PI3-kinase activity modulates apo B available for hepatic VLDL production in apobec-1<sup>−/−</sup> mice

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MATERIALS AND METHODS

Animals. All apobec-1<sup>−/−</sup> mice used in the studies were bred from a female founder mouse obtained from Washington University, St. Louis, MO. The female was bred to wild-type C57BL/6 mouse to produce an F1 generation, and F1 mice were subsequently bred to

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produce an F2 generation. Sera from F2 mice were then screened at 8 wk of age by Western blotting for B100 and B48 levels (data not shown) using rabbit anti-rat apo B antibody (4). Genetic deletion of exon 6 of the apobec-1 gene was confirmed by RT-PCR using the following primer pairs: forward: 5′-ACC ACA CGG ATC AGC GAA-3′ and reverse: 5′-TCA TGT GGA TAG TCA CAC CG-3′. B100-only mice were subsequently interbred to produce an apobec-1<sup>−/−</sup> colony at the University of Rochester. Male and female apobec-1<sup>−/−</sup> mice, aged 19 to 21 wk, were housed in single cages on a 12:12-h light-dark cycle with free access to regular chow diet (Purina 5008). In separate experiments, age-matched, male apobec-1<sup>−/−</sup> mice were fed 60% (wt/wt) fructose (Dyets, Bethlehem, PA) for 5–6 wk to induce insulin resistance. The fructose diet composition percent by weight was: 14% casein, 0.18% L-cystine, 60% fructose, 12.1% dextrose, 4.0% soybean oil, 5% cellulose, 3.5% mineral mix (#210050), 1.0% salt mix (#310025), and 0.25% choline bitartrate. All procedures performed on mice were approved by the University Committee on Animal Resources, University of Rochester.

Measurement of lipoprotein production. Mice were fasted for 16–18 h before the beginning of the kinetic experiments and then were anesthetized with 100 μl ketamine (10 mg/ml) and xylazine (1 mg/ml) and fitted with jugular (24 gauge) catheters (Becton-Dickinson, Sandy, UT). Anesthesia was maintained throughout the experimental period. The venous line was flushed with a small volume of heparinized saline (20 U/ml), and stopped with a Luer-lock injection site adapter (Baxter Healthcare, Deerfield, IL). The catheter was secured using several silk sutures (5–0), and mice were allowed 30 min to acclimate. Triton WR 1339 (Triton; Tyloxapol) was diluted in saline (100 mg/ml), warmed to 37°C, and slowly injected (1–3 min via the jugular vein (100 μl)). For glucose injections, mice were injected with 100 μl 25% (wt/vol) glucose dissolved in saline before Triton injection. For experiments involving wortmannin, mice were injected intravenously 1 h before Triton with 100 μl wortmannin (Calbiochem, San Diego, CA) in DMSO (final concentration, 0.5 mg/ml). Blood samples were collected at 0, 30, and 120 min after Triton injection. Blood was allowed to clot on ice, and serum was collected for analysis. The first 30 min after Triton injection was required for detergent equilibration and initiation of lipoprotein accumulation. Production rates for individual rats were calculated using the linear increment between 30 min and 2 h. Average production rates between groups were compared using Student’s t-test for unpaired samples.

