 g/kg body weight tyloxapol injected, after correction for plasma volume and liver weights. Panel B shows the a prolonged time course. Mice were injected with tyloxapol at 0.6 g/kg body weight, and followed for 120 min (lower curve) or for 300 min (upper curve). As can be seen, the rate of triglyceride accumulation in the plasma remains linear for a considerable period, after injection. Note that there is a variable 'lag phase' that is seen in some, but not all animals.

ously measure both the apoB species secreted by the mouse liver, B-100 and B-48. Our technique can be applied to measure the rates of apoB secretion in vivo, both in genetically altered mice, such as those available through transgenic work, and after more conventional therapies, such as diet and drugs.

Our technique makes some major assumptions. *a)* The rate of triglyceride accumulation (and apoB) in the blood, after Triton injection, is a true reflection of the rate of hepatic secretion. *b)* The chromogenicity of human LDL apoB-100 is very similar to that of mouse B-100, and the B-48 chromogenicity is also not significantly dissimilar to that of B-100. There are also three minor assumptions. *c)* All the triglyceride secreted is present in the VLDL fraction in the mouse. *d)* The molecular weights of the mouse apoB-100 and B-48 are similar to those of human B-100 and B-48. *e)* There is very little secretion of lipoproteins into the blood from the intestine during the execution of our protocol.

Regarding our first assumption, since 1951, when its effects were first described (4), Triton WR-1339 has been utilized by a number of investigators to study the triglyceride secretion rates in vivo. The Triton-induced accumulation of VLDL has been extensively used to measure the rate of triglyceride production in small animals such the rat (9, 10, 12) and the mouse (23), as well as nonhuman primates (11, 13, 15, 16), squirrel monkeys (18), dogs (5, 6), and rabbits (11, 13). Catabolism of the VLDL is thought to be blocked via a mechanism involving the inhibition of lipase and LCAT activities by the removal of the apoC-II and apoA-I from the HDL particles, which are disrupted and rapidly cleared from the circulation after Triton injection (19). Other mechanisms, such as detergent coating of the lipoprotein particles, may also contribute to the Triton effect (5–7). Injection of the Triton, therefore, leads to a time-dependent accumulation of secreted VLDL in the blood, allowing for the measurements of triglyceride and cholesterol secretion rates.

Our second assumption, the chromogenicity of apoB, has recently been examined by Kotite, Bergeron, and Havel (22), who found no significant differences between chromogenicities of rat apoB-48, human apoB-48, and human B-100. Karpe et al. (20, 21) have also reported a similar approach to quantitate simultaneously apoB-100 and B-48. In addition, we have shown that the chromogenicity of both B-100 and B-48 varies linearly with mass (Fig. 3A), and the relationship of B-100 chromogenicity to that of B-48 remains fixed over an 8-fold range (Fig. 3B). We have used human apoB as our protein standard, as this is likely to reflect the chromogenic pattern of mouse apoB. Human apoB-100 can be extracted with high purity from LDL, and the protein content can be measured, using the Lowry technique (28) or another suitable protein assay. From the limited sequence data available, mouse apoB and human apoB are also very similar in structure, hence our estimations of the mouse apoB are likely to be closer to the actual values.

