

Hepatic PTP-1B Expression Regulates the Assembly and Secretion of Apolipoprotein B–Containing Lipoproteins

Evidence From Protein Tyrosine Phosphatase-1B Overexpression, Knockout, and RNAi Studies

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Protein tyrosine phosphatase-1B (PTP-1B) plays an important role in regulation of insulin signal transduction, and modulation of PTP-1B expression seems to have a profound effect on insulin sensitivity and diet-induced weight gain. The molecular link between PTP-1B expression and metabolic dyslipidemia, a major complication of insulin resistance, was investigated in the present study using PTP-1B knockout mice as well as overexpression and suppression of PTP-1B. Chronic fructose feeding resulted in a significant increase in plasma VLDL in wild-type mice but not in PTP-1B knockout mice. Lipoprotein profile analysis of plasma from PTP-1B knockout mice revealed a significant reduction in apolipoprotein B (apoB100) lipoproteins, associated with reduced hepatic apoB100 secretion from isolated primary hepatocytes. In addition, treatment of cultured hepatoma cells with PTP-1B siRNA reduced PTP-1B mass by an average of 41% and was associated with a 53% decrease in secretion of metabolically labeled apoB100. Conversely, adenoviral-mediated overexpression of PTP-1B in HepG2 cells downregulated the phosphorylation of insulin receptor and insulin receptor substrate-1 and caused increases in cellular and secreted apoB100 as a result of increased intracellular apoB100 stability. Collectively, these findings suggest that PTP-1B expression level is a key determinant of hepatic lipoprotein secretion, and its overexpression in the liver can be sufficient to induce VLDL overproduction and the transition to a metabolic dyslipidemic state. *Diabetes* 53:3057–3066, 2004

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apoB, apolipoprotein B; FPLC, fast-protein liquid chromatography; IRS-1, insulin receptor substrate-1; MTP, microsomal triglyceride transfer protein; PI3-K, phosphatidylinositol 3-kinase; PTP, protein tyrosine phosphatase; SREBP, sterol regulatory element-binding protein.

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Protein tyrosine phosphatase-1B (PTP-1B) is a member of the protein tyrosine phosphatase family of enzymes that is localized to the endoplasmic reticulum (1,2). Overexpression studies have shown that PTP-1B dephosphorylates the insulin receptor in vitro, as well as induces the downregulation of insulin receptor substrate-1 (IRS-1) and insulin-stimulated phosphatidylinositol 3-kinase (PI3-K) activity (3–6). Tissue levels of PTP-1B have been reported to be increased in insulin-resistant diabetes in humans (7). Increased PTP-1B expression has also been observed in the *fa/fa* genetic model of insulin resistance and obesity and the ZDF/*fa/fa* model of insulin-resistant diabetes (8), whereas decreased expression of PTP-1B was seen in a model of type 1 diabetes induced by streptozotocin in the rat (9). PTP-1B knockout mice have increased sensitivity toward insulin-induced insulin receptor and IRS-1 tyrosine phosphorylation, and interestingly, these animals are resistant to obesity (10,11). Attenuation of PTP-1B expression with antisense oligonucleotides enhances hepatic insulin signaling and peripheral insulin sensitivity in vivo in *ob/ob* mice (12,13) and increases insulin signal transduction in vitro in FAO hepatoma cells (14). Single-point mutations at the amino acid or nucleotide level have been associated with both type 2 diabetes and protection from diabetes (15,16). In both transfection studies and transgenic animals, it has been shown that PTP-1B dephosphorylates the leptin receptor, growth hormone, and interferon- γ receptor-associated kinase Jak2 (17–19). PTP-1B-deficient mice thus display enhanced leptin-mediated loss of body weight (18,19). Increased PTP-1B mass and activity have also been associated with fructose-induced insulin resistance in the hamster (20). Overall, the available evidence thus suggests that increased PTP-1B can induce an insulin-resistant state; however, it is unclear how changes in insulin signaling induced by PTP-1B can influence lipid and lipoprotein metabolism and the development of metabolic dyslipidemia.

The pathophysiology of the insulin-resistant state is associated with elevated blood glucose, hyperinsulinemia, hypertension, atherosclerosis, and metabolic dyslipidemia (reviewed in 21–25). Metabolic dyslipidemia is widely

recognized as a common and important complication of insulin-resistant states and the metabolic syndrome (26). The dyslipidemia observed in the metabolic syndrome is characterized by elevated plasma triglycerides and low HDL cholesterol concentrations. There is also evidence for enhanced postprandial lipemia and the presence of small, dense LDL particles, both of which are associated with increased risk for cardiovascular disease (reviewed in 27). Mechanisms underlying the development of metabolic dyslipidemia in insulin-resistant states have been under intense investigation in recent years, and there is now ample evidence to suggest that overproduction of hepatic apolipoprotein B (apoB)-containing lipoproteins is a key underlying factor. Studies in the ApoB/BATless mice and fructose-fed hamster models have clearly shown evidence of hepatic overproduction of VLDL-apoB and shed some light on the underlying mechanisms (20,28–30). ApoB/BATless is an insulin-resistance model developed by crossing a human apoB transgenic mouse with a brown adipose tissue knockout mouse that exhibits peripheral insulin resistance (28). The resulting animal develops obesity, hypertriglyceridemia, hypercholesterolemia, and hyperinsulinemia when placed on a high-fat diet, with evidence of hepatic overproduction of VLDL-apoB. In the fructose-fed hamster model, enhanced hepatic VLDL-apoB production is closely linked to diminished insulin signaling in the liver (20). Upon fructose feeding, the hamster develops insulin resistance, obesity, and hypertriglyceridemia and exhibits a lipoprotein profile more similar to that of humans than that of other rodent models (31–36). In *ex vivo* experiments using primary hepatocytes isolated from insulin-resistant fructose-fed hamsters, elevated VLDL-apoB secretion correlated with decreased tyrosine phosphorylation of the insulin receptor, IRS-1, IRS-2, and Akt and reduced activity of IRS-associated PI3-K (20). It is interesting that these changes in hepatic insulin signaling in the fructose-fed hamster were associated with a significant increase in both mass and activity of PTP-1B (20). Treatment with the insulin sensitizer rosiglitazone restored insulin sensitivity, enhanced hepatic insulin signaling, reduced PTP-1B protein levels, and was accompanied by a significant attenuation of hepatic VLDL-apoB secretion (37). These data suggest a possible link between the impairment of intracellular signaling associated with PTP-1B and overproduction of apoB-containing lipoproteins that underlies metabolic dyslipidemia. PTP-1B may also regulate apoB secretion via the PI3-K pathway. There is evidence showing that insulin downregulates apoB secretion at least partly by activation of PI3-K signaling, leading to inhibition of VLDL maturation and secretion in rat hepatocytes (38,39). PTP-1B-mediated inhibition of IRS-1 phosphorylation can potentially block PI3-K signaling and thus affect apoB regulation.

