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Insulin suppression of VLDL apo B secretion is not mediated by the LDL receptor☆

Doru V. Chirieac,[a] Joanne Cianci,[b] Heidi L. Collins,[b] Janet D. Sparks,[b] and Charles E. Sparks[b,*]

[a] *Department of Community and Preventive Medicine, University of Rochester School of Medicine and Dentistry, P.O. Box 626, 601 Elmwood Avenue, Rochester, NY 14642, USA*

[b] *Department of Pathology and Laboratory Medicine, University of Rochester School of Medicine and Dentistry, P.O. Box 644, 601 Elmwood Avenue, Rochester, NY 14642, USA*

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Abstract

Insulin inhibits hepatic very low density lipoprotein (VLDL) apo B secretion in rats. Current studies test whether the insulin effect is LDL receptor-mediated by examining the effect of insulin on VLDL apo B secretion in hepatocytes derived from *Ldlr*-/- and control mice. Primary hepatocytes were incubated overnight with media containing ^{14}C-leucine and either 0.1 nM (basal) or 200 nM insulin. Afterwards, secreted VLDL B100 and B48 were quantitated. Insulin reduced ^{14}C-labeled B100 and B48 comparably in control and *Ldlr*-/- hepatocytes with a $62 \pm 12\%$ vs. $59 \pm 12\%$ decrease in B100, and a $56 \pm 11\%$ vs. $61 \pm 9\%$ decrease in B48. Results indicate: (1) mouse hepatocytes respond to insulin by reducing VLDL apo B output; (2) both VLDL B100 and B48 secretion are suppressed; and (3) insulin inhibition of VLDL apo B secretion is retained in *Ldlr*-/- hepatocytes.

Keywords: Insulin; VLDL; LDL; Apo B; B100; B48; LDL receptor; Hepatocytes

Very low density lipoprotein (VLDL) contains apo B as its integral protein component. As VLDL is metabolized to LDL, apo B is retained following the fate of its constitutive particle. Each VLDL particle contains one molecule of apo B; hence, apo B concentration corresponds to the number of synthesized VLDL particles [1]. In contrast to human liver, which makes only B100, rodent liver makes both B100 and B48. B48 is the form of apo B that is regulated by post-transcriptional mRNA editing and is characteristic of intestinal triglyceride-rich lipoproteins [2–4]. Metabolic pathways for regulating apo B secretion are complex with multiple levels of control, including mechanisms involving intracellular degradation of freshly synthesized apo B [5–8]. Apo B degradation may be regulated in the endoplasmic reticulum (ER) by lipid availability, as oleic acid has a protective effect on ER degradation of apo B by a pathway involving the proteasome in Hep G2 cells [7]. Insulin has a short-term regulatory effect on apo B degradation [5] that occurs after movement out of the ER [9] and most likely involves the process of fusion of a partially lipidated apo B with triglyceride that requires microsomal triglyceride transfer protein [10] and insulin-activated PI 3-kinase [11,12]. A potential mechanism for insulin-mediated post-ER degradation of apo B is by the low density lipoprotein receptor (LDLR), as there is potential interaction of partially lipidated particles with the LDLR by way of B100 or apo E ligands [13]. A role for the LDLR is supported by increased apo B secretion in hepatocytes derived from *Ldlr*-/- mice [13,14]. Recently, the LDLR has been implicated in presecretory degradation of apo B [15]. To test the hypothesis that the insulin inhibitory effect on apo B is LDLR-mediated, hepatocytes derived from *Ldlr*-/- mice

☆ *Abbreviations*: VLDL, very low density lipoproteins; LDL, low density lipoproteins; LDLR, low density lipoprotein receptor; *Ldlr*, low density lipoprotein receptor gene.

* Corresponding author. Fax: +585-756-5337.
E-mail address: Charles_Sparks@urmc.rochester.edu (C.E. Sparks).

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were studied in comparison with those derived from control mice to determine whether insulin-mediated pathways were altered. In the current studies, mouse hepatocytes responded to insulin with reduced VLDL secretion affecting both VLDL B100 and B48 particles and equivalent inhibitory effects of insulin were observed in hepatocytes derived from *Ldlr*-/- mice. The presence of insulin-mediated suppression of VLDL apo B in *Ldlr*-/- hepatocytes supports the concept that the mechanism of insulin action on VLDL apo B is independent of the LDLR.

