Apolipoprotein B Secretion Is Regulated by Hepatic Triglyceride, and Not Insulin, in a Model of Increased Hepatic Insulin Signaling

Byoung C. Moon, Antonio Hernandez-Ono, Bangyan Stiles, Hong Wu, Henry N. Ginsberg

Objective—States of insulin resistance, hyperinsulinemia, and hepatic steatosis are associated with increased secretion of triglycerides (TG) and apolipoprotein B (apoB), even though insulin targets apoB for degradation. We used hepatic-specific “phosphatase and tensin homologue deleted on chromosome 10” (Pten) knockout (hPten-ko) mice, with increased hepatic insulin signaling, to determine the relative roles of insulin signaling and hepatic TG in regulating apoB secretion.

Methods and Results—TG and apoB secretion was elevated in hPten-ko mice. When hepatic TG was reduced by inhibition of DGAT1/DGAT2 or SREBP-1c, both TG secretion and apoB secretion fell without changes in hepatic insulin signaling. Acute reconstitution of hPten reduced hepatic TG content, and both TG and apoB secretion fell within 4 days despite decreased hepatic insulin signaling. Acute depletion of hepatic Pten by adenoviral introduction of Cre into Pten floxed mice caused steatosis within 4 days, and secretion of both TG and apoB increased despite increased hepatic insulin signaling. Even when steatosis after acute Pten depletion was prevented by pretreatment with SREBP-1c antisense oligonucleotides, apoB secretion was not reduced after 4 days. Ex vivo results were in primary hepatocytes were similar.

Conclusion—Either hepatic TG is the dominant regulator of apoB secretion or any inhibitory effects of hepatic insulin signaling on apoB secretion is very short-lived. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: insulin resistance □ lipoproteins □ apolipoprotein B □ steatosis □ triglycerides

During the past 25 years, studies in cells, small animals, and humans have demonstrated a key role for hepatic insulin signaling in the regulation of very-low-density lipoprotein (VLDL) assembly and secretion. In cultured hepatocytes, acute exposure of cells to insulin inhibits the secretion of both triglyceride (TG) and apolipoprotein B (apoB) despite concomitant stimulation of TG synthesis. The acute inhibition of VLDL secretion by insulin results from direct targeting of apoB for degradation, at least in part, via phosphatidylinositol 3-kinase–mediated mechanisms. In vivo, acute glucose-stimulated hyperinsulinemia suppressed secretion of VLDL in rats, whereas short-term hyperinsulinemia with euglycemia inhibited the secretion of VLDL in normal humans. On the other hand, chronic exposure of hepatocytes to insulin increased apoB secretion, suggesting the development of insulin resistance in the pathway for apoB degradation. Mouse models of hyperinsulinemia and insulin resistance have increased VLDL secretion. Similarly, VLDL secretion was not inhibited by short-term hyperinsulinemia in obese individuals or people with type 2 diabetes mellitus, 2 states characterized by insulin resistance.

Individuals with insulin resistance typically have increased secretion of both VLDL apoB and TG, despite ambient hyperinsulinemia. However, insulin resistance is also associated with increased hepatic TG (steatosis), and hepatic TG availability is a critical determinant of the assembly and secretion of VLDL. This raises the question of whether increased apoB secretion present in people with insulin resistance results from the development of insulin resistance in the pathway of insulin-mediated targeting of apoB for degradation or is because increased availability of hepatic TG is dominant over increased hepatic insulin signaling. Relevant to this issue is our recent observation of dissociation between TG and apoB secretion in the complete absence of hepatic insulin signaling. Thus, in liver-specific insulin receptor knockout mice, apoB secretion was significantly increased, whereas TG secretion was markedly reduced. These findings suggested that reduced TG targeting of apoB for secretion was not as potent as the...
absence of insulin targeting of apoB for degradation. In the present study, we took advantage of the availability of hepatic-specific “phosphatase and tensin homologue deleted on chromosome 10” (Pten) knockout (hPTEN-ko) mice, which have markedly increased hepatic insulin signaling and steatosis, to further interrogate the relative roles of increased hepatic insulin signaling and increased hepatic TG availability in the assembly and secretion of VLDL. Of note, Stiles et al showed previously that these hPTEN-ko mice have marked steatosis despite increased TG secretion, suggesting that insulin targeting of apoB for degradation might be limiting the number of VLDL particles than can be secreted by these mice.

Materials and Methods

Animals
Liver-specific Pten knockout (hPTEN-ko) mice were generated as described. hPTEN-ko and floxed littermate mice were maintained on a 12-hour light/dark cycle and had free access to water and standard rodent chow. All mice were studied at 3 to 4 months of age if not otherwise specified. C57BL/6J mice, used for primary hepatocyte studies, were housed and fed in a similar manner. All procedures were approved by the Institutional Animal Care and Use Committee of the Columbia University College of Physicians and Surgeons.

Antisense Treatments

Dgat1 (ISI3 191761), Dgat2 (ISI3 217376), Stebp-1c (ISI3 219676) were provided by ISIS Pharmaceuticals (Carlsbad, CA). ASO treatments were carried out as described. Briefly, mice were injected intraperitoneally with ASO twice a week at a concentration of 25 μg per gram of body weight. Treatments were started at 12 weeks of age and were 4 weeks in duration.

