

Reduction of Protein Tyrosine Phosphatase 1B Increases Insulin-Dependent Signaling in *ob/ob* Mice

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Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of insulin receptor (IR) signal transduction and a drug target for treatment of type 2 diabetes. Using PTP1B antisense oligonucleotides (ASOs), effects of decreased PTP1B levels on insulin signaling in diabetic *ob/ob* mice were examined. Insulin stimulation, prior to sacrifice, resulted in no significant activation of insulin signaling pathways in livers from *ob/ob* mice. However, in PTP1B ASO-treated mice, in which PTP1B protein was decreased by 60% in liver, similar stimulation with insulin resulted in increased tyrosine phosphorylation of the IR and IR substrate (IRS)-1 and -2 by threefold, fourfold, and threefold, respectively. IRS-2-associated phosphatidylinositol 3-kinase activity was also increased threefold. Protein kinase B (PKB) serine phosphorylation was increased sevenfold in liver of PTP1B ASO-treated mice upon insulin stimulation, while phosphorylation of PKB substrates, glycogen synthase kinase (GSK)-3 α and -3 β , was increased more than twofold. Peripheral insulin signaling was increased by PTP1B ASO, as evidenced by increased phosphorylation of PKB in muscle of insulin-stimulated PTP1B ASO-treated animals despite the lack of measurable effects on muscle PTP1B protein. These results indicate that reduction of PTP1B is sufficient to increase insulin-dependent metabolic signaling and improve insulin sensitivity in a diabetic animal model. *Diabetes* 52:21–28, 2003

Type 2 diabetes is characterized by insulin resistance in tissues that are normally insulin sensitive, such as liver, fat, and skeletal muscle (1). Normal insulin action involves a complex network of signaling molecules that leads to increased glyco-

gen synthesis, glucose transport, and lipogenesis and decreased gluconeogenesis, glycogenolysis, and lipolysis (2–4). The net effect on glucose metabolism is that hepatic glucose production is reduced, while use of peripheral glucose is increased (3,4). Insulin can act through a variety of signaling pathways to produce its effects. One pathway thought to be involved in glucose and possibly lipid homeostasis leads to activation of the insulin receptor (IR), IR substrates (IRSs) such as IRS-1 and -2, phosphatidylinositol (PI) 3-kinase, and protein kinase B (PKB; also called AKT) (reviewed in 5). PKB activation increases glycogen synthesis, at least in part, through inhibition (via phosphorylation) of the PKB substrate glycogen synthase kinase (GSK)-3 (6). Inactivation of GSK-3 leads to activation of glycogen synthase (active in the nonphosphorylated state), thus increasing glycogen production in liver (7). Both PKB and PI 3-kinase activation can lead to decreased transcription of gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase, thus reducing glucose production in the liver (7,8). Pathways leading from the IR and IRS to PI 3-kinase to PKB and further downstream targets are considered to mediate the metabolic effects of insulin.

An exquisite balance is required between kinases, which are involved in transmitting the signal to downstream targets necessary for the processes described above, and phosphatases, which are required to shut down the signaling to prevent excessive or, in some cases, insufficient activation. Insulin resistance and diabetes represent states in which regulation of signaling pathways are altered so that the intracellular actions of insulin are absent or reduced (2,4,9). The mechanisms by which insulin resistance occurs and the pathways involved are still poorly understood.

Overactivation of phosphatases is one possible means of blocking insulin signaling. One key phosphatase, protein tyrosine phosphatase 1B (PTP1B), is a target for treatment of type 2 diabetes. PTP1B has been implicated in negatively regulating the IR as well as its substrates, such as IRS-1 and -2, by dephosphorylating them (10–12). PTP1B protein levels have been shown to be increased in insulin-resistant obese patients (13). In contrast, wild-type, nonobese, nondiabetic mice lacking the PTP1B gene (PTP1B^{-/-}) exhibit increased insulin sensitivity, resistance to weight gain on a high-fat diet, and an increased basal metabolic rate with no apparent deleterious effects (14,15). However, the effects of decreasing PTP1B in a

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ASO, antisense oligonucleotide; ECL, enhanced chemiluminescence; GSK, glycogen synthase kinase; IR, insulin receptor; IRS, IR substrate; PEPCK, phosphoenolpyruvate carboxykinase; PI, phosphatidylinositol; PKB, protein kinase B; PTP1B, protein tyrosine phosphatase 1B.

diabetic setting and the signaling pathways affected by reduced PTP1B levels are unknown.

