Regulation of Hepatic Apolipoprotein B-lipoprotein Assembly and Secretion by the Availability of Fatty Acids

I. DIFFERENTIAL RESPONSE TO THE DELIVERY OF FATTY ACIDS VIA ALBUMIN OR REMNANT-LIKE EMULSION PARTICLES

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The in vivo effects of increased delivery of fatty acids (FA) to the liver are poorly defined. Therefore, we compared the effects of infusing either 6 mM oleic acid (OA) bound to albumin, 0.5–20% Intralipid, or saline for 3 or 6 h into male C57BL/6J mice. Infusions were followed by studies of triglyceride (TG) and apoB secretion. Although plasma FA levels increased similarly after either 20% Intralipid or 6 mM OA, TG secretion increased only after infusion of 4–20% Intralipid; TG secretion was unchanged by 6 mM OA. By contrast, 6-h infusions of either 6 mM OA or 4–20% Intralipid increased apoB secretion. 6 mM OA and 20% Intralipid each increased secretion of apoB from primary hepatocytes ex vivo. Importantly, 0.5–2% Intralipid, which delivered more FA to the liver than 6 mM OA, did not stimulate apoB secretion. Hepatic apoB mRNA levels were unaffected by either 6 mM OA or 20% Intralipid, but microsomal triglyceride transfer protein mRNA was significantly lower after 6-h infusions with 6 mM OA versus either saline or 20% Intralipid. Lower microsomal triglyceride transfer protein mRNA levels were associated with reduced hepatic TG mass after 6-h infusions of 6 mM OA. We conclude that 1) increased FA delivery to the liver in vivo increases secretion of apoB-lipoproteins via post-transcriptional mechanisms, 2) OA-induced apoB-lipoprotein secretion occurred at least in part via mechanisms other than by providing substrate for TG synthesis, and 3) the route of delivery of FA is important for its effects on apoB secretion.

First and foremost is the question of whether increased flux of plasma fatty acids (FA) to the liver can stimulate VLDL secretion. Studies in cultured liver cell lines, primary hepatocytes, and perfused livers have provided conflicting results relative to the role of FA delivery in VLDL secretion. Thus, several (8–13), but not all (14–16) studies in cultured liver cell lines support the proposal that apoB secretion is increased by increased FA uptake. Studies in primary hepatocytes from fed rats (16–18) or hamsters (19, 20) have failed to show that exogenous FA stimulate secretion of apoB. However, we were able recently to demonstrate oleic acid (OA)-induced apoB secretion in primary hepatocytes from fasted mice (21). Results from perfused rat livers have also been mixed; OA had no effect on apoB secretion in chow-fed rats (22) but increased apoB secretion in fasted (22) or high carbohydrate diet-fed (23) rats. In vivo studies in humans have also been inconclusive. Lewis et al. (24, 25) demonstrate that, when they increased the plasma levels of FA by infusing Intralipid and heparin intravenously into normal humans, they were able to stimulate VLDL production. By contrast, Malmstrom et al. (26) were unable to increase VLDL apoB secretion when they raised plasma levels of FA by giving subjects only heparin by intravenous infusion for 8 h. Defining the role of FA in stimulating apoB secretion is critical because of the potential link between insulin resistance at the level of adipose tissues, increased release of adipocyte-derived FA into the circulation, and increased TG accumulation in non-adipose organs, including the liver (27, 28). Increased FA flux, as observed in insulin-resistant states, has been linked to increased secretion of VLDL particles in humans (5, 29–33).

Among other questions remaining unanswered are the following. 1) Is metabolic channeling of FA into VLDL TGs dependent on the origin of the FA? 2) Do FA only act as a substrate for core and surface lipids of apoB-lipoproteins, or can they act as signaling molecules, stimulating specific steps in the transport of apoB through the secretory pathway? Our recent work in cultured liver cell lines supports a signaling role for FA (36). 3) What are the mechanisms whereby the liver maintains lipid homeostasis in the face of increasing or decreasing demands related to the secretion of TG-rich apoB-lipoproteins? Will increased FA availability always lead to increased assembly and secretion of apoB-lipoproteins, and will decreased FA availability provide signals leading to decreased secretion of apoB-lipoproteins? These unanswered questions inspired us to develop an ex-
perimental approach that would allow us to study in more detail the in vivo effects of increased hepatic FA availability on both the secretion of apoB-lipoproteins from the liver and the response of the liver to changes in apoB secretion. In our first studies we addressed the following two hypotheses; first, that in vivo FA flux to the liver is an important determinant of the assembly and secretion of apoB-lipoproteins, and second, that the in vivo effects of FA on secretion of apoB, like those in cultured liver cells, are post-transcriptional. By infusing either 6 mM OA bound to albumin or varying concentrations of the lipid emulsion, Intralipid, via chronic jugular vein catheterization, we found that increasing plasma FA levels by either 6 mM OA or 4–20% Intralipid infusion increased the secretion of newly synthesized apoB through post-transcriptional mechanisms. Unexpectedly, 4–20% Intralipid increased the concomitant secretion of TGs, whereas the effect of 6 mM OA was only on apoB secretion. Importantly, 0.5–2% Intralipid, which delivered more TG-FA to the liver than 6 mM OA, did not stimulate apoB secretion. Although Intralipid is composed of TGs carrying several different FA as well as phospholipid and glycerol, the disparate results we observed suggest that the effect of albumin-bound OA on apoB secretion was related to its route of delivery. Furthermore, Intralipid at concentrations less than 4% did not stimulate either apoB or TG secretion despite the fact that those concentrations would have delivered more FA to the liver than would the infusion of 6 mM OA. These results have provided us with several new insights regarding the assembly and secretion of apoB-lipoproteins.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—The animals were male C57BL/6J mice, age 12–14 weeks, purchased from The Jackson Laboratory, Bar Harbor, ME. All mice were maintained on a 12-h light/dark cycle (light cycle was 7 a.m. to 7 p.m.) and on a regular rodent chow diet.