Adenovirus Treatments

A recombinant adenovirus containing a Pten cDNA (Pten) was kindly provided by Ramon Parsons (Columbia University, New York, NY). A recombinant adenovirus containing a Cre-recombinase cDNA (Cre) under control of the albumin promoter was kindly provided by Derek Leroith (Mount Sinai School of Medicine, New York, NY). A recombinant virus containing a green fluorescent protein (GFP) cDNA was provided by Alan Tall (Columbia University). Recombinant adenovirus was amplified in 293 cells and purified with a Vivapure Adenopack 100 column (Sartorius, Göttin-}

Liver TG

Liver TG content was measured as described. Briefly, tissues were homogenized in phosphate-buffered saline, and lipids were extracted twice with 6 volumes of chloroform and methanol (2:1). Dried lipids were resuspended in 15% Triton X-100, and TG concentration was determined by a colorimetric method (Wako Diagnostics, Richmond, VA).

Assessment of TG and apoB Secretion Rates

In Vivo
TG and apoB secretion rates were determined as described. Mice fasted for 4 hours were injected with both 15% Triton WR 1339, at a concentration of 5 μg/g of body weight, and 200 μCi of [35S]methionine per mouse through a femoral vein. Blood samples were collected just before injection and 30, 60, 90, and 120 minutes after injection by retroorbital bleeding. For TG secretion rates, the concentration of TG was determined using a commercial assay kit (Wako Diagnostics, Richmond, VA). For apoB secretion rates, plasma from 60- and 120-minute blood samples containing equal amounts of TCA-precipitable radioactivity (to correct for total protein synthesis) were separated on 4% SDS-PAGE gels. The gels were dried under vacuum and heat (65°C) for 1 hour and exposed on x-ray films to visualize apoB100 and apoB48 bands. Corresponding bands on the dried gel were cut out and counted to quantitate the rate of appearance of newly synthesized [35S]-labeled apoB in plasma.

Dual-Energy X-Ray Absorptiometry

Mice were anesthetized by intraperitoneal injection of a ketamine/xylazine mixture (100 mg and 10 mg/kg body weight, respectively) and scanned using Lunar PIXIms2 (Lunar PIXImus Corp, Madison, WI). Body composition including body weight and fat was estimated by the system software.

Quantitative Polymerase Chain Reaction

Total liver RNA was extracted with Trizol RNA extraction reagent, and cDNA was synthesized using SuperScript cDNA synthesis kits following the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Quantitative polymerase chain reaction was performed with SYBR Green in a MX 3005P (Stratagen, La Jolla, CA). Primers for quantitative polymerase chain reactions are described in Supplemental Table I, available online at http://atvb.ahajournals.org.

Western Blot Analysis

Aliquots of liver were homogenized in a lysis buffer consisting of 25 mmol/L Tris-HCL (pH 7.4), 10 mmol/L NaPO4, 10 mmol/L NaF, 10 mmol/L Na3P2O7, 1 mmol/L EGTA, 1 mmol/L EDTA, 1% NP-40, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, and 5 μg/mL aprotinin. Proteins were separated on SDS-PAGE gels, transferred onto a nitrocellulose membrane, and immunoblotted with specific antibodies. AKT, phospho-AKT (p-AKT), and PTEN antibodies were purchased from Cell Signaling (Boston, ME). Anti β-actin antibody was obtained from Sigma-Aldrich (Saint Louis, MO).

Fast Performance Liquid Chromatography

Fast performance liquid chromatography was performed as described. Briefly, a 200-μL plasma sample, pooled from 4 or 5 animals bled after a 4-hour fast, was chromatographed through 2 Superose columns connected in series using a fast performance liquid chromatography system (AKTA FPLC, UPC-900 with Unicorn software, GE Healthcare, Pittsburgh, PA). Fifty-four samples of 0.5-ml fractions were collected, and the concentrations of TG and cholesterol were determined as described above.
The difference may be due to the older age of our mice at time of sacrifice compared with the floxed mice (Table). Stiles et al. demonstrated lower plasma glucose and insulin levels, and similar findings were suggested by Stiles et al. in their Supplemental Table I. Total AKT was modestly reduced in the hPten-ko mice in this experiment, as well as in others experiments where PTEN activity was absent or reduced and p-AKT was increased; a similar finding was suggested by Stiles et al. in their Supplemental Figure IC. Total AKT was demonstrated by significantly elevated p-AKT in hPten-ko mice (Supplemental Figure IC). We also confirmed that male hPten-ko mice had lower plasma glucose and insulin levels, and similar TG levels compared with floxed mice. Stiles et al. studied only male mice. Fasting total cholesterol levels were significantly higher in both male and female hPten-ko mice, findings not noted by Stiles et al. We did not, however, confirm the findings of Stiles et al. of lower plasma levels of free fatty acids in the hPten-ko mice: this difference may be due to the older age of our mice at time of sacrifice.

**Statistical Analysis**

All data are presented as means ± SEM. Differences in means between 2 groups were evaluated for significance using a 2-tailed Student t test. A probability value < 0.05 was considered significant.