On the basis of the above observations, we hypothesized that PTP-1B expression may be a critical determinant of VLDL overproduction and metabolic dyslipidemia in insulin resistance. PTP-1B overexpression, by itself, may be sufficient to induce changes in hepatic lipoprotein metabolism, leading to development of metabolic dyslipidemia. In the present study, we examined this link by overexpression and suppression of PTP-1B in cultured hepatic cells

and by further characterizing the lipoprotein changes of PTP-1B knockout mice.

RESEARCH DESIGN AND METHODS

Cell culture. HepG2 and McArdle RH7777 cells were purchased from ATCC (Rockville, MD). HepG2 cells were maintained in α -minimum essential medium (Life Technologies, Burlington, ON, Canada) with 10% FCS (Atlanta Biologicals, Norcross, GA), and McArdle RH7777 cells were maintained in Dulbecco's modified Eagle's medium with 20% FCS. Media were supplemented with 50 units/ml penicillin and 50 μ g/ml streptomycin (Life Technologies) and maintained at 37°C, 5% CO₂.

Isolation and culture of mouse hepatocytes. Male PTP-1B knockout mice and their wild-type littermates were housed at the transgenic facility of McGill University (10). At 7–8 weeks of age (between 25 and 30 g), wild-type and PTP-1B knockout mice were fed *ad libitum* either a standard laboratory diet or a 60% fructose-enriched diet (Dyets, Bethlehem, PA) for 3 weeks. After the feeding period, primary mouse hepatocytes were isolated from PTP-1B knockout and wild-type mice as previously described (29) with minor modifications. Briefly, the primary hepatocytes were maintained in Williams E medium (Life Technologies), supplemented with 5% FBS and 1.5 ng of insulin/ml (Invitrogen, Burlington, ON, Canada) for 4 h.

Transfection of siRNA duplex. An siRNA duplex to the target sequence AAGCTGACACTGATCTCTGAA of rat PTP-1B was synthesized commercially (Qiagen, Chatsworth, CA). McArdle RH7777 cells were seeded (5×10^5 cells) in 12-well plates and were allowed to adhere for 18 h. A total of 1 μ g/ml of silencing siRNA or the same amount of nonsilencing control siRNA (cat no. 102279; Qiagen) was transfected using 13 μ l/ml RNAiFect (Qiagen) transfection reagent according to the manufacturer's instructions. After 18 h of incubation with siRNA, the medium was changed to Dulbecco's modified Eagle's medium that contained 20% FBS and antibiotics for 48 h before pulse-chase experiments were performed.

Generation of recombinant PTP-1B adenovirus. Recombinant mouse PTP-1B adenovirus (ADV1Bm) was generated by using AdenoVator Vector System (Qiagen). Briefly, 1.3 kb of mouse PTP-1B cDNA was cloned into pShuttle-CMV at *Bam*HI/*Xba*I sites. After recombination with pAdEasy-1 plasmid DNA, two clones with the PTP-1B insert were selected and designated as ADV1Bm. After two rounds of viral plaque purification, the clone ADV1Bm (A3) revealed successful integration of the PTP-1B gene into the adenovirus and was amplified in HEK293 cells for further experiments. A control adenovirus encoding β -galactosidase (Ad β -gal) was amplified and purified in the same way as the PTP-1B.

Infection of cell cultures with recombinant adenoviruses. HepG2 (5×10^5) cells were seeded onto collagen-coated six-well plates. Four hours after seeding, cells were infected with ADV1Bm (multiplicity of infection [MOI] 2.5–40). Two hours after infection, the medium was removed and replaced with complete cell medium that contained 5% FCS, and cells were maintained for 48 h before pulse-chase experiments.

Metabolic labeling of adenovirus-infected or siRNA-transfected cells. Cell cultures were preincubated in serum-free, methionine/cysteine-free α -minimum essential medium for 1 h and labeled with 100 μ Ci/ml [³⁵S]methionine/cysteine for 1 h. After the pulse, both cell and medium were harvested for immunoprecipitation to detect apoB100.

Analysis of lipoproteins by fast-protein liquid chromatography. At 7–8 weeks of age, wild-type and PTP-1B knockout mice were fed *ad libitum* either a standard laboratory diet or a fructose-enriched diet for 3 weeks. At the end of the feeding period, animals were killed and blood was collected. Lipoproteins from the plasma of wild-type and PTP-1B knockout mice were separated by automated gel filtration chromatography on a Pharmacia (Amersham Pharmacia Biotechnology, Uppsala, Sweden) fast protein liquid chromatography (FPLC) system. Plasma samples (1 ml) were manually transferred to a 2-ml sample loop with two washes of 0.5 ml of saline solution. Samples were programmed (Liquid Chromatography Controller LCC-500 Plus) to be loaded and separated on a 50-cm column (16-mm internal diameter) packed with cross-linked agarose gel (Superose 6 prep grade; Pharmacia, Piscataway, NJ). Cholesterol and triglyceride were measured enzymatically in every second FPLC fraction on an autoanalyzer (Cobas Mira; Roche, Montclair, NH).

Immunoblot analysis. Immunoblotting was carried out essentially as described (20). An anti-mouse monoclonal antibody was used for detection of PTP-1B (Oncogene). Evaluation of tyrosine phosphorylation state of insulin signaling proteins was carried out as described previously (20). HepG2 cells were insulin- and serum-starved for 5 h, and cells were incubated in the absence or presence of 100 nmol/l insulin for 10 min. Cell lysates were subjected to immunoprecipitation with a specific antibody against either the insulin receptor β -subunit or IRS-1 (1 μ g antibody/500 μ g protein from cell