**Surgical Procedures**—Mice were anesthetized with 3.3 μg/kg body weight of ketamine (15 mg/ml) and xylazine (3 mg/ml). The incision, location of the jugular vein, insertion of the cannula, and closing of the wound were carried out with a dissection microscope after the operative procedure developed for rats (37). In brief, silicone rubber tubing (catalog number 11-189-15A, 0.51-mm inner diameter, 0.94-mm outer diameter, Fisher) filled with saline was inserted into the jugular vein, and the outer portion of the tubing was tunneled subcutaneously, exiting at the nape of the neck. The length of tubing inserted into the jugular vein was 5 mm (38). Once the tubing was in place, it was filled with saline to maintain patency and was closed at the end with a metal stopper. The mice were allowed to recover for 2–3 days before the experiment was performed. The tubing was flushed several times during that period.

**Lipid, Glucose, and Insulin Determinations**—Total plasma TG concentrations were measured using commercial kits (INFINITY™ Triglyceride Reagent, Sigma). Plasma-free FA levels were measured by a colorimetric method using a commercial kit (number 994-75409) from Wako Chemicals (Richmond, VA). Plasma insulin concentrations were measured by enzyme-linked immunosorbent assay using a commercial kit (Merodia, ultrasensitive rat insulin ELISA, American Laboratory Products Co., Windham, NH). Glucose levels were measured using an enzymatic kit (No. S15-106; Sigma). The fatty acid composition of the soybean oil was: C16:0, 10.4%; C18:0, 4.0%; C18:1, 23.5%; C18:2, 53.5%; C18:3, 8.3%.

On the morning of the experiment, food was removed, and the silicone tubing was flushed with saline and connected through polyethylene tubing to a Harvard Compact Infusion Pump (Harvard Apparatus, Holliston, MA). The animals were fasted for the duration of the experiment. The infusion line was inserted subcutaneously and tunneled to the back of the mouse. The infusion line was connected to a syringe pump that delivered a 2% solution of Triton WR 1339 (44) for the determination of TG mass. [14C]Triton X-100 (150 mg) were homogenized and extracted twice with a chloroform:methanol (2:1 v/v) solution. The organic layer was dried under nitrogen gas and resolubilized in chloroform. An aliquot of this chloroform solution was reconstituted in an aqueous solution containing 2% Triton X-100 (44) for the determination of TG mass. [14C]Triton X-100 (150 mg) were homogenized and extracted twice with a chloroform:methanol (2:1 v/v) solution. The organic layer was dried under nitrogen gas and resolubilized in chloroform. An aliquot of this chloroform solution was reconstituted in an aqueous solution containing 2% Triton X-100 (44) for the determination of TG mass. [14C]Triton X-100 (150 mg) were homogenized and extracted twice with a chloroform:methanol (2:1 v/v) solution. The organic layer was dried under nitrogen gas and resolubilized in chloroform. An aliquot of this chloroform solution was reconstituted in an aqueous solution containing 2% Triton X-100 (44) for the determination of TG mass. [14C]Triton X-100 (150 mg) were homogenized and extracted twice with a chloroform:methanol (2:1 v/v) solution. The organic layer was dried under nitrogen gas and resolubilized in chloroform. An aliquot of this chloroform solution was reconstituted in an aqueous solution containing 2% Triton X-100 (44) for the determination of TG mass.
obtained from Promega (Madison, WI) and Invitrogen. DNA sequences of these clones were verified by DNA sequencing. The amplified products were cloned into a PCRII vector using a TA cloning kit obtained from Invitrogen. We used reverse transcription (RT)-PCR to generate cDNA from total RNA (male C57 BL/6J mice) by reverse transcription-PCR. The probe for apoB (46) was generated by amplification of the target gene from liver mRNA. 5 mg of liver mRNA was hydridized to a test riboprobe and a reference riboprobe in a hybridization solution. The hybridization solution contained 5 mg of liver mRNA, 30% formamide, 0.05% xylene cyanol, 0.05% bromphenol blue, 20 mM EDTA and separated on 5% PAGE, 8% urea gels. Dried gels were exposed to x-ray films overnight at −80 °C. For quantification, protected RNA fragments were cut out and counted in a liquid scintillation counter.