**Results**

**Liver-Specific Pten Knockout Mice Have Increased Hepatic Insulin Signaling, Increased Hepatic De Novo Lipogenesis, and Hepatic Steatosis**

To evaluate the importance of increased hepatic insulin signaling relative to hepatic TG content in the regulation of VLDL apoB secretion, we studied the hPten-ko mouse. The hPten-ko mouse was characterized in some detail previously by Stiles et al., but we felt it was necessary to validate those original findings in our laboratory before extending studies in this model. Supplemental Figure IA confirms the obvious steatotic liver present in an hPten-ko mouse. The steatosis was confirmed quantitatively in Supplemental Figure IB, which shows that hepatic TG levels were increased almost 10-fold in hPten-ko mice. The steatotic liver present in an hPten-ko mouse was characterized in some detail previously by Stiles et al.

**TG and apoB Secretion Rates Are Increased in hPten-ko Mice**

Increased TG secretion had previously been observed, and we confirmed that finding; TG secretion in hPten-ko mice was double that in floxed mice (Figure 1A and 1B). We...

**Figure 1.** Hepatic-specific Pten knockout (hPten-ko) mice have increased secretion of triglycerides (TG) and apolipoprotein B (apoB) in vivo and apoB100 secretion ex vivo. TG and apoB turnover studies were performed after a 4-hour fast. Mice were injected with Triton WR 1339 and [35S]methionine, and blood samples were obtained during the next 2 hours. A. TG secretion was higher over the 2-hour time course. B. TG secretion rate, in mg/dL per hour, was greater in hPten-ko (Ko) mice. C. apoB secretion rates were plotted as the percentage of the rates in floxed mice 60 and 120 minutes after injection of [35S]methionine, apoB100 secretion rates were increased in hPten-ko mice. D. apoB-48 secretion rates were increased in hPten-ko mice. All data (A–D) are expressed as mean ± SEM. n = 7 to 8. *P < 0.05, **P < 0.01. E. apoB100 secretion was increased in primary hepatocytes isolated from hPten-ko mice. All data are normalized to cell apoB100 in cells. n = 4. **P < 0.01.

**Table.** Characteristics of hPten-ko and Floxed Mice

<table>
<thead>
<tr>
<th></th>
<th>Male Flox</th>
<th>Male hPten-ko</th>
<th>Female Flox</th>
<th>Female hPten-ko</th>
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</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>24.5 ± 0.9</td>
<td>22.6 ± 0.9</td>
<td>17.6 ± 0.5</td>
<td>18.7 ± 0.7</td>
</tr>
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<td>Fasting TC, mg/dL</td>
<td>88.8 ± 21.8</td>
<td>122.0 ± 43.7*</td>
<td>76.2 ± 3.6</td>
<td>118.3 ± 2.8*</td>
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<tr>
<td>Fasting TG, mg/dL</td>
<td>41.6 ± 2.3</td>
<td>43.9 ± 5.0</td>
<td>31.6 ± 0.5</td>
<td>48.9 ± 6.5†</td>
</tr>
<tr>
<td>Fasting FFA, mmol/L</td>
<td>0.70 ± 0.06</td>
<td>0.65 ± 0.08</td>
<td>0.62 ± 0.06</td>
<td>0.72 ± 0.05</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>139.2 ± 4.8</td>
<td>110.3 ± 7.9†</td>
<td>142.4 ± 5.2</td>
<td>137.2 ± 7.9</td>
</tr>
<tr>
<td>Insulin, ng/mL</td>
<td>0.43 ± 0.04</td>
<td>0.09 ± 0.02*</td>
<td>0.55 ± 0.08</td>
<td>0.25 ± 0.03†</td>
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</table>

All measurements were from blood samples obtained after a 4-h fast. All values are mean ± SEM. Differences in means between 2 groups were evaluated for significance using a 2-tailed Student t test. A probability value < 0.05 was considered significant.
hypothesized that despite steatosis and increased hepatic TG secretion, there would be increased insulin-mediated targeting of apoB into a degradation pathway, with decreased VLDL apoB secretion. We tested our hypothesis directly by injecting [35S]methionine intravenously, together with Triton WR1339, to quantitate the appearance of newly synthesized, radiolabeled apoB in plasma. In contrast to our expectations, rates of secretion of newly synthesized apoB100 and apoB48 were increased 50% to 100% in hPten-ko mice (Figure 1C and 1D). Furthermore, ex vivo studies of primary hepatocytes from hPten-ko mice confirmed increases in both cellular and secreted apoB100 compared with cells from floxed mice (Figure 1E). We did not observe differences in apoB48 in cells or media between hPten-ko and floxed mice. Thus, rather than finding evidence of increased insulin-mediated targeting of apoB for degradation in hPten-ko mice, we had in vivo data supporting increased secretion of both apoB100 and apoB48, as well as ex vivo data demonstrating increased apoB100 secretion in these mice.

We could not, however, rule out the possibility that increased insulin signaling in the hPten knockout mouse actually limited what would have been an even greater response in apoB secretion to the prevailing level of steatosis. Therefore, we designed experiments to reduce hepatic steatosis, with the idea that the targeting of apoB for degradation by increased hepatic insulin signaling in hPten-ko mice would be unmasked.