Materials and methods

Animals. Six- to seven-week-old male *Ldlr*-/- [16] and male wild-type control (C57Bl/6) mice were purchased from Jackson Lab (Bar Harbor, ME) and maintained at the University of Rochester Animal Care Facility. The mice were fed Purina 5008 rodent chow and water ad libitum and at 10 weeks of age (25 ± 3 g) were used to prepare heptocytes.

Primary mouse hepatocytes. Age-matched *Ldlr*-/- and control mice were fasted overnight (16–18 h) and anesthetized by intraperitoneal injection with ketamine (800 mg/kg body weight, 0.2 ml) and xylazine (80 mg/kg body weight 0.1 ml). After clamping the superior vena cava, a 50-ml solution of Krebs–Ringer bicarbonate, Hepes, penicillin, streptomycin, and gentamicin [17] was infused at 8 ml/min into the inferior vena cava to perfuse the liver. Then, 50 ml solution containing 84.5 U/ml collagenase (Type 2, Worthington, Lakewood, NJ) was infused at 5 ml/min to digest the liver. The digested liver was removed and minced in Waymouth's 752/1 media (Gibco-BRL, Grand Island, NY) containing 0.2% (w/v) bovine serum albumin (BSA) containing antibiotics [17], hereafter referred to as Waymouth's media. The mixture was then screened with Nitex mesh (Sefar America, Depew, NY) and isolated hepatocytes were pelleted by centrifugation twice at 50*g* for 2 min, followed by purification using Percoll [18]. Viable hepatocytes were washed in Waymouth's media and seeded onto 60 mm petri dishes (10^6 cells/ml, 2 ml/dish) previously coated with rat tail collagen [17,18]. After 2–4 h, non-adherent cells were removed by rinsing culture dishes three times with Hanks' balanced salt solution containing 0.2% (w/v) BSA. Cells were then re-incubated in Waymouth's media containing 0.8 μCi/ml ^{14}C-leucine (specific activity 93.5 mCi/mmol) plus unlabeled leucine (final concentration 14.3 μM) and either 0.1 nM (basal) or 200 nM insulin at 37 °C in a humidified atmosphere of 95% air/5% CO_2 (v/v). After overnight incubation (16–18 h), media were collected from 3 to 6 plates per condition and pooled, and protease inhibitor cocktail I was added as preservative (final concentration, 1% v/v) (Calbiochem, San Diego, CA).

Apo B measurements. To each pooled media sample, 0.5 mg of freshly isolated rat serum VLDL protein ($d < 1.006$ g/ml) was added as carrier. Mouse VLDL was isolated from media by density ultracentrifugation ($d < 1.006$ g/ml) for 18 h at 14 °C at 50,000 rpm using a Ti 70 rotor (Beckman–Coulter Instruments, Fullerton, CA) [19]. The top VLDL fraction (1 ml) was removed, mixed in methanol:chloroform:diethyl ether (5:5:10, v/v/v) to delipidate the VLDL, and stored at −20 °C overnight [20]. Precipitated apoproteins were pelleted by centrifugation, washed in cold diethyl ether, and air-dried. Pellets were then dissolved in Laemmli's buffer containing freshly added DTT [21], heated at 95 °C for 5 min, and B100 and B48 were separated by electrophoresis on 3.5–24% (w/v) Acrylaide/acrylamide gradient gels cast on Gelbond PAGE film (FMC Bioproducts, Rockland, ME) [22]. After electrophoresis, gels were heat-fixed at 170 °F in a convection oven for 45 min and ^{14}C-labeled B100 and B48 bands were visualized and quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). To determine absolute label incorporation, B100 and B48 bands were cut out from gels and digested with perchloric acid (0.2 ml 60%, v/v) and hydrogen peroxide (0.1 ml 30%, v/v) as previously described [23,24]. Samples were radioassayed by liquid scintillation counting after mixing with 5 ml Hionic Fluor (Packard Instrument Company, Meriden, CT) and equilibrating samples overnight in the dark. ^{14}C-labeled VLDL B100 and B48 were normalized to mg cell protein per dish by assay of protein content of cell lysates using a modified Lowry method [25].