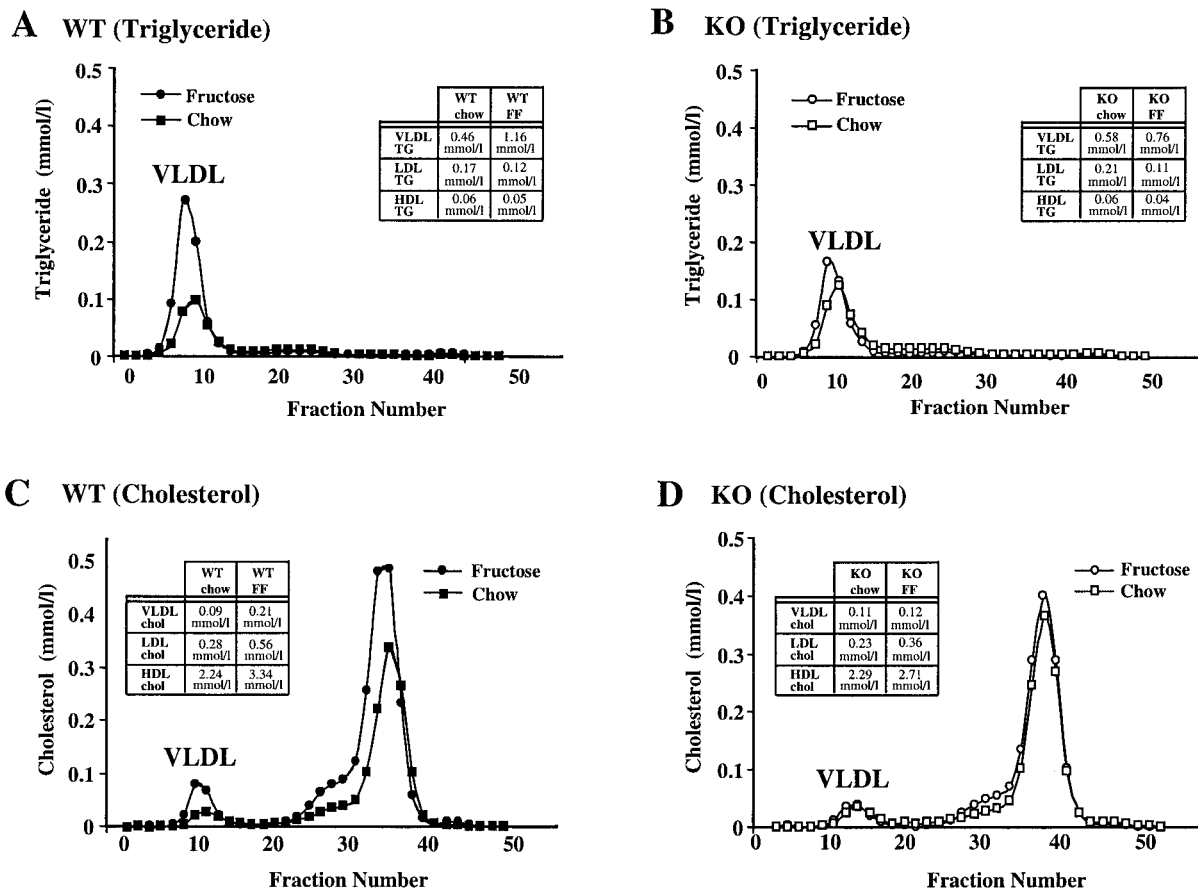


FIG. 1. PTP-1B knockout mice are resistant to fructose-induced dyslipidemia and plasma VLDL-triglyceride elevation. Wild-type (WT) and PTP-1B knockout (KO) mice were fed a fructose-enriched diet for 3 weeks. Pooled plasma from three pairs of wild-type and knockout mice was subjected to FPLC analysis to fractionate lipoproteins. Total cholesterol and triglyceride levels were measured in all FPLC lipoprotein fractions. *A* and *B*: Triglyceride distribution in plasma lipoproteins of wild-type and knockout mice. *C* and *D*: Cholesterol distribution in plasma lipoproteins of wild-type and knockout mice. *Inset*: Total triglyceride and cholesterol in lipoprotein subclasses. FF, fructose fed.

lysate). Immunoprecipitates were analyzed by immunoblotting using an anti-phosphotyrosine, *p*-Tyr (py99), antibody.

RESULTS

PTP-1B knockout mice are resistant to fructose-induced dyslipidemia and plasma VLDL-triglyceride elevation. PTP-1B knockout mice display enhanced insulin sensitivity and are resistant to fat-induced obesity (10,11). In the present study, we carried out further characterization of the lipid and lipoprotein profiles of PTP-1B knockout mice. For examining differences in plasma lipid and apoB lipoproteins, wild-type and PTP-1B knockout mice were fed a fructose-enriched diet for a period of 3 weeks. FPLC analysis of plasma lipoproteins was then carried out (Fig. 1). The high-fructose diet induced significant increases in VLDL-triglyceride as well as LDL cholesterol and HDL cholesterol in the wild-type mice (Fig. 1*A* and *C*) but not in PTP-1B knockout mice (Fig. 1*B* and *D*). Total plasma triglyceride was significantly increased in wild-type mice upon fructose feeding (Table 1); however, there was no significant increase in knockout mice that were fed the same fructose-enriched diet. Total plasma cholesterol increased significantly in both wild-type and knockout mice; however, the increase observed in wild-type mice was greater and highly significant (Table 1).

Plasma levels of VLDL-triglyceride and VLDL-apoB are decreased and hepatic apoB production is reduced in PTP-1B knockout mice. We next examined whether lipoprotein levels were also decreased in the plasma of PTP-1B knockout mice. Immunoblot analysis of plasma apoB levels in mouse plasma indicated a twofold reduction in total circulating levels of apoB100 and apoB48 in PTP-1B knockout mice (Fig. 2*A*, $n = 3$, $P < 0.05$) compared with that in wild-type mice. A three- to fourfold reduction in total lipoprotein-associated apoB was observed when comparing the immunoblots of various lipoprotein fractions (Fig. 2*B*; measured as area under

TABLE 1

Lipid profile of wild-type and PTP-1B knockout mice on chow versus fructose-enriched diet

	<i>n</i>	Triglyceride (mmol/l)	Cholesterol (mmol/l)
Wild type + chow	8	1.08 ± 0.22	2.91 ± 0.11
Wild type + fructose	7	1.44 ± 0.13	4.05 ± 0.30
<i>P</i>		0.0002	0.00028
Knockout + chow	6	0.92 ± 0.27	3.14 ± 0.30
Knockout + fructose	7	1.03 ± 0.05	3.90 ± 0.58
<i>P</i>		0.27	0.017

Data are means ± SD.