**ASO Knockdown of Dgat1 and Dgat2 in hPten-ko Mice Decreased Hepatic TG Levels and TG Secretion But Did Not Unmask Insulin-Mediated Targeting of apoB for Degradation**

To reduce hepatic steatosis without altering hepatic insulin signaling, we treated hPten-ko mice with ASO to both Dgat1 and Dgat2, twice weekly for 4 weeks. Administration of the 2 ASOs did not alter hepatic insulin signaling (data not shown) but resulted in marked reductions in Dgat1 and Dgat2 mRNA (Supplemental Figure II A). Hepatic TG levels and the rate of TG secretion were concomitantly and dramatically reduced by 50% compared with hPten-ko mice receiving control ASO (Supplemental Figure IIB and IIC). However, the secretion rates of apoB100 and apoB48 were not significantly decreased in Dgat ASO-treated mice compared with control ASO-treated groups (Supplemental Figure IID and IIE).

Even though these treatments dramatically reduced hepatic TG levels, livers of hPten-ko mice treated with DGAT ASOs still contained very high TG levels (743 μg/mg protein); in our laboratory, C57BL/6J mice on chow diets have liver TG concentrations between 50 and 150 μg/mg protein. We could not rule out, therefore, that this high liver TG content might have been adequate to protect apoB from insulin-mediated degradation in hPten-ko mice. Thus, we hypothesized that further reduction of hepatic TG might be needed to unmask the effects of increased insulin signaling on apoB secretion in hPten-ko mice.

**Srebp-1c ASO Treatment of hPTEN-ko Mice Decreased Hepatic TG and TG Secretion but Only Minimally Affected apoB Secretion**

To test this hypothesis, we targeted Srebp-1c, a global lipogenic transcription factor in the liver, for ASO treatment. Srebp-1c ASO treatment of hPten-ko mice twice weekly for 4 weeks did not alter hepatic insulin signaling (data not shown) but successfully decreased Srebp-1c mRNA more than 50% (Supplemental Figure III A), and this was paralleled by a reduction in hepatic TG content of 70%, to 338 μg/mg protein, compared with hPten-ko mice treated with control ASO (Supplemental Figure III B). TG secretion was decreased concomitantly by approximately 40% (Supplemental Figure III C). These Srebp-1c ASO-mediated changes did not, however, affect the secretion rate of apoB100 (Supplemental Figure III D). Secretion of apoB 48 was reduced by 25%, and this was significant only at the 2-hour time point (Supplemental Figure III E).

Overall, the results of these 2 experiments with specific ASO-treatments that reduced hepatic TG levels dramatically, indicated that increased hepatic insulin signaling in hPten-ko mice did not appear to significantly target apoB for degradation even when hepatic TG was only modestly elevated; there was no change in apoB100 secretion, whereas apoB48 secretion may have tracked with TG content and secretion.

**Primary Hepatocytes From hPten-ko Mice Are Not Sensitive to the Acute Effects of Insulin on apoB Secretion or Cellular apoB Content**

Our findings to this point were unexpected and indicated that we needed to demonstrate well-known effects of insulin to target apoB100 for degradation and thereby reduce apoB secretion. We confirmed this well-established observation both with primary hepatocytes isolated from C57BL/6J wild-type mice and with hepatocytes isolated from floxed control mice (Figure 2A and 2B). In both wild-type and floxed hepatocytes, treatment with 10 nmol/L insulin for 16 hours reduced cellular and secreted apoB100 by 40% to 60%. As reported in prior literature, insulin treatment did not affect apoB48 cell or media content. By contrast, primary hepatocytes from hPten-ko mice did not respond to acute insulin treatment with a decrease in either apoB secretion or cellular content (Figure 2C). Interestingly, and as reported previously, longer term insulin treatment (72 hours) of primary hepatocytes from floxed control mice was associated with a loss of insulin-mediated inhibition of apoB secretion (Figure 2B), suggesting that insulin resistance had developed during that period of time. Importantly, prolonged insulin treatment of primary hepatocytes isolated from hPten-ko mice did not alter either apoB secretion or cellular content; thus, these cells were insulin resistant at both 16 and 72 hours (Figure 2C). Overall, our results indicate that primary hepatocytes from hPten-ko mice, which had been exposed chronically in vivo to increased insulin signaling, did not respond to acute insulin treatment by reducing apoB100 secretion. Because prior studies had suggested that chronic hyperinsulinemia could reduce the ability of insulin to acutely target apoB for degradation, we next attempted to acutely alter hepatic insulin signaling in vivo.
Figure 2. Apolipoprotein B (apoB) 100 secretion is inhibited by insulin treatments in primary hepatocytes from C57BL/6J and floxed control mice, but not from hepatic-specific Ptten knockout (hPtten-ko) mice. Primary hepatocytes from wild-type, floxed, and hPtten-ko mice were treated with or without 10 nmol/L insulin for either 16 or 72 hours and then labeled with [35S]methionine at steady state for 2 hours. Immunoprecipitated [35S]methionine-labeled apoB100 and apoB48 were separated (left panels), and the amount of newly synthesized protein in cells or media was quantitated by scintillation counting of the bands (right panels). A, Acute insulin treatment reduced apoB100 in cells and media of primary hepatocytes from C57BL/6J. B, Acute insulin treatment reduced apoB100 in cells and media of primary hepatocytes from floxed control mice, whereas chronic treatment did not reduce apoB in cells or media. C, Neither acute nor chronic insulin treatment affected apoB100 in cells or media of hPtten-ko primary hepatocytes. Insulin treatment did not affect apoB48 in cells or media of primary hepatocytes from any of the 3 groups. n=3 for each type of mice. *P<0.05, **P=0.001.
Acute Restoration of PTEN in hPten-ko Mice Reduced Insulin Signaling, Hepatic TG Content, and Both TG and apoB Secretion Rates