Pulse-chase experiments. Mouse hepatocytes were isolated by collagenase perfusion (5), and viable cells were purified on Percoll gradients (30). Cells (2 × 10⁶ cells/ml, 2 ml/60-mm dish) were seeded onto dishes previously coated with rat tail collagen and incubated in a humidified atmosphere of 95%/5% (vol/vol) air/CO₂ at 37°C for 2–4 h. For long-term labeling with <sup>14</sup>C]leucine, dishes were rinsed three times in 0.2% (wt/vol) BSA/HBSS to remove nonadherent cells and were incubated overnight (14–16 h) in <sup>14</sup>C]-labeling media that consisted of leucine-free Waymouth’s medium containing 2 μCi/ml <sup>14</sup>C]-l-leucine (Spec. Act. ~315 mCi/mmol), 15 μl M L-leucine, and 0.1 mM insulin. After incubation, media were removed, cells were washed once in 0.2% (wt/vol) BSA/HBSS, <sup>14</sup>C]-labeling media containing 1 μM wortmannin or equivalent DMSO were added, and incubation was continued. After 2 h, cells were washed three times in cold (4°C) chase media consisting of Waymouth’s medium containing 5 nmol t-leucine and reincubated in chase medium containing 1 μM wortmannin or equivalent DMSO for 1 and 2 h. Pulse-chase studies using <sup>35</sup>S label, employed hepatocytes incubated in Waymouth’s medium containing 0.1 mM insulin overnight. Cells were then rinsed three times in Waymouth’s medium lacking t-methionine, t-cysteine, and t-cysteine (depletion medium) and reincubated in depletion medium for 45 min containing wortmannin (1 μM final) or DMSO equivalent. To each dish was then added 130–175 μCi (Spec. Act. 1175 Ci/mmol) EXPRE<sup>35</sup>S<sup>35</sup>S-protein labeling mix (NEG-072, Perkin-Elmer Life Sciences, Boston, MA), and cells were incubated for 30 min or 3 h. Label incorporation into protein was terminated by diluting the labeling medium with an equivalent volume of cold (4°C) Waymouth’s medium containing 20 mM l-methionine and 5.0 mM l-cysteine. Monolayers were then washed three times and reincubated in Waymouth’s chase medium containing 10 mM l-methionine and 2.5 mM l-cysteine with 1 μM wortmannin or equivalent DMSO added. At various time periods thereafter, media samples were collected, and cells were washed three times in ice-cold HBSS and frozen in liquid nitrogen until analysis. Immunoprecipitation of <sup>35</sup>S-labeled B100 from cells and media samples were carried out as previously described (33). Immunoprecipitates were collected by incubation with protein A-Sepharose for 3–4 h at 4°C and washed extensively as described by Lodish and Kong (18). Immunoprecipitated <sup>35</sup>S- or <sup>14</sup>C]-labeled B100 was eluted by addition of 2× Laemmli buffer (17) and incubated at 95–100°C for 5 min. Labeled B100 was separated by SDS-PAGE on 4% (wt/vol) acrylamide/Acrylaide minigels cast on GelBond film. After electrophoresis, gels were rinsed in distilled water and heat fixed, and labeled B100 was analyzed using the PhosphorImager and quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Biochemical analyses. Serum glucose was measured by a colorimetric assay employing glucose oxidase, and serum TG was measured by assay of glycerol released following lipase treatment, both being determined using commercial kits (Sigma, St. Louis, MO). Mouse serum VLDL B100 was quantitated after isolation by ultracentrifugation of serum for 18 h at 14°C at 50,000 rpm in an L-70 ultracentrifuge using a 70.1 Ti rotor (Beckman Instruments, Palo Alto, CA). Isolated lipoproteins were delipidated in chloroform/methanol/diethylether (5:5:10, vol/vol/vol), and apoproteins were dissolved in 2× Laemmli buffer (17) containing 25 mM dithiothreitol. Apoproteins were separated by SDS-PAGE alongside of mouse B100 standards purified by SDS-column chromatography (32). After separation, gels were stained in Coomassie blue and photographed, and images were digitized. B100 concentration was determined using ImageQuant software compared with B100 standards. VLDL B100 concentration was corrected for losses during ultracentrifugation by using the ratio of serum TG to VLDL TG recovered as >90% of serum TG in apobec-1<sup>−/−</sup> mice was present in the d < 1.006 g/ml density fraction as previously reported (22).

Statistics. Values are presented as the means ± SD or ± SE when means between groups were compared. Differences were evaluated by Student’s t-test.