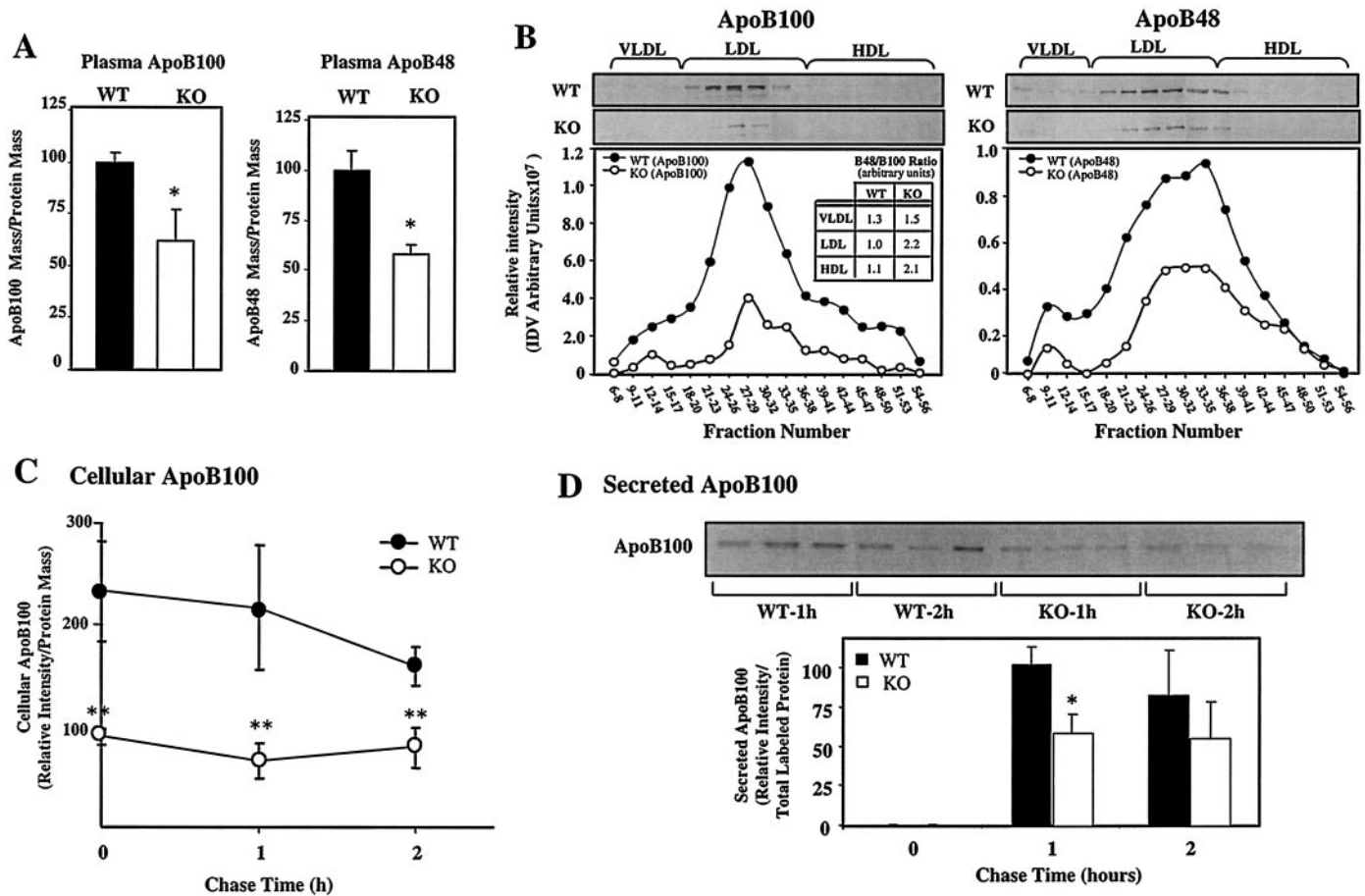


FIG. 2. Plasma levels and hepatocyte production of apoB in PTP-1B knockout mice. **A:** Total plasma levels of apoB100 and apoB48 were assessed in both wild-type (WT) and PTP-1B knockout (KO) mice by quantitative immunoblotting using a polyclonal anti-hamster apoB antibody. Plasma was obtained and pooled from four pairs of wild-type and PTP-1B knockout animals ($P < 0.05$). **B:** ApoB100 and apoB48 levels were quantified in lipoprotein fractions obtained after FPLC analysis of mouse plasma obtained from wild-type and PTP-1B knockout mice by quantitative immunoblotting. *Inset:* Ratio of apoB48/apoB100 in lipoprotein subclasses. **C and D:** Pulse-chase analysis of mouse apoB100 in primary hepatocytes derived from wild-type and PTP-1B knockout mice. Primary mouse hepatocytes from wild-type and PTP-1B knockout mice were radiolabeled with [35 S]methionine/cysteine and chased for the indicated times. ApoB100 was immunoprecipitated from the cell lysates (**C**) and medium (**D**). Graph shows a representative experiment performed in triplicate using primary hepatocytes isolated from one pair of wild-type and PTP-1B knockout animals ($n = 4$ pairs; * $P < 0.05$; ** $P < 0.01$).

the curve). FPLC fractionation of plasma lipoproteins, followed by immunoblotting for apoB, showed significantly lower levels of both apoB100 and apoB48 associated with plasma lipoprotein fractions of PTP-1B knockout mice ($n = 3$, $P < 0.05$) compared with those in wild-type littermates (Fig. 2B). It is interesting that there was an appreciable increase in the ratio of apoB48 to apoB100 mass detected in denser lipoprotein fractions (see inset in Fig. 2B), suggesting differential effect of PTP-1B gene deletion on plasma levels of apoB isoforms.

Ex vivo pulse-chase experiments using isolated hepatocytes from wild-type and PTP-1B knockout mice were also performed to determine whether absence of the PTP-1B gene affected apoB secretion. In primary hepatocytes, cells were pulsed for 45 min, and radioactivity was chased for 1 and 2 h. As with primary hamster hepatocytes, a longer pulse period was required to allow significant recovery of labeled apoB from primary mouse hepatocytes (29). There was a significant decrease in the cellular levels of apoB100 accumulated at the end of the pulse in hepatocytes from PTP-1B knockout mice ($n = 4$, $P < 0.01$), suggesting either a significant reduction in apoB100 synthesis or a rapid cotranslational degradation of apoB100 during the pulse

(Fig. 2C). Hepatocytes that were isolated from the PTP-1B knockout mice displayed a significant decrease in apoB100 secreted into the medium at 1 h chase time ($n = 4$, $P < 0.05$), but the change at 2 h chase did not reach statistical significance (Fig. 2D). There was also a decrease in apoB48 visible at $t = 0$ h in the cell fraction (data not shown) but no significant change in apoB48 secretion.

Transfection with siRNA reduces intracellular PTP-1B protein mass and attenuates the secretion of apoB100. Because McArdle RH7777 cells express relatively high levels of PTP-1B when compared with other cell lines such as HepG2 and are less insulin sensitive (data not shown), they were used as an in vitro model for siRNA inhibition studies. A series of siRNA duplexes were constructed and used to examine the effect of attenuated PTP-1B expression on apoB-lipoprotein production. After screening a number of siRNA duplexes, an optimal concentration of 1 μ g/ml exhibited a significant decrease of 41% ($P < 0.01$) in intracellular PTP-1B mass (Fig. 3A) compared with transfection using the same quantity of a nonsilencing siRNA. Steady-state metabolic labeling was next carried out in McArdle RH7777 cells under the same conditions, and apoB100 in the medium was quantified.