To acutely reduce hepatic insulin signaling, we introduced wild-type Pten via adenoviral infection into hPten-ko mice. As expected, administration of Pten adenovirus restored hepatic PTEN protein within 4 days and was associated with decreased phosphorylation of AKT compared with GFP adenovirus-treated mice (Figure 3A). Decreased hepatic insulin signaling was associated with a significant reduction in the expression of Fas and a trend toward decreased expression of Srebp-1c (Figure 3B). Hepatic TG content decreased in mice receiving Pten adenovirus (Figure 3C). TG secretion was lower in mice with restored hepatic PTEN protein (Figure 3D). Secretion rates of apoB100 and apoB48 were lower after restoration of hepatic PTEN (Figure 3E and 3F). Acute reconstitution of PTEN in hPten-ko mice did not alter plasma levels of lipids, glucose, or insulin and did not affect body weight or body fat compared with control GFP virus-treated mice (Figure 3G and 3H). These data indicate that despite reduced hepatic insulin signaling, which might have been expected to result in increased secretion of apoB, secretion of apoB fell in parallel with the reductions in hepatic TG content and secretion.

Acute Liver-Specific Knockout of Pten in Floxed Mice Increased Insulin Signaling, Hepatic TG Content, and Both TG and apoB Secretion Rates

Next, we acutely increased hepatic insulin signaling by deletion of the Pten gene in floxed mice using a Cre recombinase adenovirus. Four days after infection of floxed mice with adenovirus under control of the albumin promoter, PTEN protein was dramatically reduced, and insulin signaling, assessed by the level of p-AKT, was significantly increased compared with GFP adenovirus-treated mice (Figure 4A). Increased hepatic insulin signaling was accompanied...
by trends toward increased expression of the lipogenic genes Srebp-1c and Fas (Figure 4B) and significantly increased hepatic TG mass (Figure 4C). Concomitantly, TG secretion was 2-fold higher in the floxed mice in whom hepatic \textit{Pten} had been acutely knocked down compared with floxed mice that had received \textit{Gfp} adenovirus (Figure 4D). Although we expected that the acute increase in insulin signaling in mice with acute \textit{hPten} knockout would target apoB for degradation, there was no change in the secretion rate of apoB100, and secretion of apoB48 actually increased by 50% compared with floxed mice that had received \textit{Gfp} adenovirus (Figure 4E and 4F).

**Acute Liver-Specific Knockout of Floxed \textit{Pten} Mice That Were Pretreated With Srebp-1c ASO Did Not Result in Inhibition of apoB Secretion Despite Increased Insulin Signaling Without An Increase in Hepatic TG Content**

In the previous experiment, acute increases in hepatic insulin signaling resulted in significant increases in hepatic TG, somewhat confounding interpretation of the results. Therefore, we repeated the acute knockout experiment in floxed mice that had been treated with Srebp-1c ASO for 4 weeks to block de novo lipogenesis and steatosis. Acute knockout of hepatic \textit{Pten} resulted in a decrease in PTEN protein and increased insulin signaling confirmed by increased levels of p-AKT (Figure 5A). However, Srebp-1c expression did not increase at all, whereas FAS expression trended to increase only slightly in the mice pretreated with Srebp-1c ASO (Figure 5B). TG content rose modestly (compare Figure 5C with Figure 4C) and was still within the normal range for our laboratory for chow-fed C57BL/6J mice. TG secretion also increased only slightly (compare Figure 5D with Figure 4D). Thus, we had created a model of increased insulin signaling without steatosis and expected, therefore, to demonstrate insulin-mediated targeting of apoB for degradation. However, 4 days of increased hepatic insulin signaling in the acute \textit{hPten} knockout mice, in the absence of steatosis, had no effect on either apoB100 or apoB48 secretion (Figure 5E and 5F). These data provide very strong evidence that increasing insulin signaling over a period as short of 4 days led to resistance in the pathway of insulin-mediated targeting of apoB for degradation.

**Discussion**

The assembly and secretion of VLDL is 1 of the key pathways, together with fatty acid (FA) oxidation and de novo lipogenesis, by which the liver maintains control over TG content and attempts to avoid steatosis.\textsuperscript{26–28,36,37} It is not surprising, therefore, that although the regulation of the assembly of VLDL is complex,\textsuperscript{3,26,28,29} the availability of hepatic TG is a potent factor regulating TG secretion. Increased delivery of either albumin-bound FA or TGFA-enriched remnant lipoproteins to cultured hepatocytes,\textsuperscript{29,38–40} perfused livers,\textsuperscript{51,42} or livers in vivo\textsuperscript{43} can increase the secretion of both TG and apoB. Increased numbers of VLDL particles that are either the same size or larger are secreted. In contrast to increasing hepatic TG by delivery of FA or TGFA from plasma, increasing TG availability by increasing hepatic lipogenesis has been reported to increase secretion of TG but not apoB. For example, diets high in carbohydrates increased hepatic lipogenesis and TG secretion in VLDL without...
increasing apoB secretion, indicating assembly and secretion of larger VLDL. Pharmacological stimulation of the liver X receptor in mice increased lipogenesis and secretion of VLDL TG, but apoB secretion was unaffected. Similarly, overexpression of SREBP-1c resulted in secretion of very large VLDL, suggesting more TG but not more apoB secretion. On the other hand, a recent study in hamsters suggested increases in lipogenesis can stimulate both TG and apoB secretion.