RESULTS

Effect of wortmannin and glucose injection on VLDL production in apobec-1<sup>−/−</sup> mice. Hepatic VLDL production rates were determined on fasted male and female apobec-1<sup>−/−</sup> mice by measuring TG accumulation in serum following injection of Triton WR 1339 to block VLDL catabolism. Over 90% of serum TG in apobec-1<sup>−/−</sup> mice is present as VLDL (22), and therefore, fasting TG production reflects mainly VLDL TG secretion by the liver. Glucose-injected male and female mice had, on average, a 28% and 33% reduction, respectively, in hepatic VLDL TG produced over a 120-min period compared with saline-injected controls (Table 1), which was a similar reduction to that observed in male rats using similar methodologies (4). In contrast, wortmannin injection significantly increased VLDL TG production by an average of 52% in males and by 89% in females compared with saline-injected controls (P < 0.05).

Effect of wortmannin and glucose injection on VLDL B100. To distinguish between increased TG production due to an increase in TG content per VLDL particle versus secretion of an increased number of particles, serum VLDL B100 at the
Table 1. Triglyceride production rates (µg·ml⁻¹·min⁻¹) in fasted apobec-1⁻/⁻ mice under various test conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Saline</th>
<th>Wortmannin</th>
<th>Glucose</th>
</tr>
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<tbody>
<tr>
<td>Males</td>
<td>23.3 ± 4.4</td>
<td>35.5 ± 4.8*</td>
<td>16.8 ± 1.3*</td>
</tr>
<tr>
<td>Females</td>
<td>19.9 ± 3.5</td>
<td>37.7 ± 5.6*</td>
<td>13.4 ± 2.6*</td>
</tr>
<tr>
<td>Fructose-fed males</td>
<td>36.9 ± 10*</td>
<td>38.9 ± 14</td>
<td>36.7 ± 13</td>
</tr>
</tbody>
</table>

Results are the average ± 1 SD of 4 mice per group. *Differences in means compared with saline-injected mice at a probability level of at least \( P < 0.05 \).

120-min time point was measured as a reflection of particle number because there is one apo B molecule per VLDL particle (10). Serum VLDL (d < 1.006 g/ml) from male mice was analyzed by SDS-PAGE following isolation by ultracentrifugation, and B100 was quantitated using stained gels and coelectrophoresed mouse B100 as standards (Fig. 1). Compared with saline-injected mice, wortmannin increased VLDL B100 by 80% (Fig. 2). These data demonstrate that in vivo inhibition of PI3-kinases by wortmannin stimulated the secretion of increased numbers of VLDL B100 particles by liver.

**In vivo wortmannin increases serum glucose levels.** PI3-kinase activation by insulin plays a key role in glucose utilization by muscle and adipose tissue and in the regulation of blood glucose (reviewed in Refs. 7 and 38). To confirm that wortmannin in the presence of Triton is actively inhibiting PI3-kinase activation by insulin resistance, we measured serum glucose changes following wortmannin injection. At the 120-min time point, serum glucose levels in wortmannin-injected mice were 74% higher than in saline-injected mice (424 ± 51 vs. 244 ± 48 mg/dl; \( P < 0.05 \)). This increase demonstrates wortmannin effects on glucose transport in vivo in the presence of Triton. Glucose levels in saline-injected mice at 120 min were higher than control levels following fasting, suggesting a small independent effect of Triton on serum glucose as has been demonstrated in previous studies (4).

**Effect of insulin resistance on wortmannin- and glucose-mediated changes in hepatic TG production.** Insulin resistance states are characterized by deficits in PI3-kinase activation by insulin (28). To determine whether insulin resistance in glucose pathways alters the ability of wortmannin to stimulate VLDL B100 production, we performed similar injection studies in male, apobec-1⁻/⁻ mice fed a 60% (wt/wt) fructose-enriched diet as has been described for hamsters (35, 36). Fructose has been shown to induce insulin resistance and attenuate hepatic insulin signaling in rodents. After 5–6 wk on diet, fructose-fed mice weighed more than control mice (26.8 ± 2.5 g vs. 20.3 ± 2.4 g; \( P < 0.05 \)), had higher serum TG levels (91.9 ± 20.5 vs. 66.2 ± 24 mg/dl; \( P < 0.05 \)), cholesterol (151.91 ± 32.1 vs. 69.5 ± 12.2 mg/dl; \( P < 0.05 \)), and glucose (279.2 ± 30.5 vs. 127 ± 16 mg/dl; \( P < 0.05 \)). These results are consistent with metabolic changes associated with insulin resistance. With the use of the Triton strategy, fructose feeding increased hepatic TG production in male mice by 58% compared with Chow-fed males (Table 1). In fructose-fed mice, TG production rates following saline, wortmannin, and glucose injections were all similarly elevated, averaging 36.9 ± 10, 38.9 ± 14, and 36.7 ± 13 µg·ml⁻¹·min⁻¹, respectively. Serum VLDL B100 levels of fructose-fed mice 120 min after injection were correspondingly increased in saline, wortmannin, and glucose-injected groups (Fig. 2) averaging 28.5 ± 6.8 (n = 4), 27.7 ± 8.6 (n = 4), and 30.0 ± 4.5