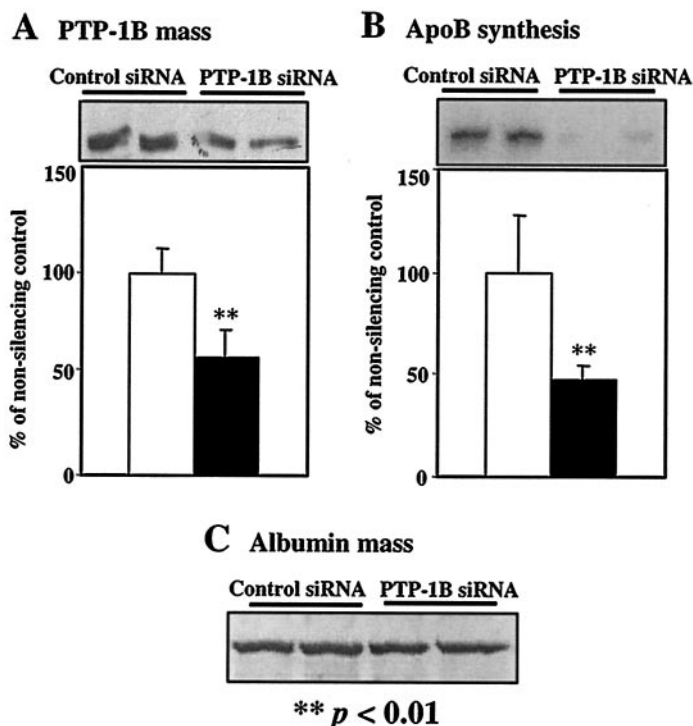


FIG. 3. Transfection with PTP-1B siRNA significantly decreases PTP-1B mass and downregulates apoB100 secretion in rat hepatoma McArdle RH7777 cells. Rat hepatoma McArdle RH7777 cells were transfected with 1 $\mu\text{g}/\text{ml}$ of either PTP-1B siRNA or nonsilencing control siRNA. **A:** A total of 30 $\mu\text{g}/\text{lane}$ cellular proteins were resolved on 8% SDS-PAGE and immunoblotted using a monoclonal antibody against PTP-1B. **B:** Cells were radiolabeled with [^{35}S]methionine/cysteine for 30 min and chased for 1 h. Medium apoB100 was immunoprecipitated using a polyclonal anti-hamster antibody. **C:** A total of 30 $\mu\text{g}/\text{lane}$ total protein was resolved on 8% SDS-PAGE and immunoblotted using a polyclonal antibody against albumin. Values given are representatives of two independent experiments performed in duplicate.

There was a significant 53% ($P < 0.01$) decrease in apoB100 in cells that were transfected with the PTP-1B siRNA (Fig. 3B) compared with cells that were transfected using nonsilencing siRNA. These data suggest that a decrease in PTP-1B can directly affect the level of apoB secreted into the medium. There was no detectable change in the control protein albumin (Fig. 3C) and no change in [^{35}S] incorporation into TCA-precipitable total protein (data not shown) under any of the transfection conditions.

Infection with PTP-1B adenovirus results in a dose-dependent overexpression of PTP-1B in HepG2 cells.

The effect of PTP-1B overexpression on hepatic lipoprotein production was also examined using a recombinant adenoviral vector encoding the full-length mouse PTP-1B. Immunoblot analysis showed a significant increase in PTP-1B protein mass after a total of 48 h of infection of HepG2 cells with MOI 10 of PTP-1B recombinant adenoviruses (ADV1Bm; Fig. 4A). A small amount of endogenous PTP-1B was observed in noninfected HepG2 cells (no virus control) or those that were infected with the control, Ad β -gal. PTP-1B was overexpressed with increasing dose in cells that were infected with ADV1Bm (up to 5.06 \pm 0.17-fold; $P < 0.01$; Fig. 4B).

Overexpression of PTP-1B in HepG2 cells increased tyrosine phosphorylation but not protein mass of the insulin receptor and IRS-1. For assessing the activity of overexpressed PTP-1B on insulin receptor and IRS-1,

HepG2 cells that were infected with PTP-1B adenovirus were stimulated with 100 nmol/l insulin for 10 min. Cell lysates were immunoprecipitated with an antibody against the insulin receptor or IRS-1. The immunocomplexes then were analyzed by SDS-PAGE and then probed for tyrosine phosphorylation. Tyrosine phosphorylation of the insulin receptor and IRS-1 were decreased by $68.5 \pm 0.8\%$ ($P < 0.05$) and by $51.3 \pm 6.4\%$ ($P < 0.05$), respectively (Fig. 4C and E) in cells that were infected with PTP-1B adenovirus (ADV1Bm) in comparison with cells that were infected with the control adenovirus (Ad β -gal). The decrease in phosphorylation was not due to any change in protein mass (Fig. 4D and F).

PTP-1B overexpression increased the stability and secretion of apoB100 in HepG2 cells. An important underlying cause of metabolic dyslipidemia is thought to be the hepatic overproduction of apoB-containing lipoprotein particles. To directly examine our hypothesis that PTP-1B expression in hepatocytes can drive apoB particle secretion, we performed metabolic labeling studies of HepG2 cells overexpressing PTP-1B. HepG2 cells first were infected with ADV1Bm, which resulted in PTP-1B overexpression (Fig. 5A), and then were metabolically labeled with [^{35}S]methionine/cysteine. After the pulse, the cells and the medium were collected and immunoprecipitated with anti-human apoB antiserum. As shown in Fig. 5B and C, in untreated cells, no changes were observed in the amount of apoB100 associated with the cells or secreted into the medium. By contrast, however, in the presence of 100 nmol/l insulin stimulation, the amount of apoB100 was significantly increased in both the medium $198.8 \pm 54.8\%$ ($P < 0.05$) and the cells $260.7 \pm 4.8\%$ ($P < 0.01$) after infection with ADV1Bm compared with cells that were infected with Ad β -gal (Fig. 5D and E). Furthermore, PTP-1B overexpression did not affect secretion of a control protein, albumin, upon stimulation with 100 nmol/l insulin, arguing against a global effect on hepatic protein secretion (Fig. 5F).