Published studies in which TG synthesis was increased without directly increasing FA delivery or hepatic lipogenesis indicate that additional complexity is possible. Thus, 2 studies in which short-term overexpression of Dgat 1 or Dgat 2 was achieved using recombinant adenovirus demonstrated increased hepatic TG content but had differing effects on TG and apoB. In one study, short term overexpression of Dgat1 increased both TG and apoB secretion, whereas overexpression of Dgat2 had no effects. In the second study, there was no effect of overexpression of either of these enzymes on TG or apoB secretion. Consistent with the latter report, mice transgenic for Dgat 2 developed fatty liver without increases in TG secretion. In contrast, overexpression of Dgat1 in McAdle RH7777 cells increased cellular TG content and the secretion of both TG and apoB.

Some of the experiments noted above could have been confounded by alterations in hepatic insulin signaling. For example, high-carbohydrate feeding, with concomitant hyperinsulinemia, could have targeted apoB for degradation, resulting in secretion of the same number of VLDL particles each containing more TG. Similarly, increases in liver X receptor signaling may increase hepatic insulin sensitivity, although a recent study indicates significant complexity in this regard. It is also very likely that the time course of insulin-mediated targeting of apoB for degradation is complex; acute hyperinsulinemia targets apoB for degradation, whereas longer exposure to insulin results in a loss of such targeting. Therefore, the background sensitivity of the liver to insulin will also be a critical determinant of the response of apoB to any alterations in ambient insulin levels in plasma or hepatic insulin signaling.

Our findings in mice specifically lacking hepatic PTEN provide significant, new insights regarding the relative importance of hepatic TG content and hepatic insulin-signaling for the secretion of both TG and apoB. First, our studies demonstrate clearly that in the presence of longstanding increases in both hepatic insulin signaling and hepatic TG, the availability of TG for assembly into VLDL is the dominant stimulus for apoB secretion. Thus, in the hPten-ko mice, where markedly increased insulin signaling should have

Figure 5. Treatment of floxed mice with Srebp-1c ASO before acute depletion of hepatic Pten blocks the increase in hepatic triglycerides (TG) but does not result in decreased apolipoprotein B (apoB) secretion. Floxed mice were pretreated for 4 weeks with Srebp-1c ASO before being injected with recombinant adenovirus containing either Cre (Cre) or Gfp (GFP). Studies were performed 4 days after injection of virus. **A**, Insulin signaling was increased significantly in mice receiving Cre adenovirus as assessed by the phospho-AKT (p-AKT)/AKT ratio. **B**, Srebp-1c and Fas gene expression were not increased in ASO-pretreated mice receiving Cre adenovirus. **C**, Hepatic TG levels were not significantly increased and were in the normal range in ASO-pretreated mice receiving Cre adenovirus. **D**, TG secretion was not significantly increased in ASO-pretreated mice receiving Cre adenovirus. **E** and **F**, Secretion rates of apoB100 and apoB48 were not affected in ASO-pretreated mice receiving Cre adenovirus. n=3 for each group. *P<0.01.
reduced apoB secretion, the latter was actually increased in parallel with increased TG secretion. This was true both in vivo and ex vivo, the latter in primary hepatocytes isolated from hPten-ko and floxed mice. In hPten-ko mice where hepatic TG levels were reduced by either inhibiting TG synthesis with Dgat ASOs or inhibiting de novo lipogenesis with Srebp-1c ASO, apoB secretion fell modestly and appropriately concomitant with reductions in hepatic TG content and TG secretion; we had expected more significant reductions in apoB secretion in the presence of high levels of insulin signaling but less hepatic TG-associated stimulation of VLDL assembly. More importantly, when hepatic PTEN activity was acutely restored in the hPten-ko mice, and insulin signaling was significantly reduced, apoB secretion did not increase but actually fell in parallel with reductions in hepatic TG content and secretion. The strongest evidence for the importance of hepatic TG was apparent in studies where we acutely knocked out hepatic Pten and insulin signaling acutely increased; in studies performed only 4 days after adenoviral infection, apoB48 secretion increased in parallel with increased TG secretion, whereas apoB100 secretion was unchanged. Even when we acutely knocked out hepatic Pten but prevented the subsequent acute increase in TG content by pretreating the mice with Srebp-1c ASO, apoB secretion was appropriate for the level of TG secretion. The acute knockout studies indicated that either hepatic TG is always dominant over hepatic insulin signaling as a determinant of apoB secretion, or that 4 days of increased insulin signaling is long enough for development of resistance to insulin-mediated apoB degradation.5,15-17,20,21 Our ex vivo studies showing loss of insulin-mediated inhibition of apoB secretion in primary hepatocytes from floxed mice after incubation with insulin for 72 hours confirm those earlier results. Notably, Pten knockout hepatocytes had no response to incubation of insulin at either 16 or 72 hours.