![Fig. 1. Effect of wortmannin on B100 content of VLDL (d < 1.006 g/ml) 120 min following Triton WR 1339 (Triton) injection of male apobec-1⁻/⁻ mice. Mouse sera obtained after saline (C1, C2, C3) or wortmannin injections (W1, W2, W3) were ultracentrifuged to isolate VLDL. After delipidation, VLDL apoproteins were separated by SDS-PAGE, and gels were stained with Coomassie blue (A). Serum VLDL B100 concentration was determined using coelectrophoresed mouse B100 standards and standard curves generated by densitometry (B).](image-url)
mg/dl \( (n = 8) \), respectively. The similarity of TG production rates in fructose-fed mice and those observed with wortmannin-injected chow-fed mice suggested a maximum level of VLDL production had been achieved. The induction of insulin resistance by fructose feeding also eliminated effects of glucose and wortmannin injection on VLDL TG and B100 production observed in chow-fed mice.

**The effect of wortmannin on long-term \(^{14}\)C-labeled B100 in primary hepatocytes derived from apobec-1\(^{-/-}\) mice.** Long-term (14–16 h) labeling experiments were performed to evaluate the kinetics of more uniformly labeled B100 (Fig. 3). After 2 h of wortmannin (0 time), there was significantly more \(^{14}\)C-labeled B100 in wortmannin-treated hepatocytes compared with control \((32.5\% \pm 8.0\%, n = 5, P < 0.015)\). The majority of the increased B100 was secreted into the medium by 1 h, resulting in an increase of medium B100 of 30.6\% \(\pm\) 9.4\% \((P < 0.05)\) at 1 h and 50.4 \(\pm\) 14.9\% at 2 h \((P < 0.01)\) of chase. There was little additional secretion between 1 and 2 h either in DMSO or wortmannin-treated cells. A significant proportion of B100 remained in the cell after 1 h of chase in wortmannin and DMSO-treated hepatocytes. This cellular B100 pool disappeared slowly over the next hour of incubation but did not appear in the media, indicating that it was degraded. At all time points, there was increased total B100 label with wortmannin compared with DMSO-treated cells. Media accumulation of \(^{14}\)C-labeled B100 decreased between 2 and 3 h of chase (data not shown), suggesting reuptake of secreted B100, and uptake by the LDL receptor has been reported in primary mouse hepatocytes derived from apobec-1\(^{-/-}\) mice \((22)\). Uptake of secreted B100 by the LDL receptor was confirmed in a separate experiment by incubating hepatocytes for 5 h with medium containing heparin (10 mg/ml), which increased the accumulation of \(^{14}\)C-labeled B100 by 38\% \(\pm\) 9.6\% by hepatocytes derived from apobec-1\(^{-/-}\) mice.