We performed a series of pulse-chase experiments in HepG2 cells overexpressing PTP-1B. Infected HepG2 cells were pretreated with 100 nmol/l insulin and then pulsed for 15 min and chased for 1 and 2 h. Overexpression of PTP-1B resulted in significant increases in cellular accumulation and secretion of apoB100 at both 1- and 2-h chase times (Fig. 6A and B). Significantly higher levels of total labeled apoB100 could be recovered from cells and medium after PTP-1B overexpression (at both 1-h chase [$P < 0.01$] and 2-h chase [$P < 0.01$], in comparison with cells that were infected with the control adenovirus, Ad β -gal), suggesting an enhancement in intracellular stability of apoB100 (Fig. 6C). Increasing the dose of PTP-1B adenovirus caused further increases in apoB secretion, suggesting that the effect was dose dependent (data not shown).

DISCUSSION

The first evidence of a link between PTP-1B-induced changes in insulin signaling and alterations in lipid and lipoprotein metabolism were provided by studies in PTP-1B knockout mice, demonstrating a resistance to dietary fat-induced obesity and a reduction in plasma triglyceride concentration (10). Characterization of the lipid profile of PTP-1B knockout mice in the present report

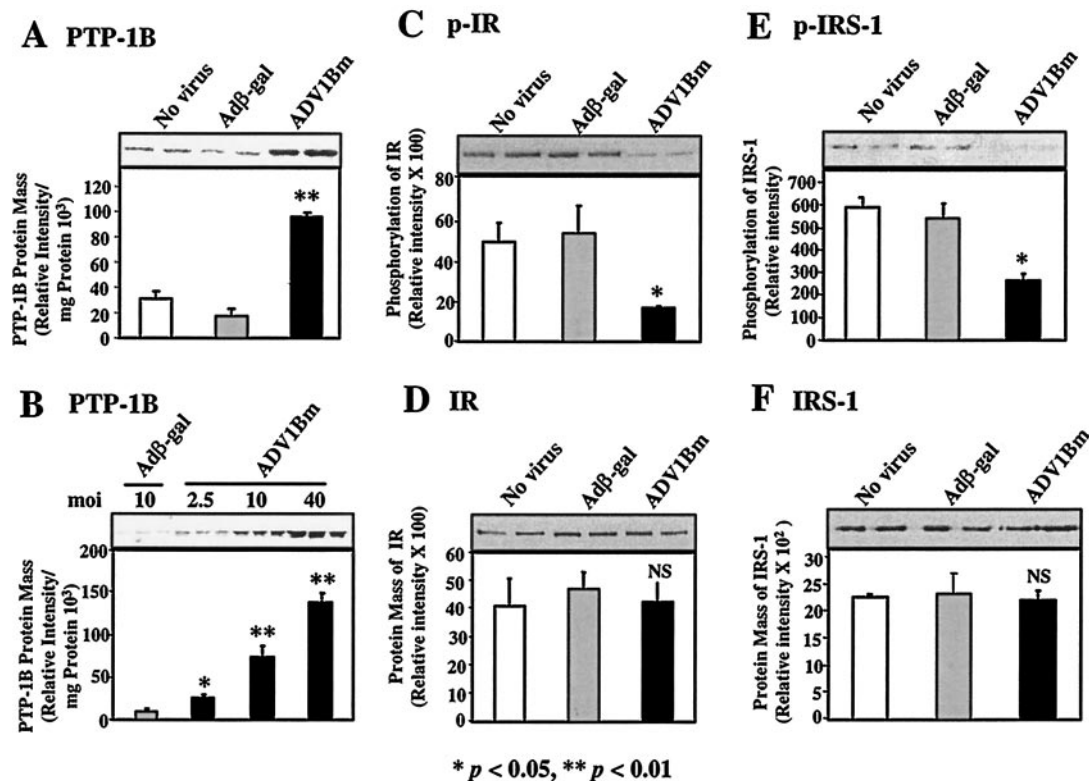


FIG. 4. Overexpression of PTP-1B in HepG2 cells downregulates insulin receptor and IRS-1 phosphorylation. HepG2 cells were infected with MOI 10 of PTP-1B recombinant adenovirus ADV1Bm (A) or infected with MOI 2.5, 10, and 40 of ADV1Bm (B). The recombinant adenovirus encoding β -galactosidase (Ad β -gal) was used as negative control. Equal amounts of protein (20 μ g) from cell lysates were subjected to SDS-PAGE and immunoblotting using a PTP-1B monoclonal antibody. For determining insulin receptor and IRS-1 phosphorylation, cells were lysed, and 500 μ g of cell protein was immunoprecipitated with an anti-insulin receptor β -subunit rabbit polyclonal antibody (C and D) or an anti-IRS-1 rabbit polyclonal antibody (E and F). Samples were subjected to 8% SDS-PAGE and probed with an anti-phosphotyrosine antibody, *p*-Tyr (py99; C and E). After stripping, the blots were reprobed with a polyclonal antibody to the β -subunit of the insulin receptor (D) or a polyclonal anti-IRS-1 antibody (F). The values given are representative immunoblots from three independent experiments from A and B and two independent experiments from C to F.

has shown that there is a significant reduction in the number of atherogenic, apoB-containing lipoproteins accompanied by a reduction in plasma triglycerides, in the absence of PTP-1B expression. Fructose feeding of wild-type and PTP-1B knockout mice further confirmed the ability of reduced PTP-1B expression to protect against dyslipidemia. As fructose feeding induces hypertriglyceridemia and dyslipidemia in animal models (40–42), the present data suggest that these diabetogenic and lipogenic effects of fructose may at least in part be mediated by PTP-1B. These observations also suggest that reduction in PTP-1B expression may not only protect against fat-induced insulin-resistant dyslipidemia (43,44) but also can prevent carbohydrate-induced changes in insulin sensitivity and the associated abnormalities in lipid and lipoprotein metabolism.

The mechanism by which PTP-1B may influence lipid and lipoprotein metabolism and play a role in the development of metabolic dyslipidemia is most likely regulated indirectly via changes in insulin sensitivity. Insulin is a well-known regulator of lipid and lipoprotein metabolism (45), and hepatic lipogenesis and lipoprotein production are tightly controlled by insulin action (45). Studies in the fructose-fed model of insulin resistance and the ApoB/BATless mice model have clearly demonstrated that development of an insulin-resistant state is closely associated with changes in hepatic lipogenesis and the

assembly and secretion of VLDL-apoB (20,28,30). These studies suggested that insulin resistance brings about changes in hepatic insulin signaling that lead to deregulation of the assembly and secretion of apoB-containing lipoproteins. In accordance with this notion, we recently found a significant increase in hepatic protein mass and activity of PTP-1B in insulin-resistant hamsters (20). Increased hepatic expression of PTP-1B was accompanied by attenuation of hepatic insulin signaling and a significant overproduction of apoB-containing lipoprotein particles (20). Studies using the peroxisome proliferator-activated receptor γ agonist rosiglitazone have shown that insulin sensitization can significantly ameliorate VLDL secretion in the fructose-fed hamster model, both in vivo and ex vivo, an effect associated with a rosiglitazone-induced reduction in hepatic levels of PTP-1B levels that were initially increased by fructose feeding (37). These observations were the first to suggest that hepatic PTP-1B expression may be subject to diet-induced modulation with potential impact on hepatic insulin sensitivity and lipoprotein metabolism. They also suggested that modulation of PTP-1B gene expression may induce a metabolic dyslipidemic state.