RNA Probe Preparation and RNase Protection Assays—Total cellular RNA was isolated from livers using TRIzol reagent following the protocol provided by the company (Invitrogen). The RNA probe for mouse apoB (46) was generated by amplification of the target gene from liver RNA (male C57BL/6J mice) by reverse transcription-PCR. The probe for MTP was described previously (39). PCR primers used and the size of amplified products for each probe are shown in Table I. The PCR products were cloned into a PCRiI vector using a TA cloning kit obtained from Invitrogen. DNA sequences of these clones were verified by DNA sequencing using a PerkinElmer ABI 377 automatic DNA sequencer. Antisense probes were synthesized using an in vitro transcription kit obtained from Promega (Madison, WI) and [α-32P]CTP (800Ci/mmol) (Amersham Biosciences). Mouse cyclophilin (Ambion Co., Austin, TX) was used as reference RNA to normalize for variation in RNA loading in RNase protection assays. RNase protection assays were carried out as described previously (39). Briefly, total cellular RNA (10 μg) was hybridized to a test riboprobe and a reference riboprobe in a hybridization buffer (30 μl) and incubated at 48 °C overnight. After overnight hybridization, 20 units of RNase T2 (Invitrogen) was added to the mix. Control incubations with probe plus yeast mRNA with or without RNase T2 were used as controls. After incubation at 37 °C for 2 h, RNase T2 was removed by phenol extraction, and protected RNA fragments were ethanol-purified and resuspended in 5 μl of loading buffer (95% formamide, 0.05% xylene cyanol, 0.05% bromphenol blue, 20 mM EDTA) and separated on 5% PAGE, 8% urea gels. Dried gels were exposed to x-ray films overnight at −80 °C. For quantification, protected RNA fragments were cut out and counted in a liquid scintillation counter.

### RESULTS

We conducted preliminary studies to determine the optimal concentration of Intralipid as well as the time course of changes in plasma FA and TG in response to infusions of OA and Intralipid. Our goal was to increase plasma FA levels to high physiological levels, about three times above base line, for several hours. We infused Intralipid at concentrations ranging from 0.5 to 20% for 6 h through a cannulated jugular vein. The results, depicted in Fig. 1A, demonstrate that concentrations of Intralipid between 0.5 and 10% did not increase plasma FA levels significantly; infusion of 20% Intralipid, which delivered 172 mg of TG-FA into the circulation over 6 h, raised plasma FA levels more than 2-fold compared with saline (0.80 ± 0.16 versus 0.90 ± 0.12 mmol/liter; p < 0.01). The finding that infusion of 0.5% Intralipid was the highest concentration we could use without adversely affecting the mice, and that concentrations satisfied our need in terms of raising plasma FA levels. Indeed, a 6-h infusion of 6 mM OA raised plasma FA levels to 1.08 ± 0.32 mmol/liter (n = 9) (Fig. 1B), and this was significantly greater than the FA level achieved by saline. Plasma FA concentrations tended to be higher after 6 mM OA versus 20% Intralipid infusions, but the levels were not significantly different from each other (p = 0.07). The finding that infusion of 0.5–10% Intralipid over 6 h, which delivered between 4.3 and 86 mg of TGFA into circulation (compared with the 1.5 mg of FA delivered by infusion of 6 mM OA), did not increase plasma FA levels suggested that 1) much of the FA generated by peripheral lipolysis of Intralipid TG was internalized in the adipose and muscle beds, where lipoprotein lipase was active, and/or 2) most of the Intralipid TG was taken up by tissues together with the whole Intralipid particle.

After choosing 20% Intralipid, we determined plasma FA and TG changes during 6-h infusions of 6 mM OA, 20% Intralipid, and saline. Plasma FA and TG levels were measured at base line and 1, 2, 3 and 6 h after the start of each infusion (n = 3/group). Plasma FA levels increased in all three groups of mice during the first hour of study, suggesting both effects of stress as well as FA delivery (Fig. 1B). In the saline-infused mice, FA levels fell rapidly back toward base line, reaching base-line concentrations by 3 h. In mice infused with OA or 20% Intralipid, FA levels drifted down between 1 and 2 h of infusion but then maintained relatively constant levels thereafter. Of note, only 20% Intralipid infusion induced a significant increase in plasma TG levels over time (Fig. 1C). Based on the above studies we chose to carry out most of our infusions for 6 h. We also conducted some studies with 3-h infusions.

There were no differences in the baseline (pre-infusion) concentrations of TG, FA, glucose, or insulin among the 6 mM OA-
20% Intralipid-, and saline infusion-treated mice (Table II). In particular, base-line plasma FA levels were comparable in all the groups and were ~0.2–0.3 mmol/liter. After 6-h infusions, FA levels were significantly increased in 6 mM OA and 20% Intralipid groups to 1.08 and 0.8 mmol/liter, respectively (p < 0.05 both versus saline). Lower concentrations of Intralipid did not increase plasma FA levels significantly versus saline. The time course for changes in plasma FA during the 6-h infusions of OA and 20% Intralipid was examined next. Plasma FA levels were measured at base line and 1, 2, 3, and 6 h after starting the infusions (B). Plasma FA levels increased during the first 1 h of infusion of either OA or Intralipid; FA levels remained stable during the OA infusion but drifted down slightly during the last few hours of the Intralipid infusion. There was also an increase in FA levels during the first 1 h of the saline infusion, but those levels returned to base line between 3 and 6 h. Only Intralipid infusion caused a significant increase in plasma TG levels over time (C). TG levels rose during the first hour of 20% Intralipid infusion and remained constant thereafter, TG levels during infusion of 6 mM OA were not different from saline. IL, Intralipid. The asterisk denotes significant differences (p < 0.05) compared with saline at the end of 6-h infusion.

20% Intralipid-, and saline infusion-treated mice (Table II). In particular, base-line plasma FA levels were comparable among the three groups (OA, 0.24 ± 0.15 mmol/liter; Intralipid, 0.20 ± 0.08 mmol/liter; saline, 0.23 ± 0.14 mmol/liter). Although human studies have indicated that acute elevations of plasma FA can stimulate insulin secretion (47) and cause insulin resistance (48), there were no significant changes in either plasma insulin or plasma glucose levels at the end of the infusions in the mice receiving either OA or Intralipid compared with mice receiving saline (Fig. 2).