Our results must be compared with a recent report by Qiu et al53 in which a different liver-specific Pten knockout mouse model was found to have steatosis and increased expression of lipogenic genes but decreased hepatic levels of apoB100 and apoB48. Furthermore, in that article,53 acute expression of a dominant negative Pten vector in HepG2 cells, McA-RH7777 cells, and primary hamster hepatocytes resulted in increased insulin signaling and reduced secretion of apoB100 and apoB48 because of increased proteasomal degradation. Differences in strains (we studied hPten-ko mice on a C57Bl/6 background, whereas the knockout mouse in the article by Qiu et al54 was on a mixed background) could have resulted in different phenotypes, but we believe that differences in methodological approaches may be the basis for the contrasting findings in this case. Qiu et al53 conducted only limited studies in their Pten knockout mouse, and did not perform any in vivo studies of TG and apoB secretion. Their finding of decreased hepatic levels of apoB does not rule out the possibility of increased secretion of apoB; more efficient secretion could result in reduced steady-state levels of hepatic apoB. The remainder of the studies by Qiu et al53 used cultured hepatocytes. They found that reduced PTEN activity secondary to overexpression of a dominant negative Pten resulted in decreased secretion of apoB; these observations are consistent with previously published work demonstrating the acute effects of increased insulin signaling on apoB degradation.1 Indeed, we also demonstrated that insulin treatment of primary hepatocytes isolated from either wild-type C57BL/6J or floxed mice resulted in reductions in the accumulation of newly synthesized apoB100 in cells and media. In our studies, apoB48 cell and medium levels were not affected by acute insulin treatment, also in accordance with published data.1 As noted above, we demonstrated that after incubation of floxed primary hepatocytes with insulin for 72 hours, apoB100 secretion was no longer inhibited. On the other hand, were unable to demonstrate altered apoB100 secretion after acute insulin treatment (either 16 or 72 hours) in hepatocytes isolated from hPten-ko mice. These results are in agreement with studies showing that “insulin resistance” develops in the pathway of insulin-mediated targeting of apoB100 for degradation after prolonged exposure of cultured liver cells to the hormone.6,16,17,35 Overall, however, we cannot explain all of the differences between our results and those of Qiu et al.53

MTP plays a critical role in the assembly and secretion of VLDL, and insulin plays a key role in the regulation of MTP at the transcriptional level,55,56 working via the MAPK/ERK pathway57,58 and FoxO1.59 MTP has a long half-life, so insulin signaling must be increased chronically to result in reduced levels of MTP,55,57 and Qiu et al53 did see reduced MTP and activity, albeit without altered mRNA levels in their hPten knockout mouse. We did not see changes in the levels of either MTP mRNA (Supplemental Table II) or MTP (data not shown) in our hPten-ko mice. Studies from several laboratories have identified sites upstream of the apoB gene where transcriptional regulation may occur;60,61 however, there appears to be very little in vivo regulation of transcription of the apoB gene. Expression of apoB, as measured by its mRNA level, was the same in hPten-ko as in floxed mice. Severe insulin deficiency can inhibit translation of apoB mRNA,62 as can inhibition of MTP55; there are no data suggesting increased translational efficiency in the presence of increased insulin signaling.

Of note, hPTEN-ko mice had significantly increased plasma levels of cholesterol, which were the result of increased high-density lipoprotein cholesterol concentrations (data not shown). This was an unexpected finding that may provide novel insights into the role of hepatic insulin signaling in the regulation of high-density lipoprotein metabolism, particularly in view of our prior observation that mice with severe hepatic insulin resistance, because of the absence of hepatic insulin receptors, had very low high-density lipoprotein cholesterol levels.60 Additional studies will be needed to better define the mechanisms underlying these observations.

In summary, despite evidence for markedly increased hepatic insulin signaling in hPten-ko mice, there was no evidence for specific targeting of apoB for degradation. In contrast, apoB secretion paralleled hepatic TG content. Our results indicate that although there is a compelling literature from in vitro, ex vivo, and in vivo studies demonstrating that acute increases in insulin signaling can reduce apoB secretion by targeting the protein for intracellular degradation, this effect appears to dissipate by 4 days in vivo, consistent with
long-term in vitro studies. Our results are also supported by observations in mouse models of insulin resistance and increased VLDL secretion,18,19 as well as demonstrations that VLDL secretion is not inhibited by insulin in humans with insulin resistance.20,21 The loss of insulin-mediated regulation of apoB secretion leaves hepatic TG as the major determinant of VLDL assembly and secretion.22 Thus, in humans with insulin resistance–associated increases in VLDL secretion, the concomitant steatosis that is commonly present must result from other abnormalities that can limit apoB secretion.23

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**Disclosures**
None.

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41. Salam WH, Wilcox HG, Heimberg M. Stimulation by oleic acid incorporation of L-[(4,5-3H) leucine into very low density lipoprotein apoprotein by the isolated perfused rat liver. *Biochim Biophys Acta*. 1985;132:28–34.