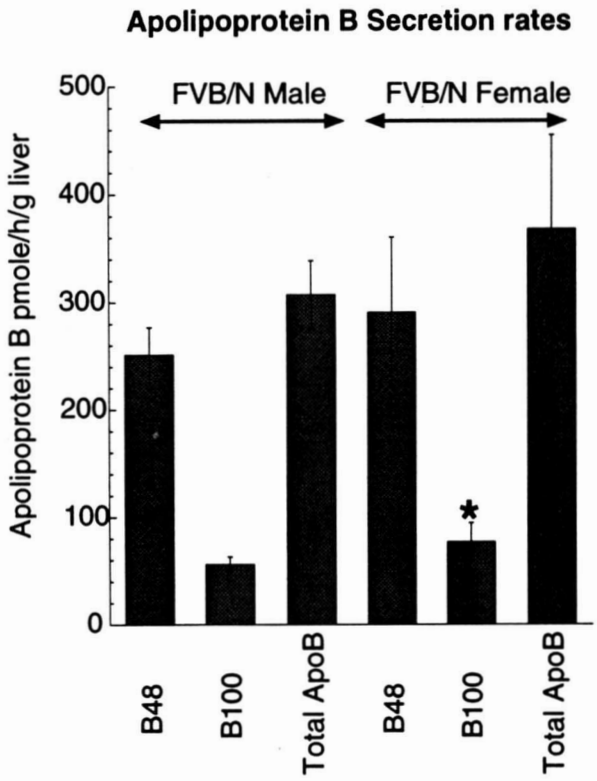

Fig. 5. Apolipoprotein B secretion rates in FVB/N male and female mice. ApoB-100 and apoB-48 rates of secretion were quantitated as described in Methods. The data are expressed in pmol/h per gram liver weight, and the error bars indicate ± SD. Males secrete 56 ± 7.3 pmol/h • g apoB-100 versus 77 ± 1.7 pmol/h •g for females (P = 0.035). For B-48, the values are 251 ± 25.8 and 290 ± 70 pmol/h •g liver respectively (P = 0.24).

Third, our fractionation procedures result in the isolation of lipoprotein particles with a density of 1.02 g/ml or less. This would allow us to collect all the VLDL and most, if not all, of the LDL from the mouse plasma (33). Our measurement of the apoBs relies on the quantitative recovery of VLDL, and we have assumed that all the secreted triglyceride is present in this fraction. This, our third assumption, is reasonable, as apoB-containing lipoproteins are the major, if not the only, vehicles for triglyceride secretion from the liver and the intestine. The infranatant fraction, after removal of the VLDL portions, contained between 5 and 10% of the total triglyceride loaded onto the gradient (data not shown). We estimate that some of this 'loss' represents the adherence of VLDL to the sides of the tubes during centrifugation, hence our assumption may be valid.

During the execution of our protocol, the intestine could have contributed to the triglyceride/apoB pool. We did not fast these animals totally, but allowed them access to a fat-free diet for 2 h prior to the Triton injection and 5 h post-injection. Under these circumstances, intestinal secretion of triglyceride-rich lipoproteins should be minimal. Any lipoproteins secreted prior to this period would be rapidly cleared from the blood in the 2 h prior to the injection. A fat-free diet was used, in preference to no food, because prolonged fasting can affect lipoprotein metabolism (23). A fasting time of as little as 4 h in mice has been shown to affect lipoprotein metabolism. It is possible that the intestine continued to produce B-48-containing lipoprotein particles during our experimental period. Although in humans, very little apoB-48 is detected in the plasma in the fasted state, rodents may continue secreting apoB-48-containing lipoproteins during this period. Hence, in rats with lymph fistulae, the amount of apoB protein did not decline under fasting conditions, although there was a dramatic fall in the triglyceride content (34). Hence, although the triglyceride contribution may have been minimal, the apoB contribution could be significant. Similar data for the mouse are not available, but the discrepancy in the ratio of B-48 to B-100 secreted and the ratio of edited to unedited mRNA seen in our animals could be explained if the intestine continued to secrete B-48-containing lipoproteins. If this is the case, our analyses allow us to estimate that the intestine contributed 17–22% of apoB-48, by assuming that our measured levels of hepatic mRNA editing reflect the proportions of hepatic apoB-48 and B-100 secreted.