**Effect of wortmannin on net synthesis of \(^{35}\)S-labeled B100 in hepatocytes derived from apobec-1\(^{-/-}\) mice.** When hepatocytes were incubated with \(^{35}\)S label for 30 min, significantly more B100 was \(^{35}\)S labeled following wortmannin treatment compared with control. In paired experiments, the increase in synthesis after incubation (0 time) was 11\%, 52\%, and 51\% in 3 independent experiments. After 30 min of chase (the time necessary to fully elongate B100), more label was incorporated into B100 than at 0 time with wortmannin treatment. To better estimate this “net increase” in B100 synthesis, we increased the labeling period to 3 h so as to allow full labeling of B100 \((36)\). As shown in Fig. 4, after a 3-h labeling period, wortmannin significantly increased \(^{35}\)S-label incorporation into B100 compared with DMSO-treated cells in four independent experiments \((70\%, 60\%, 39\%, and 31\%)\) averaging 50 \(\pm\) 18\% \((P < 0.01)\). After 90 min of chase, wortmannin-treated hepatocytes had secreted 1.9 \(\pm\) 0.30 times as much B100 as DMSO-treated hepatocytes \((P < 0.03)\). Results from \(^{14}\)C labeling and from 30- and 180-min \(^{35}\)S-labeling protocols indicated that wortmannin treatment increased B100 available for secretion as

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**Fig. 3.** Effect of Wort on long-term \(^{14}\)C-labeled B100 in primary cultures of hepatocytes derived from apobec-1\(^{-/-}\) mice. **A:** mouse hepatocytes were incubated in medium containing \(^{14}\)C-labeled leucine overnight (14–16 h). Hepatocytes were reincubated in fresh labeling medium containing 1 \(\mu\)M Wort or DMSO for 2 h followed by incubation in chase media for 1 and 2 h. Dotted lines indicate incubation was carried out in labeling medium. **B:** at each time point of chase, cells and media were collected and \(^{14}\)C-labeled B100 was isolated by immunoprecipitation and analyzed by SDS-PAGE. C: results were quantified by Phosphorimage analysis, corrected for plate protein, and plotted against time of chase. Results represent the average \(\pm\) SE in 5 independent experiments where duplicate plates were analyzed in each experiment. **AU,** arbitrary units; \(\bigcirc\), Wort-treated cells; \(\bullet\), DMSO-treated cells. \(*\)Results differ from the DMSO-treated hepatocytes \((P < 0.05)\).
DISCUSSION

Insulin is a potent anabolic hormone responsible for the synthesis and storage of metabolic products derived from carbohydrates, lipids, and proteins. Intracellular effects of insulin depend on phosphorylation of downstream proteins altered by the insulin receptor including the IRS family of proteins (reviewed in Refs. 37 and 38). Metabolic effects of insulin appear to involve mainly downstream activation of PI3-kinase (28, 38). We have previously shown that insulin inhibits VLDL secretion in vivo and in vitro and that PI3-kinase is necessary (23, 31) through mechanisms that regulate the synthesis and intracellular degradation of apo B (33). With the use of wortmannin, it was shown that insulin inhibits the maturation phase of VLDL assembly by preventing lipid transfer to pre-VLDL particles (3). Without complete assembly, the degradation of pre-VLDL apo B is favored. Current studies addressed the hypothesis that in vivo fluctuations in PI3-kinase activity modulate hepatic VLDL production. We used apobec-1-/- mice whose hepatocytes synthesize and secrete only B100 as our model of human liver VLDL metabolism, and our experiments were conducted in fasted mice to minimize the intestinal contribution. Our results demonstrate that in male and female mice, glucose-stimulated insulin secretion suppresses hepatic VLDL production, and wortmannin inhibition of PI3-kinase maximizes hepatic VLDL production.

We have previously shown in rats that insulin has a direct effect on the liver in controlling VLDL secretion in vivo (4) and that insulin dysregulation leads to overproduction of VLDL in insulin-resistant ZDF rats (6). Rodent liver synthesizes both VLDL B100 and VLDL B48, and the B100/B48 ratio depends on ambient levels of insulin (24), which are known to alter apobec-1 expression (8). Thus the effects of insulin on VLDL B100 production in rodents may be complicated by overlapping metabolic pathways for B48. Human liver does not express apobec-1 and therefore does not synthesize B48. The apobec-1-/- mouse affords the opportunity to study a rodent model more closely resembling human liver, because hepatocytes derived from these mice synthesize and secrete VLDL B100 (22). To evaluate the effects of insulin resistance on the PI3-kinase pathway, male apobec-1-/- mice were fed a fructose-enriched diet and were characterized by hyperlipidemia and fasting hyperglycemia, consistent with previous studies (36). Significant increases in hepatic VLDL output are demonstrated in mice under insulin-resistant conditions induced by fructose. Data suggest that the increase observed is related to loss of regulation of PI3-kinase by insulin and reversal of insulin suppression of apo B secretion seen physiologically.