Measurement of ^{14}C-leucine incorporation into total cell protein. Cells were scraped into lysis buffer (0.5% (v/v) Triton X-100, 0.05 M barbital, pH 8.6). After brief sonication, lysates were cleared by centrifugation at 4 °C. Lysates (0.1 ml) were adsorbed onto GF/C glass filters. One set of filters was washed twice with 10% TCA (v/v) at 60 °C for 5 min. After rinsing in absolute ethanol for 2 min, the filters were air-dried [26]. Unwashed (total counts) and washed (TCA-precipitatable counts) filters were mixed with 5 ml Ecoscint A (Packard Instrument Company, Meriden, CT), analyzed by liquid scintillation counting, and TCA precipitatable cell protein radioactivity was used to calculate ^{14}C-leucine incorporation into total protein, and results were normalized to mg cell protein per dish.

Statistical analysis. Results are expressed means ± 1SD and differences between means were determined by Student's t test with the level of significance at $p < 0.05$. N indicates the number of independent mouse hepatocyte preparations.

Results and discussion

LDLR is known to have a central role in plasma lipoprotein catabolism [27]. Recently, there has been a strong interest in testing the role of LDLR in modulating hepatic production of apo B-containing lipoproteins as knowledge of hepatic lipoprotein regulatory pathways expands. Hepatic apo B secretion was examined in vitro in studies using primary hepatocytes derived from *Ldlr*-/- mice, as these animals have been reported to show an increase in hepatic triglyceride and VLDL apo B output, suggesting that the LDLR might alter the proportion of apo B that escapes presecretory degradation [13]. One mechanism may involve ER-localized LDLR [15], while another possible mechanism may involve the re-uptake of newly secreted apo B-containing lipoproteins at the cell surface [28]. In vivo studies performed with Triton WR 1339; however, demonstrate no difference in hepatic VLDL triglyceride and apo B production rates in *Ldlr*-/- mice compared with controls, even though these animals had higher plasma cholesterol and triglyceride levels [29]. Similar results were obtained when studies were conducted in mice lacking the *apobec-1* gene and producing only B100. The B100-only model overcomes potential differences in the affinity of B100 and B48 for the LDLR and more closely parallels human liver VLDL metabolism.

In the current studies, secretion of VLDL apo B was studied in primary cultures of mouse hepatocytes. In rat hepatocytes, apo B is predominantly secreted into the VLDL fraction, which is the fraction susceptible to inhibition by insulin [30]. We therefore isolated VLDL

from culture media to study insulin effects. Similar to rat hepatocytes, over 60% of apo B was secreted into the VLDL fraction by mouse hepatocytes and similar results were observed for control and *Ldlr*-/- mice. In control and *Ldlr*-/- mice, ^{14}C-leucine incorporation into VLDL B48 and B100 was virtually identical (Tables 1 and 2), indicating that there was no significant increase in VLDL apo B production in hepatocytes derived from *Ldlr*-/- mice.

Since insulin inhibits VLDL apo B secretion by rat liver and by rat hepatocytes through a mechanism involving intracellular degradation of freshly synthesized apo B [30], studies were performed in the *Ldlr*-/- hepatocytes to test the hypothesis that the LDLR was responsible for the insulin effect. VLDL was isolated from the media by ultracentrifugation and, following delipidation, B100 and B48 were separated by SDS–PAGE and quantitated (Fig. 1). Insulin (200 nM) reduced B100 and B48 as well as total VLDL apo B secretion. PhosphorImager analysis showed reductions in B100 and B48 that were, respectively, $62 \pm 12\%$ and $56 \pm 11\%$ in control hepatocytes ($N = 5$) and in *Ldlr*-/- hepatocytes, results were similar with $59 \pm 12\%$ decrease in B100 and $61 \pm 9\%$ decrease in B48 ($N = 7$). Insulin treatment resulted in a small increase (5–10%) in cell protein and absolute label incorporation into cell protein. Absolute dpm were also calculated in experiments where B48 and B100 were eluted from gels and radioassayed and results are shown in Tables 1 and 2 expressed as dpm per mg cell protein. Similar results were obtained in hepatocytes derived from control and *Ldlr*-/- mice (Table 1 compared to Table 2), suggesting that mouse hepatocytes respond to insulin similarly to rat hepatocytes with reductions in both VLDL B100 and B48 secretion. In agreement with Rader's laboratory [29], no difference was observed in label incorporation into VLDL apo B in hepatocytes derived from control vs. *Ldlr*-/- mice. We conclude that the suppressive effect of insulin on hepatic VLDL secretion is not mediated by the LDL receptor.