To determine the effects of elevations of plasma FA on the assembly and secretion of VLDL, TG and apoB secretion rates were measured after completion of the infusions using Triton WR1339, a surfactant that coats lipoprotein particles and inhibits their clearance from plasma. With lipoprotein catabolism suppressed, increases in plasma TG over time are indicative of the rate at which TG is secreted from the liver (41). Fig. 3A shows the data for 3-h infusions of saline, 20% Intralipid, and 6 mM OA, and Fig. 3B shows the data from 6-h infusions. The linear graphs on the left show the plasma TG levels before and during a 120-min period after injection of Triton. At the end of the 3- or 6-h infusions (which are labeled as 0 min in the figure), plasma TG levels were, as expected (see Fig. 1C), significantly higher in the mice infused with 20% Intralipid than in either the OA- or the saline-infused mice. After Triton injection, plasma TG levels increased steadily over the next 120 min in all groups; the rate of rise was greater in the mice infused with Intralipid versus the mice infused with OA or saline. The rates of increase in plasma TG after the 6-h infusions followed by Triton were 5.3 ± 1.5 versus 2.9 ± 1.1 and 2.2 ± 0.7 mg/h for Intralipid, OA, and saline, respectively (p < 0.001). Similar rates were seen after the Triton studies after 3-h infusions. The absolute increase in plasma TG concentration between 30 and 120 min was about twice as great after Intralipid infusion versus either OA or saline infusions in both 3- and 6-h infusion studies (507 ± 62 versus 280 ± 58 and 254 ± 67 mg/dl, p < 0.05,
after 3-h infusions and 458 ± 123 versus 227 ± 76 and 188 ± 54 mg/dl, p < 0.05 after 6-h infusions) (bar graphs on the right of Fig. 3, A and B). Of note, although the absolute increases in plasma TG concentration between 30 and 120 min after Triton in the mice infused with OA were slightly greater than the increases seen in mice infused with saline for either 3 or 6 h, the differences were not statistically significant. Thus, the infusion of Intralipid, but not OA, led to increased TG secretion relative to the mice infused with saline for both 3 and 6 h.

To quantify the number of apoB-lipoproteins secreted from the liver after each infusion, the appearance in plasma of newly synthesized apoB was estimated using [35S]methionine and the liver after each infusion, the appearance in plasma of newly synthesized and secreted (radiolabeled) apoB100 (Fig. 4A) and apoB48 (Fig. 4B) levels at either 60 or 120 min after Triton injection in either OA- or Intralipid-infused mice compared with saline-infused mice. By contrast, after 6-h infusions (Fig. 5), secretion of newly synthesized apoB100 during the 60 min after Triton was increased by 3-fold in both the OA and Intralipid groups as compared with saline (341 ± 145 and 331 ± 145 versus 100 ± 145%, p < 0.05) (Fig. 5A). At 120 min after Triton, the differences in labeled apoB100 levels between the OA and saline and between Intralipid and saline groups remained statistically significant. A similar pattern of appearance in plasma of newly synthesized apoB48 was observed, except that accumulation in plasma was significantly greater after both OA and Intralipid infusions relative to saline both at 60 min and at 120 min post-Triton injection (Fig. 5B). Thus, in contrast to what was observed for TG secretion, infusion of either Intralipid or OA led to increased apoB secretion relative to the mice infused with saline.

To confirm the effects of OA and Intralipid on apoB secretion at the cellular level, we isolated primary hepatocytes immediately at the end of infusions of saline, 6 mM OA, or 20% Intralipid and performed stable metabolic labeling studies with [35S]methionine. We did three separate, independent experiments and pooled the data for statistical analysis. In Fig. 6A, the data, expressed as percent of apoB secreted from hepatocytes isolated from saline-infused mice, indicate that the secretion of both apoB100 and apoB48 from hepatocytes was significantly greater in the mice infused with either OA or Intralipid versus the mice infused with saline (apoB100, 162 ± 40 and 223 ± 67 versus 100 ± 20%, p < 0.05, both comparisons; apoB48, 227 ± 39 and 153 ± 15 versus 100 ± 10%, p < 0.05, both comparisons). There were no differences in the cellular levels of newly synthesized apoB100 between either the OA and saline groups or the Intralipid and saline groups (OA (72 ± 17%)- and Intralipid (112 ± 38%)- versus saline (100 ± 25%); not significant, both comparisons), although cell levels of apoB100 were lower in the OA-infused versus the Intralipid-infused group (p < 0.05) (Fig. 6B). Cell levels of newly synthesized apoB48 were higher in hepatocytes isolated from OA-infused mice compared with hepatocytes from mice infused with saline (159 ± 25 versus 100 ± 18%, p < 0.05), but no differences were observed in cell apoB48 levels between the Intralipid and saline infusion groups (112 ± 19 versus 100 ± 18%). Overall, these results indicate that primary hepatocytes from mice in-

![Fig. 2. Neither OA nor Intralipid infusion caused significant changes in plasma insulin and glucose levels compared with saline.](http://www.jbc.org/)