Finally, the potential site of inaccuracy of our system comes from our estimation of the baseline apoB meas-

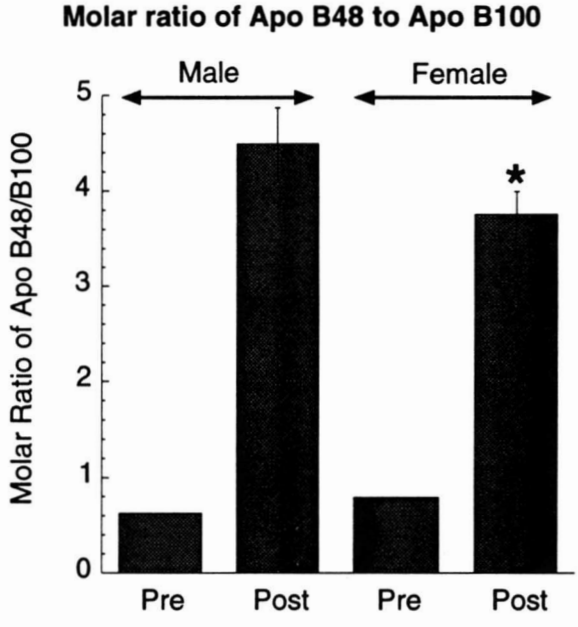

Fig. 6. Pre- and post-injection ratios of apoB-48 to B-100 in VLDL. The molar ratio of B-48 to B-100 was determined at baseline or after 5 h post-injection of tyloxapol in male and female FVB/N mice. Compared to a pre-injection ratio of 0.63, the ratio increased to 4.50 ± 0.37 (SD) in the males, compared with a baseline value of 0.79 in the females that rose to 3.75 ± 0.24 in the females. Analysis of the post-injection ratios between the males and females showed a statistically significant difference (P = 0.0098). See text for discussion.

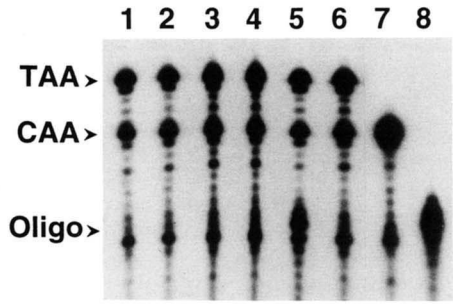

Fig. 7. Primer extension analysis of total liver RNA. To determine the proportions of steady state levels of edited apoB mRNA, cDNA was synthesized from total RNA extracted from male and female livers, amplified for the apoB sequence spanning the edited base, and analyzed for the extent of editing by primer extension analysis as described in Methods. The positions of migration of the products from edited (TAA) or unedited (CAA) mRNA are indicated. Each track (1–3, males and 4–6, females) represents RNA from one animal. Track 7 is a control for read-through, using cloned mouse apoB DNA containing CAA only. In the absence of reverse transcription of the RNA, no extension products were seen (a representative sample shown, track 8).

urements. Despite our ability to measure apoB in the range of 5 μg/ml, the average mouse plasma has an apoB level of 1.6–2 μg/ml, hence we have to pool plasma from at least three animals to obtain this value. Whilst we routinely correct for losses, using the plasma and VLDL triglyceride values, an error of more than 10% could easily affect our calculations of the VLDL-apoB fractional turnover rates. The calculation of apoB secretion rates is less affected than the fractional turnover rates because the amount of apoB secreted is far greater than the baseline values. With an assumed error rate of 100% in our baseline apoB determinations, the mouse still has a turnover that is orders of magnitude faster than in the human. For example, in humans the VLDL apoB pools may have a turnover of between 2- and 10 pools per day, and for comparison the LDL pools may be between 0.2 to 2 pools per day (35). In contrast, the turnover for apoB-48-containing VLDL in the mouse is approximately 1800 pools per day (both sexes), and for B-100-containing VLDL, it is 260 and 390 pools per day in males and female mice, respectively.