Table 1
Effect of insulin on secreted VLDL ^{14}C-apo B in control mice

Insulin	–	+	% Reduction
VLDL B100	88.0 ± 17.8	37.0 ± 6.5	53.2 ± 7.9
VLDL B48	353.4 ± 46.4	188.5 ± 33.8	44.2 ± 8.0

Values expressed as dpm per mg cell protein ($N = 6$).

Table 2
Effect of insulin on secreted VLDL ^{14}C-apo B in *Ldlr*-/- mice

Insulin	–	+	% Reduction
VLDL B100	73.3 ± 15.3	32.6 ± 4.5	50.0 ± 11.7
VLDL B48	318.4 ± 32.5	133.3 ± 13.7	55.8 ± 8.4

Values expressed as dpm per mg cell protein ($N = 4$).

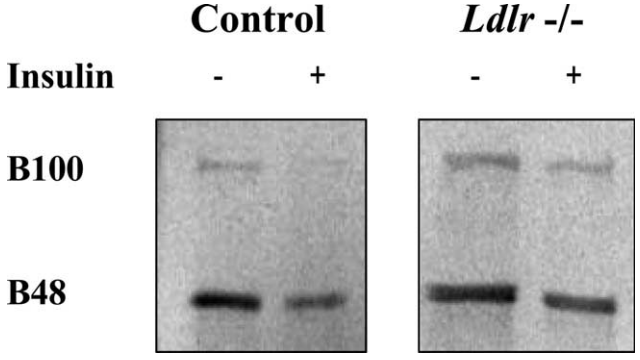

Fig. 1. Effect of insulin on VLDL-apo B secretion in the presence and absence of a functional LDLR. Hepatocytes from wild-type and *Ldlr*-/- mice were labeled with ^{14}C-leucine for 16–18 h in the presence of either 0.1 nM (basal) or 200 nM insulin. Following isolation of VLDL by ultracentrifugation ($d < 1.006$), ^{14}C-labeled B100 and B48 were separated by SDS–PAGE. Gels were heat-fixed and B100 and B48 were quantitated by PhosphorImager analysis. Results shown are representative of results from six independent hepatocyte preparations for wild type and four independent hepatocyte preparations for *Ldlr*-/- mice.

Our laboratory has focused on short-term regulation of hepatic VLDL apo B by insulin. In vivo and in vitro suppression of VLDL apo B by insulin in rats has been well documented [31]. In the post-prandial period, when portal insulin concentrations are high, hepatic VLDL production is reduced, minimizing competition with lipoproteins of intestinal origin for catabolism. As each VLDL particle contains one molecule of apo B and insulin inhibits apo B secretion, insulin also reduces the number of VLDL apo B lipoprotein particles produced. Insulin resistance, such as that occurs with the Zucker obese rat, results in the loss of ability of insulin to regulate hepatic apo B secretion in the short term [32]. The resultant net increase in hepatic VLDL apo B production may have implications for the hypertriglyceridemia observed with insulin resistance, especially during the post-prandial period. Results suggest that increasing LDLR expression, as it occurs with statin therapy, would not restore insulin-regulated hepatic VLDL secretion that is lost in insulin resistance states. The focus of future studies will be to define the mechanism responsible for insulin-mediated degradation of apo B in the liver. Since hypertriglyceridemia is an important component of the newly defined metabolic syndrome, understanding the biochemistry of insulin regulation of apo B metabolism has importance.

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