**Table II**

Plasma FA, TG, insulin, and glucose concentrations at baseline in three groups of mice

|                  | Saline | 6 mM OA | 20% Intralipid | p  |
|------------------|--------|---------|----------------|----|
| Body weight (g)  | 24.4 ± 1.2 | 26.0 ± 2.2 | 23.6 ± 2.1 | 0.091 |
| FA (mmol/liter)  | 0.24 ± 0.16 | 0.20 ± 0.08 | 0.23 ± 0.14 | 0.755 |
| TG (mg/dl)       | 40.0 ± 17.0 | 48.0 ± 22.0 | 33.0 ± 16.0 | 0.220 |
| Insulin (ng/ml)  | 0.44 ± 0.01 | 0.46 ± 0.05 | 0.45 ± 0.02 | 0.408 |
| Glucose (mg/dl)  | 249 ± 34 | 235 ± 29 | 225 ± 22 | 0.900 |

**FIG. 2.** Neither OA nor Intralipid infusion caused significant changes in plasma insulin and glucose levels compared with saline. Plasma insulin (A) and glucose (B) concentrations were measured in three groups (saline, n = 11; 6 mM OA, n = 9; 20% Intralipid, n = 6) at base line and at the end of infusions. The values represent the average concentrations of plasma insulin (ng/ml) and glucose (mg/dl) in each group. Open bars, at base line. Closed bars, at the end of infusion. IL, Intralipid.
Fused with either OA or Intralipid secreted more newly synthesized apoB-containing lipoproteins compared with the mice infused with saline. Increased rates of apoB secretion after OA or Intralipid infusion could have resulted from increased transcription of the apoB gene and a subsequent increase in apoB synthesis (2).

**FIG. 3.** Infusion of 20% Intralipid, but not 6 mM OA, increased TG secretion in both 3- and 6-h infusions. The mice were infused with saline, 6 mM OA, and 20% Intralipid for 3 (A) or 6 h (B). At the end of the infusions, Triton WR1339 was injected intravenously. Blood samples were collected at base line, the end of infusion (just before injection of Triton, 0 min), and 30, 60, 120 min after Triton injection to measure plasma TG concentrations. In the graphs on the left of A and B, the mean plasma TG concentrations (mg/dl) were plotted against time. At the end of the infusions (0 min; before injection of Triton), the plasma TG levels were significantly higher in the Intralipid group than in the OA and saline groups. Plasma TG levels increased steadily in all three groups over the 120 min after the injection of Triton, but only 20% Intralipid significantly increased TG secretion compared with saline infusion. The same data expressed as the absolute increase in plasma TG levels between 30 and 120 min after Triton are shown in bar graphs on the right. TG base line, TG at base line before infusions. TG 0, TG at the end of the infusions just before Triton injection. The asterisk denotes significant differences \( p < 0.05 \) between 20% Intralipid and both OA and saline infusions after 3- and after 6-h infusions.

**FIG. 4.** There was no significant increase in secretion of \( ^{35} \)S-labeled apoB100 and apoB48 after 3 h of infusion of either 6 mM OA or 20% Intralipid. The effect of 3-h infusions of 6 mM OA or 20% Intralipid on secretion of newly synthesized apoB100 (A) and apoB48 (B) into plasma is depicted relative to saline (n = 6/group). Plasma \( ^{35} \)S-labeled apoB levels at 60 and 120 min after Triton and \( ^{35} \)S-methionine injection were determined by 4% SDS-PAGE, autoradiography, and densitometry scan. The relative levels of newly secreted plasma \( ^{35} \)S-labeled apoB are presented as a percentage of newly secreted plasma apoB in saline-infused mice.
However, neither 6 mM OA nor 20% Intralipid infusions affected hepatic apoB mRNA levels compared with saline infusions (Fig. 7). These data indicate that the increased secretion of apoB in mice infused with either OA or Intralipid originated at a posttranscriptional level.

The discordant effects of OA and Intralipid suggested that hepatic lipid content would be affected differently by each infusion. Therefore, we measured liver TG content at the end of the 3- and 6-h infusions without Triton WR 1339 (Fig. 8). There were no significant differences in liver TG content at 3-h infusions of saline, 6 mM OA, or 20% Intralipid (189 ± 32 μg/mg of protein and 199 ± 19 μg/mg of protein versus 242 ± 64 μg/mg of protein, respectively; n = 3/group, p > 0.05) even though Intralipid-infused mice tended to have higher liver TG content relative to OA- and saline-infused mice. Interestingly, we found that at the end of the 6-h infusions, livers from mice infused with OA had less TG than the mice infused with either Intralipid or saline (OA, 109 ± 32 μg/mg of protein, n = 11, versus Intralipid, 202 ± 38 μg/mg of protein, n = 11, and versus saline, 162 ± 54 μg/mg of protein, n = 13; p < 0.05 both comparisons). Again, there was a trend toward more hepatic TG in the livers of Intralipid-infused mice compared with saline-infused mice.

Infusion of 20% Intralipid had the potential to deliver much more FA (from TG-FA) to the liver than 6 mM OA. Indeed, as noted above, 6-h infusions of 20% Intralipid could potentially deliver about 172 mg of FA to the liver, whereas 6 mM OA had the potential to deliver only about 1.5 mg of FA to the liver. To gain insight into the actual amounts of FA actually delivered during the infusions, we conducted additional studies. We injected Intralipid labeled with [14C]triolein (labeled in OA) or...
[3H]OA bound to albumin through the jugular vein immediately after 4.5-h infusions of 20% Intralipid (n = 3) and 6 mM OA (n = 3), respectively (see “Experimental Procedures”). We found that ~50% of the albumin-bound OA and 25% of the triolein-derived OA had been taken up by liver 5 min after injection of each tracer (Fig. 9). More triolein-derived OA was found in the lungs, spleen, and blood. Thus, infusion of 6 mM OA for 6 h would deliver about 0.75 mg of OA to the liver, whereas a 6-h infusion of 20% Intralipid would deliver about 43 mg of TG-FA to the liver.