Hepatic secretion of triglycerides and apoB in the mouse reveal striking differences in fatty acid and triglyceride metabolism between mice and humans. Comparison of absolute secretion rates for VLDL triglycerides and VLDL apoB, corrected for total body weight, are enormously higher in mice than humans. Triglyceride secretion rates in FVB/N mice averaged 290 mg/kg • h (body weight), compared to an estimated rate of 10–30 mg/kg • h for humans (36). In humans, most of the fuel supplied to the muscle and other peripheral tissues is derived from glucose and circulating free fatty acids (FFA). Of the FFA, under fasting conditions, most of this is supplied by the adipose stores, whereas on feeding, dietary fatty acids are unloaded from chylomicrons. Hepatic VLDL, in humans, makes a relatively small contribution to FFA delivery. In the mouse, in contrast, VLDL triglyceride secretion rates are proportionately much higher than in humans and represent a major source of FFA for the periphery. Because mice contain relatively small stores of adipose tissue, the bulk of the fatty acids for VLDL triglycerides is synthesized in the liver, presumably from carbohydrate, and delivery of VLDL triglycerides becomes the major source of fuel for the periphery. The higher relative basal metabolic rates and the frequent feeding habits are pertinent to this. One could speculate that the rapid turnover of VLDL apoB is therefore a 'necessity' for such animals, otherwise the accumulation of apoB in the plasma, as is seen in humans, would lead to rapid atherosclerosis and premature death. Total apoB secretion in the mouse is between 13 and 16 nmol/kg • h (body weight), compared with humans where these rates have been estimated to range from 2 to 4 nmol/kg • h (35, 37–39). These values are expressed in molarity, as opposed to mass, because murine VLDL contains both B-48 and B-100.

Our methodology can be applied to the study of a variety of animal models, transgenic as well as animals on dietary and drug regimens, to measure the hepatic rates of VLDL, apoB-48 and B-100 secretion. For example, it is well established that in the apoE and LDL-re-

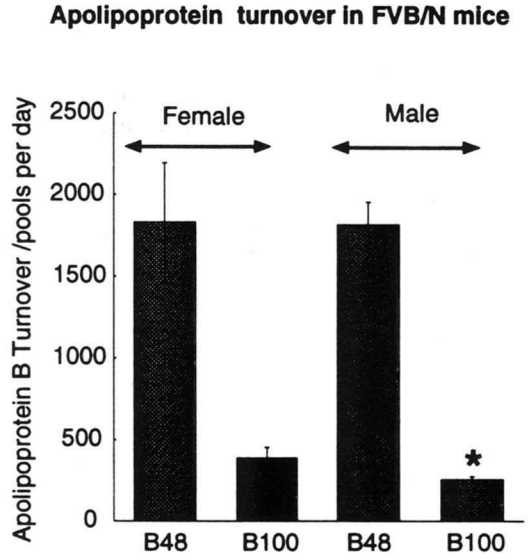

Fig. 8. VLDL apoB turnover rates in male and female FVB/N mice. Turnover rates for apoB-48 and B-100 were computed from the steady state baseline values and from the rates of secretion of apoB after tyloxapol injection. A pool is defined as the total amount of apoB in the plasma at baseline. The error bars indicate SD. Hence, for apoB-48-VLDL, males have a turnover of 1814 ± 139 pools per day compared with 1831 ± 365 pools per day in the female (P = 0.92). For B-100-VLDL, males have a turnover of 255 ± 19 pools per day compared with 386 ± 66 pools per day for the female (P = 0.0055).

ceptor knockout animals, there is a profound block in the clearance of the secreted B/E lipoproteins. However, it is unknown whether the rates of secretion from the liver are also affected under these circumstances. Similarly, it has been hypothesized that obesity, genetic or acquired, may lead to an overproduction of apoB from the liver. Although indirect measurements, both in human and animal studies, suggest this to be true, our methodology should allow us to directly measure secretory rates to test such hypotheses. In addition, our method has the advantage of simultaneously measuring both apoB-48 and B-100, thus allowing us to determine the influence of experimental manipulation on their secretory rates (as well as mRNA editing).

The reported data here show that the turnovers of apoB-100 and B-48 in the VLDL fractions in vivo are very rapid for the mouse. Indeed, assuming that the turnover of apoB in the mouse is entirely within the VLDL compartment, apoB-48 has an estimated turnover of about 1800 pools per day, with no significant sex differences, but the turnover of B-100 is both much slower and shows significant sex differences (255 ± 19 pools per day in the male versus 386 ± 65 pools per day in the female, $P = 0.006$).

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