Data reported here seem to support this notion. First, adenoviral overexpression of PTP-1B in cultured HepG2 cells attenuated insulin signaling (diminished phosphorylation of insulin receptor and IRS-1) and induced the

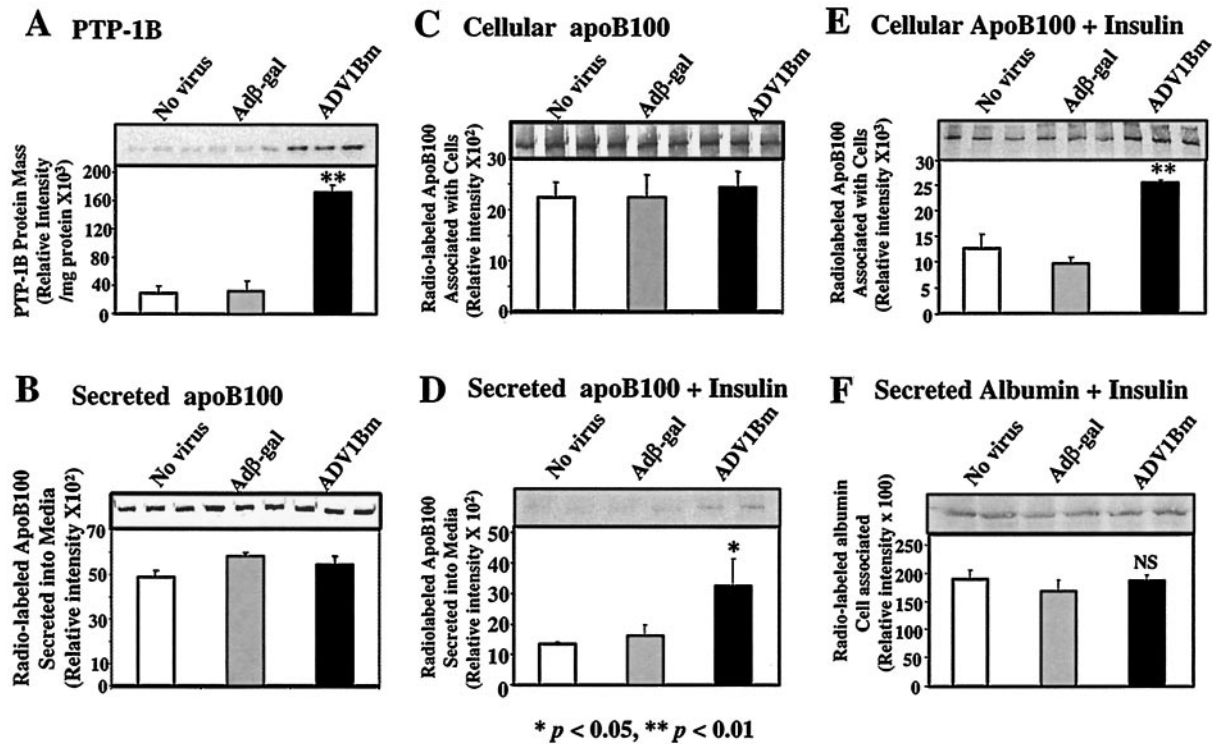


FIG. 5. PTP-1B overexpression increases apoB100 accumulation in HepG2 cells stimulated with insulin. HepG2 cells were infected with MOI 10 of ADY1Bm or Adβ-gal. Cells were treated without (B and C) or with (E and F) 100 nmol/l insulin for 2 h and pulsed with [³⁵S]methionine/cysteine. After a 1-h pulse, the cells and the medium were collected and immunoprecipitated with a polyclonal anti-human apoB antibody. A total of 20 μg of cell lysates from insulin-treated samples was resolved by 8% SDS-PAGE to assess PTP-1B expression levels (A). The apoB100 depleted media were immunoprecipitated with anti-human albumin antiserum and then resolved in 8% SDS-PAGE (F). The values given are mean ± SD and are representative of two independent experiments.

secretion of apoB-containing lipoproteins. Second, there was attenuation of apoB100 secretion after siRNA-mediated suppression of intracellular PTP-1B protein mass. These data are the first to demonstrate a direct link between PTP-1B expression and the rate of hepatic apoB production. Because the effect of PTP-1B overexpression on apoB production was observed only in the presence of insulin stimulation, the data suggest that PTP-1B's effect on apoB secretion is indirect and most likely related to

blocking insulin-mediated inhibition of apoB secretion. Increased apoB secretion in the presence of PTP-1B overexpression seemed to result from enhanced intracellular stability of apoB in HepG2 cells. These data compare well with recent observations in the fructose-fed hamster model that clearly showed reduced apoB degradation and higher intracellular stability of apoB in fructose-fed hamster hepatocytes (20,29,30). Insulin has been shown to inhibit apoB secretion by inducing intracellular apoB