As noted above, when 0.5–20% Intralipid was infused for 6 h, only 20% Intralipid raised plasma FA levels. We therefore infused mice with Intralipid, 0.5–10% for 6 h, followed by Triton and [35S]methionine injection to determine the effects of these lower concentrations on apoB and TG secretion. The increase of plasma TG between 30 and 120 min after Triton was no significant further increase in either TG or apoB secretion as a consequence of infusing Intralipid and heparin to raise FA levels, but Malmstrom et al. (26) were unable to show increased apoB secretion after raising plasma FA levels with heparin alone. The different results observed by these two groups may have resulted from different levels of FA flux, with higher flux in the study by Lewis et al. (24). On the other hand, both of these investigations utilized tracer kinetic methods to estimate apoB secretion during non-steady state situations; analysis of tracer kinetics during non-steady state conditions is very complex, and assumptions regarding whether production or fractional clearance are affected by the perturbation are required.

In the studies reported here we directly infused either OA or Intralipid to increase delivery of plasma FA to the liver. Our results demonstrate for the first time in vivo a direct link between increased plasma FA delivery and the stimulation of apoB secretion into plasma. Thus, 6-h intravenous infusions of either 6 mM OA or 20% Intralipid in C57BL/6J mice, which raised plasma FA concentrations to similar, high physiologic levels, increased the secretion of newly synthesized apoB100 and apoB48 about 3 times compared with infusions of saline. The effects of both OA and Intralipid on in vivo secretion rates were confirmed by ex vivo experiments with primary hepatocytes that were isolated and studied immediately at the end of the 6-h infusions. The increased in vivo secretion of newly synthesized apoB was not associated with changes in apoB mRNA levels. This latter result indicates that the effects of OA and Intralipid on assembly and secretion of VLDL were post-transcriptional, supporting data generated from cultured liver cells (2–4, 49).

**DISCUSSION**

Hepatic VLDL production is primarily substrate-driven, the most important regulatory substrate being FA (25, 34, 49). The total pool of FA in hepatocytes can be derived from three sources, 1) plasma FA delivered bound to albumin, 2) plasma TG-FA delivered via lysosomal hydrolysis of internalized remnant lipoproteins, and 3) de novo lipogenesis. The contribution of FA from each of these sources to TG secreted on nascent lipoproteins is unclear (34, 50). In particular, the role of plasma albumin-bound FA as a direct stimulus of the assembly and secretion of apoB-lipoproteins has remained controversial despite numerous investigations. In vivo studies in humans support an important role of plasma FA delivery to the liver as a stimulus for apoB and TG secretion (29, 30, 33, 51–53), but none of those studies directly tested the effect of increasing FA delivery to the liver. Lewis et al. (24) report increased apoB secretion as a consequence of infusing Intralipid and heparin to raise FA levels, whereas Malmstrom et al. (26) were unable to show increased apoB secretion after raising plasma FA levels with heparin. The different results observed by these two groups may have resulted from different levels of FA flux, with higher flux in the study by Lewis et al. (24). On the other hand, both of these investigations utilized tracer kinetic methods to estimate apoB secretion during non-steady state situations; analysis of tracer kinetics during non-steady state conditions is very complex, and assumptions regarding whether production or fractional clearance are affected by the perturbation are required.
The exact site(s) where the delivery of plasma FA effects apoB secretion remains to be determined. Beyond demonstrating the link between delivery of plasma FA to the liver and apoB secretion, our results offered several additional insights into the roles that hepatic FA and TG availability play in regulating the assembly and secretion of apoB-lipoproteins. First, among these new insights was that delivery of only a very small quantity of albumin-bound FA (less than 1 mg, based on the quantity of OA delivered into the circulation over 6 h by infusion of 6 mM OA and the data in Fig. 9) could stimulate increased secretion of apoB without affecting secretion of TG. This finding indicates that in vivo apoB secretion can be uncoupled from the delivery of large quantities of substrate for hepatic TG synthesis. The uncoupling between increased apoB secretion, and the availability of increased TG for co-secretion was associated with a loss of TG from the liver; hepatic TG mass was reduced by ~30% in livers from mice infused with OA for 6 h compared with saline-infused mice. The importance of uncoupled apoB secretion as the cause of reduced hepatic TG content is supported by our finding that, after 3 h of 6 mM OA infusion, at a time when apoB secretion had not yet been increased, the TG content of the liver had not yet been reduced. We were surprised that, in the absence of increased TG secretion (as measured by the Triton method), hepatic TG content was reduced between 3 and 6 h of OA infusion (when increased apoB secretion occurred). We must assume that the Triton methodology was not sensitive enough to pick up small changes in TG secretion that accompanied increased secretion of apoB-lipoprotein. Alternatively, increased oxidation of hepatic FA or decreased hepatic lipogenesis during the 6-h infusions of OA together with increased secretion of apoB-lipoproteins could have reduced hepatic TG mass. Preliminary studies

**FIG. 8.** After 6-h infusions, livers from mice infused with 6 mM OA had less TG than livers from mice infused with either 20% Intralipid or saline. At the end of 3- or 6-h infusions (without Triton WR 1339), mice infused with 6 mM OA, 20% Intralipid, and saline were sacrificed, and the livers were collected for the measurement of liver TG content. The liver TG content from 3-h infusions (n = 3/group) is shown in A, and that from 6-h infusions (n = 13, 11, 11 in saline, OA, and Intralipid groups, respectively) is shown in B. The mean values of liver TG content are expressed as μg of TG/mg of liver total protein. The asterisk denotes significant differences (p < 0.05) in both comparisons to Intralipid and saline infusion.
of the expression of genes involved in FA oxidation (CPT1, AOX) or lipogenesis (ACC, FAS), however, showed no effects of infusion of either 6 mM OA or 20% Intralipid.