48. Moon et al. Regulation of apoB secretion by TG and insulin.
Apolipoprotein B Secretion Is Regulated by Hepatic Triglyceride, and Not Insulin, in a Model of Increased Hepatic Insulin Signaling
Byoung C. Moon, Antonio Hernandez-Ono, Bangyan Stiles, Hong Wu and Henry N. Ginsberg

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Apolipoprotein B secretion is regulated by hepatic triglyceride, and not insulin, in the liver-specific PTEN knockout mouse, a model of increased hepatic insulin signaling.

Byoung C. Moon¹, Antonio Hernandez-Ono¹, Bangyan Stiles², Hong Wu³,⁴, and Henry N. Ginsberg¹∗.

Supplemental Figure I.
Supplemental Figure II.
Supplemental Figure III.
Supplemental Table I
Supplemental Table II
Supplemental figure I: Liver-specific deletion of PTEN causes steatosis, increased hepatic insulin signaling, induction of lipogenic genes in the liver and lipodystrophy in male mice. (A) Liver phenotypes of Flox and hPten-ko mice showing steatosis in hPten-ko liver (Male 12 weeks old). (B) Liver TG was extracted from 12 weeks old male mice (n=7-8). *p<0.01 (C) Western blot analysis of PTEN, phospho-AKT and AKT for Flox and hPten-ko male mice showing absence of PTEN is associated with increased P-AKT. (D) Lipogenic gene expression for Flox and hPten-ko male mice (n=3-4). *p<0.01. (E) DEXA scan analysis using Lunar PIXImus2 (Lunar PIXImus corp., Madison, WI) was performed at 12 weeks of age. Body weight and body fat in male (n=7 for Flox; n=4 for hPten-ko mice) and female mice (n=3 for Flox; n=7 for hPten-ko mice). *p=0.03, **p=0.004.
Supplemental figure II: Antisense knockdown of DGAT 1 and DGAT 2 mRNA decreased hepatic TG content and TG secretion, but did not affect apoB secretion. 

hPon-ko mice were treated with either control ASO or the combination of Dgat1 and Dgat2 ASO for 4 weeks at 25 μg/g body weight twice/week. (A) DGAT ASO treatment reduced both DAG1 and DAG2 gene expression compared to a control ASO treated group. (B) Hepatic TG levels were decreased in the mice treated with DGAT ASO compared to Control ASO-treated mice. (C) TG secretion was reduced in DGAT ASO-treated mice. (D and E) There was a non-significant trend toward lower apoB100 and apoB48 secretion in DGAT ASO-treated mice. Data are expressed as the mean ± SEM; N = 4 or 5 per group. *p<0.05, **p<0.01.
Supplemental figure III. Antisense knockdown of Srebp-1c mRNA decreased hepatic TG content, TG secretion, and apoB48 secretion. Lep/ko mice were treated with either control ASO or Srebp-1c ASO, respectively for 4 weeks at the dose of 25 μg/g BW twice a week. (A) Srebp-1c ASO treatment reduced Srebp-1c expression. (B) Hepatic TG levels were decreased in the Srebp-1c ASO treated mice. (C) TG secretion rate was reduced in Srebp-1c ASO-treated mice. (D) ApoB100 secretion rates were not affected by Srebp-1c ASO treatment. (E) ApoB48 secretion rates were reduced in Srebp-1c treated mice. Data are represented as mean ± SEM (n = 3 per group). *p < 0.01.
### Supplemental table I

Primer sequences for qPCR

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<th>Gene</th>
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<th>Primers</th>
<th>Sequence (5'----&gt;3')</th>
<th>Product size</th>
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<td>Srebp-1c</td>
<td>NM_011480.3</td>
<td>Forward: GAAGCTGTGGGGTAGGTCT</td>
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<td>Fas</td>
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<tr>
<td>Scd1</td>
<td>NM_009127.4</td>
<td>Forward: TGC GAT ACA CTC TGG TGC TC</td>
<td>Reverse: AGG ATA TTC TCC CGG GAT TG</td>
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<tr>
<td>Pparγ2</td>
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<td>Cd36</td>
<td>U23108</td>
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<td>Lxra</td>
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<td>Dgat1</td>
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<td>Reverse: TATCGTTTCTGGCTGAGGT</td>
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Supplemental table II

Expression of genes in hPTEN-ko and Floxed mice

Total mRNA was prepared from the livers of hPTEN-ko and Flox control mice (12 weeks old male). Data are expressed as the mean ± SEM. N=3-4 * p < 0.01.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Male Flox Mean ± SEM</th>
<th>Male hPTEN-ko Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCD1</td>
<td>100 ± 16</td>
<td>796 ± 127 *</td>
</tr>
<tr>
<td>PPARγ2</td>
<td>100 ± 7</td>
<td>1468 ± 134 *</td>
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<tr>
<td>CD36</td>
<td>100 ± 8</td>
<td>687 ± 44 *</td>
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<td>LXRα</td>
<td>100 ± 26</td>
<td>92 ± 19</td>
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<td>DGAT1</td>
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<td>DGAT2</td>
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<td>108 ± 15</td>
</tr>
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<td>110 ± 9</td>
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<tr>
<td>MTP</td>
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