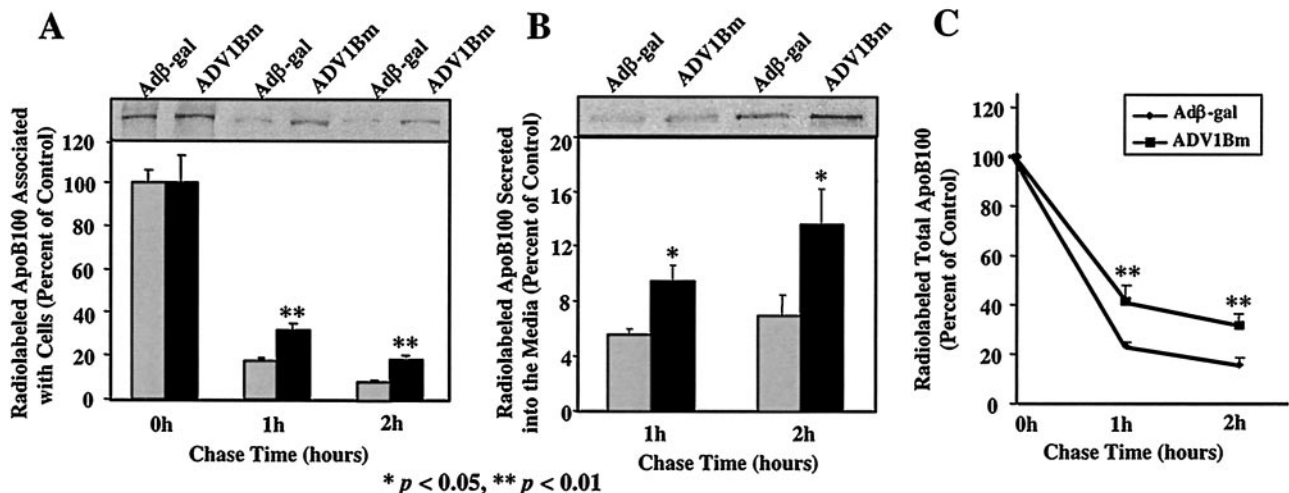


FIG. 6. PTP-1B overexpression increases intracellular stability of apoB in HepG2 cells. HepG2 cells were infected with MOI 10 of ADY1Bm or Adβ-gal. Cells were treated with 100 nmol/l insulin for 2 h and pulsed with 100 μCi/ml [³⁵S]methionine/cysteine, and the radioactivity was chased for 1 and 2 h. The cells (A) and the medium (B) were collected and immunoprecipitated with a polyclonal anti-human apoB antibody. The samples were subjected to 5% SDS-PAGE, transferred onto polyvinylidene fluoride membrane, and measured by fluorography.

degradation (46,47), and it is thought that this effect can be blocked with the induction of hepatic insulin resistance (48). Recent studies have clearly shown that treatment of FAO hepatoma cells or *ob/ob* mice with antisense oligonucleotides against PTP-1B can significantly enhance insulin sensitivity and insulin-mediated glucose utilization (12–14). These reports, however, did not examine changes in lipid and lipoprotein metabolism associated with inhibition of PTP-1B expression. Our present data seem to extend these observations and further suggest that reducing PTP-1B expression can significantly attenuate hepatic lipoprotein secretion most likely as a result of enhanced hepatic insulin signaling.

The mechanisms that link hepatic insulin sensitivity to the assembly and secretion of apoB-containing lipoproteins have been under intense investigation and implicate a number of potential factors, including microsomal triglyceride transfer protein (MTP), ER-60, and sterol regulatory element-binding protein-1c (SREBP-1c). Hyperlipidemia in an animal model of type 2 diabetes with visceral fat obesity, the Otsuka Long-Evans Tokushima fatty rat, is also associated with elevated hepatic MTP mRNA (49). Fructose-induced metabolic dyslipidemia was shown to be accompanied by a significant upregulation of MTP protein mass (30), as well as MTP mRNA and lipid transfer activity (unpublished observations). These changes, however, were not observed in the ApoB/BAT-less mouse model of insulin resistance (28). Recent *in vitro* studies also indicate a direct positive correlation between PTP-1B overexpression and both SREBP-1 gene expression and fatty acid synthase mRNA levels (50). Thus, PTP-1B can potentially influence the rate of *de novo* lipogenesis, leading to alterations in the secretory pool of lipid substrates available for VLDL assembly and secretion. There is also evidence that chronic modulation of apoB and VLDL secretion can be achieved via changes in MTP expression and activity.

Previous work has demonstrated that insulin-suppressive effects on apoB secretion require PI3-K activation (51). Phung et al. (51) demonstrated that short-term insulin stimulation increased PI3-K activity associated with IRS-1 and significantly increased the IRS-1/PI3-K mass in low-density microsomes, the site of apoB synthesis. Localized activation of PI3-K was thought to mediate the inhibition of apoB secretion possibly via generation of phosphatidylinositol-3,4,5-trisphosphate (51). PTP-1B overexpression has also been shown to reduce PI3-K activity in L6 myocytes, FAO hepatoma cells, and adipocytes (52,53). It thus is plausible to suggest that PTP-1B may potentially attenuate the suppressive effects of insulin on apoB secretion by blocking PI3-K activation via dephosphorylation of IRS-1. Further direct evidence is needed, however, to test this hypothesis. It has also been shown that PI3-K may modulate VLDL assembly and apoB secretion independent of IRS-1 (54). It is possible that other isoforms of IRS (e.g., IRS-2) may be regulated by PTP-1B and may modulate apoB secretion. Alternatively, PTP-1B may act on the insulin receptor directly, and this may regulate apoB production via the mitogen-activated protein kinase arm of the insulin-signaling pathway. Recent evidence has shown that in HepG2 cells, insulin regulates MTP mRNA through transcriptional regulation. Transcrip-

tional regulation was shown to occur through the mitogen-activated protein kinase pathway (ERK and p38) but not the PI3-K pathway (55).

Interestingly, we have also found that modulation of PTP-1B expression can significantly influence intracellular apoB degradation. This effect either could be indirect via changes in lipid availability or may be mediated by direct alteration in the activity of proteolytic machinery involved in apoB degradation. Work in our laboratory has shown that in HepG2 cells, cellular apoB and ER luminal apoB-containing lipoproteins are associated with ER-60, an ER-localized cysteine protease (56,57). In the insulin hamster model, livers of fructose-fed hamsters expressed a lower level of ER-60, compared with control hamsters fed with standard laboratory diet (20). Changes in insulin sensitivity brought about by modulation of PTP-1B expression can thus potentially influence the assembly and secretion of VLDL via changes in the expression and/or activity the ER-60 and intracellular stability of apoB. It is important to note that ER-60 may be one of several pathways that may mediate the effect of PTP-1B on apoB production, and the activity of other yet-unknown protease systems operating in the secretory pathway may be modulated by PTP-1B. It is also noteworthy that the focus of the current study was the link between PTP-1B expression and apoB100 production, and differences may potentially exist between the regulation of apoB100 versus apoB48. Indeed, there is evidence that apoB48 may be less sensitive to the suppressive effects of insulin compared with apoB100 (58) and thus may be potentially less sensitive to changes in PTP-1B expression levels. The data in PTP-1B knockout mice seem to support this hypothesis as there was a preferential reduction in apoB100 over apoB48 compared with wild-type littermates.

In summary, the body of evidence presented in this report supports the notion that upregulation of PTP-1B can play an important role in the induction of metabolic dyslipidemia in insulin-resistant states. Further studies are needed to determine the molecular basis of the link between PTP-1B expression in hepatocytes and the process of VLDL assembly and secretion.

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