We previously demonstrated in cultured HepG2 and Mc RH7777 cells that OA could stimulate the assembly and secretion of VLDL even though TG synthesis and MTP activity were inhibited (36), leading us to conclude that OA acted as a signaling molecule to allow completion of the translocation of lipid-poor apoB and its subsequent fusion with a pre-existing lipid droplet in the secretory compartment (1, 36, 58, 59). Our present data support a signaling role for OA in vivo but differ from the tissue culture results in that OA was unable to stimulate the concomitant secretion of TG. We initially thought that the different experimental protocols used in the present study versus our previous work (36) might account for the differing outcomes. In the previous cell culture experiments, we exposed those cells to OA for a relatively short period of time. Thus, we only looked at a small window of time during which pre-existing lipid droplets within the secretory pathway would have been available for fusion with the increased numbers of newly synthesized apoB molecules moving through the secretory pathway. By contrast, in the present in vivo experiments we exposed the livers to OA for several hours, raising the possibility that the secretion of VLDL was increased during the early part of the OA infusion (mimicking our prior in vitro results), but that later, during the majority of the infusion period, the lack of an adequate supply of FA to stimulate TG synthesis led to an isolated increase in apoB secretion. However, the finding that 3-h infusions of OA did not stimulate apoB secretion does not support this hypothesis, although we cannot rule out the possibility that secretion of TG increased transiently at some point between 3 and 6 h of OA infusion.

We gained several additional insights regarding regulation of the assembly and secretion of TG-rich apoB-lipoproteins from our studies with a wide range of Intralipid. First, those studies indicated that the way FA is delivered to the liver is an important determinant of its effect on apoB secretion. Thus, when 0.5, 1, or 2% Intralipid were infused for 6 h, −1, 2, or 4 mg of TG-FA was delivered to the liver. ApoB secretion, however, was not stimulated. This result strongly suggests that the delivery of TG-FA by Intralipid was not qualitatively equal to the delivery of less than 1 mg of albumin-bound OA, at least in terms of the ability to stimulate apoB secretion. Only when 4–20% Intralipid were infused for 6 h (which would have delivered between −8.5 and 43 mg of TG-FA to the liver) was apoB secretion-stimulated. We believe that the most likely explanation for these observations is that lipolysis of Intralipid TG-FA by lipoprotein or hepatic lipase was only a minor source of FA delivery to the liver. If ~0.75 mg of albumin-bound OA

![Fig. 9. Differential hepatic uptake of OA and Intralipid.](image1)

![Fig. 10. Dose response of Intralipid infusion.](image2)
was required to stimulate apoB secretion, then it appears that this critical quantity of albumin-bound FA was not available for hepatic uptake until at least 4% Intralipid, which delivered a total of about 8.5 mg of TG-FA to the liver, was infused. This would mean that only about 10% of Intralipid TG-FA delivered to the liver during that infusion arrived as albumin-bound FA after lipolysis in the circulation by either lipoprotein or hepatic lipase. This is compatible with the finding that plasma FA levels did not rise until 20% Intralipid was infused. Although it is likely that lipoprotein lipase-associated lipolysis occurred during infusion of Intralipid (as indicated by the increase in plasma FA when 20% Intralipid was used), it appears that such lipolysis was associated with efficient targeting of the liberated FA to the local tissue bed. It is also likely that hepatic lipase generated FA in the liver circulatory bed, leaving us to suggest that either this is a minor pathway or that hepatic lipase-associated lipolysis of Intralipid generated FA that were taken up by a pathway that was not linked to apoB secretion. Future studies in which lipoprotein and hepatic lipase are inhibited are planned to address these issues.

If very little Intralipid TG-FA reached the liver as albumin-bound FA, then the bulk of the TG-FA that was taken up by the liver must have been internalized as particle-associated lipid, probably after the emulsion particles had acquired apoE to become “remnant-like.” Why, then, was this source of FA unable to stimulate apoB secretion until the amounts taken up reached were about 10× the amount of albumin-bound OA that was taken up? A possible explanation is that lysosomal hydrolysis of internalized particulate TG generates FA that are unable to act as signaling molecules in the apoB secretory pathway. It has been demonstrated both in cell culture (60) and in vivo (61) that remnant lipoproteins can increase apoB secretion from the liver. In those studies, however, the delivery of TG-FA probably exceeded a critical level, similar to the conditions in which 4–20% Intralipid was infused. Indeed, our finding that 6-h infusions of 4–20% Intralipid stimulated both apoB and TG secretion suggest that Intralipid TG-FA were preferentially targeted for incorporation as a core lipid into apoB-lipoproteins.