Hepatic VLDL production in chow-fed mice can be reduced by as much as 30% by glucose and stimulated by 52% in males and 89% in females by wortmannin. This demonstrates potential for significant fluctuations in VLDL under different physiological conditions. Findings suggest that hepatic VLDL production in vivo may be partially suppressed under basal conditions by wortmannin-sensitive PI3-kinases and that liver is capable of significantly modulating VLDL secretion with increases or decreases. Lipogenesis has likely remained stable within the 2-h time frame of the Triton experiments, indicating that synthesis of B100 appears to be a major determinant in the export of available TG. Data support that wortmannin facilitates the transfer of existing lipid to pre-VLDL B100 to form VLDL by inhibiting PI3-kinase.

The finding that the effects of glucose and wortmannin disappear following fructose-feeding suggests that the maximum levels of hepatic VLDL production attained are also unregulated. In contrast to chow-fed mice, in fructose-fed mice, increased VLDL export occurs in a background of enhanced lipogenesis. Despite this, hepatic B100 and TG output in fructose-fed mice are not increased significantly above wortmannin-stimulated secretion observed in chow-fed mice, suggesting TG export rates are determined by B100 as our model of human liver VLDL metabolism, and our experiments were conducted in fasted mice to minimize the intestinal contribution. Our results demonstrate that in male and female mice, glucose-stimulated insulin secretion suppresses hepatic VLDL production, and wortmannin inhibition of PI3-kinase maximizes hepatic VLDL production.
formed in fructose-fed hamsters, which demonstrate significant increases in B100 synthesis and secretion due to stabilization of hepatic B100 (36). In human studies, fructose feeding has also been shown to increase VLDL production (11).

In recent studies using apobec-1Δ mice derived hepatocytes, multiple cellular pools of B100 were defined kinetically including an incomplete translation pool, a fully translated, full-length, presecretory B100 pool, and a cellular B100 pool subject to slow degradation (22). Using various times of label incorporation, our data are consistent with wortmannin stimulating the “net translation” of apo B peptides to full-length B100. This, in turn, increased the availability of full-length B100 in the presecretory pool as most of the increased labeled cellular B100 appeared in the medium within 1 h and contributed to B100 media increases at 1 and 2 h. A number of investigators have described proteasomal degradation of B100 in the endoplasmic reticulum (reviewed in Refs. 12 and 13), and current studies were not performed to rule out the possibility that wortmannin treatment increased recovery of B100 subject to proteasomal degradation. Further experiments will be necessary to separate effects of synthesis and degradation on availability of nascent B100. However, it is reasonable to conclude from our studies that wortmannin treatment increased freshly translated apo B within a presecretory pool.

In conclusion, current studies demonstrate, in vivo and in vitro, that VLDL assembly and secretion are regulated by PI3-kinase activation state and that fluctuations in activity are a determinant in VLDL output. Results suggest that fructose feeding prevents the modulatory effects of PI3-kinase on B100 and TG assembly similarly to wortmannin inhibition, both conditions leading to “pathway-specific” resistance and increased VLDL secretion. The present study also suggests a direct relationship between insulin resistance and maximized hepatic VLDL production. Together, these in vivo and in vitro results support that heptacically derived VLDL B100 lipoproteins contribute to the development of hypertriglyceridemia typical of insulin resistance and are consistent with previous observations in ZDF rats (6). We speculate that maximizing VLDL output under conditions of insulin resistance could be “protective” against the toxicity of liver lipid accumulation. Considerable future experimentation will be necessary to place the current findings into pathophysiologic context, considering the potential effects of secretory VLDL on the arterial wall in the process of atherogenesis.

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