A final, related insight from our studies relates to what has been referred to as the two-step pathway of VLDL assembly (1, 2, 58, 59). Our data support this scheme and suggest that the first step of initial apoB transport and limited lipidation may be regulated differently than the second bulk lipidation step. Specifically our studies suggest that although only a small increase in the flux of albumin-bound OA to the liver was required to stimulate apoB secretion, much larger quantities of
FA had to be delivered before TG secretion was stimulated. Although the stimulation of apoB secretion by Intralipid only occurred at concentrations of the emulsion that concomitantly stimulated TG secretion, we do not believe that this contradicts two separate and unique mechanisms for stimulating the assembly and secretion of apoB-lipoproteins. Indeed, a 3-h infusion of 20% Intralipid also stimulated TG secretion but did not affect apoB secretion. Integrating all of these data, we believe that Intralipid was inefficient at delivering FA via a pathway that mimicked albumin-bound OA in terms of effects on apoB secretion; higher concentrations of Intralipid achieved delivery of adequate FA via that pathway (or into the same pool to which albumin-bound OA was targeted) and thereby stimulated apoB secretion. The additional TG-FA delivered to the liver by higher concentrations of Intralipid did not further stimulate apoB secretion but were available for incorporation into the additional apoB-lipoproteins that were being secreted. Whether the Intralipid-derived FA that was resecreted with apoB entered the liver as albumin-bound FA after lipolysis of Intralipid TG by lipoprotein or hepatic lipase, as TG that was hydrolyzed in hepatic lysosomes, or via both pathways remains to be determined.

We appreciate that Intralipid differs in several ways from albumin-bound OA and that a potential role of the other FA, such as palmitic acid and linoleic acid, must be considered. We cannot rule out that palmitic and/or linoleic acid are targeted for incorporation as TG into newly assembled VLDL. On the other hand, if soybean oil is about 25% OA, then 2% Intralipid, which did not stimulate apoB secretion, would have delivered about 1 mg of OA to the liver. Thus, our contention that albumin-bound OA was preferentially linked to the apoB secretory pathway seems strong. We also agree that the roles of the glycerol and phospholipids provided by infusion of Intralipid need to be defined. However, examination of the composition of 20% Intralipid suggests that neither the phospholipid component, of which about 2.5 mg would have been delivered to the liver, or the glycerol content, of which about 5–6 mg would have been delivered to the liver, would have a significant impact relative to the 43 mg of FA delivered over 6 h. Furthermore, 2% Intralipid, which delivered 0.25 mg of phospholipid, 0.5 mg of glycerol, and 4.3 mg of FA to the liver did not stimulate apoB secretion, whereas 0.75 mg of OA did.

The potential role of insulin action, or resistance, in our studies also needs to be addressed in more detail in future studies. Insulin inhibits VLDL secretion acutely in cultured hepatocytes (62, 63) and in normal humans (62, 64, 65), despite increases in TG synthesis. However, insulin-resistant rodents (66) and humans (64, 67) do not exhibit this insulin-induced inhibition of apoB secretion. Our 6-h infusions of 6 mm OA or 20% Intralipid might have produced an insulin-resistant state (48), and this could have contributed to the increased apoB secretion we observed. Neither OA nor Intralipid infusions, however, caused significant changes in plasma insulin or glucose levels. Although we did not determine directly whether increased FA flux caused either global or hepatic insulin resistance, we believe that it is unlikely that the observed effects of either OA or Intralipid infusion on apoB-lipoprotein secretion were mediated by the development of hepatic insulin resistance.

Finally, a preliminary observation that deserves comment was that MTP mRNA was reduced in livers from mice infused with OA for 6 h. A critical player in the early post-transcriptional regulation of apoB secretion (68, 69), MTP appears to function in VLDL assembly by catalyzing both the initiation of lipoprotein, as well as the formation of triglyceride-rich droplets in the smooth endoplasmic reticulum. These droplets may then fuse with nascent apoB particles to produce mature lipoproteins. Hepatic MTP gene expression can be up-regulated by both high fat (70) and high sucrose (71) diets in hamsters. Ob/Ob mice have increased levels of MTP mRNA and activity (72). In each of these conditions hepatic steatosis and elevated rates of VLDL secretion are present, suggesting that the increases in MTP gene expression are part of a response to maintain hepatic lipid homeostasis. This hypothesis is supported by our finding that hepatic MTP mRNA levels were lower in mice infused with OA for 6 h; a condition where increased apoB secretion in the absence of adequate TG availability led to reductions in hepatic TG content. By contrast, we did not see changes in MTP mRNA after infusion of OA for only 3 h, when apoB secretion had not yet increased, or with Intralipid infusions for either 3 or 6 h, where there were modest increases in both hepatic TG content and secretion. Because MTP protein has a long half-life (54), we do not believe that the changes in mRNA had any effect on the outcomes we observed. The molecular basis of the reduction in MTP gene expression to OA, which would over time be expected to reduce cellular MTP protein and activity, will require further study.

In conclusion, increased FA flux to the liver in vivo can increase secretion of apoB-lipoproteins through post-transcriptional mechanisms. Infusion of a very small quantity of albumin-bound OA stimulated apoB secretion without concomitant stimulation of TG; this uncoupling between increased apoB secretion and the availability of additional TG for co-secretion was associated with reduced hepatic TG mass and decreased hepatic MTP mRNA levels. Studies with a wide range of concentrations of Intralipid indicated that the route of delivery of FA to the liver may be critical for its effects on apoB secretion and supported unique effects for FA and TG on the assembly and secretion of apoB-lipoproteins. Our results, based on in vivo perturbations, could be relevant to the observations that secretion of apoB-lipoproteins is increased in individuals with insulin resistance or with combined hyperlipidemia, a syndrome that has been linked to increased plasma FA flux, whether or not they have hypertriglyceridemia (29–33).

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J. Biol. Chem. 2004, 279:19362-19374.
doi: 10.1074/jbc.M400220200 originally published online February 17, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400220200

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