Diabetic obese (*ob/ob*) mice exhibit many of the same phenotypic and insulin signaling abnormalities associated with type 2 diabetes (16,17). They have elevated plasma glucose and insulin levels, are insulin resistant, have reduced metabolic functions, and exhibit altered protein levels and phosphorylation levels of insulin signaling pathway elements, such as IRS-1 and -2, in the absence and presence of insulin stimulation (16). In this study, we sought to identify the effects of PTP1B reduction on signaling pathways in liver of *ob/ob* diabetic mice in the absence and presence of insulin stimulation and whether these changes could increase insulin sensitivity and attenuate the diabetic phenotype.

RESEARCH DESIGN AND METHODS

Animal studies. Identification of antisense oligonucleotide (ASO) inhibitors, including ISIS-113715 (Isis Pharmaceuticals, Carlsbad, CA), and details of animal studies have been described elsewhere (18). Briefly, obese diabetic C57Bl/6J *ob/ob* mice (6–7 weeks of age; obtained from The Jackson Laboratories, Bar Harbor, ME) were weighed and glucose levels determined by the glucose oxidase method (Precision G glucose meter; Abbott Laboratories, North Chicago, IL) from tail snip blood samples. The animals were randomized based on glucose levels and body weight, and baseline plasma insulin samples were determined by enzyme-linked immunosorbent assay (ELISA; ALPCO Diagnostics, Windham, NH). The treatment groups were 25 mg/kg PTP1B ASO and saline control ($n = 30$) for 6 weeks. All mice were dosed two times per week intraperitoneally. At the end of each week, glucose and insulin levels were determined under nonfasting conditions by 10:00 A.M. Before necropsy, 15 saline- and 15 ASO-treated animals were intraperitoneally administered insulin at 2 units/kg in saline with 0.1% BSA or vehicle control (saline with 0.1% BSA) after a 5-h fast. Tissue samples from liver, fat, and muscle (1 and 5 min posttreatment) were taken from both vehicle- and insulin-treated animals and flash frozen in liquid nitrogen.

Lysate preparation and protein assays. Lysis buffer (1 ml) (18) was added to 50 mg frozen liver or muscle. Tissue was disrupted by sonication (for liver) or by use of a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and sonication (for muscle), mixed by inversion at 4°C and then pelleted at 14,000 rpm. Lipid layers were removed and then supernatants were split into two portions. One portion was mixed with Laemmli loading buffer (Bio-Rad Laboratories, Hercules, CA) and used for gel electrophoresis and Western blotting. The second portion was used for immunoprecipitations and analysis of protein content. Protein assays were performed according to the method of Bradford (19).

Immunoprecipitation and Western blotting. For immunoprecipitation, IRS-1 antibody (6 μ l NH₂-terminal antibody; Upstate Biotechnology, Lake Placid, NY) or IRS-2 antibody (10 μ l NH₂-terminal antibody; Santa Cruz Biotechnology, Santa Cruz, CA) was added to 500 μ g liver lysate from saline- and PTP1B ASO-treated *ob/ob* mice. Volume was adjusted to 500 μ l with lysis buffer and then mixed by inversion for 2 h at 4°C. Protein A (IRS-1) or protein G (IRS-2) Agarose beads (Life Technologies, Rockville, MD) were added (40 μ l of a 50:50 slurry in lysis buffer) to the lysate, and the mixture was inverted at 4°C for an additional 2 h. Beads were briefly pelleted in a microfuge at 4°C and then washed three times with 1 ml lysis buffer followed by addition of 40 μ l Laemmli buffer (Bio-Rad Laboratories, Hercules, CA). The mixture was boiled for 5 min, the beads were pelleted, and 10 μ l supernatant was used for Western blotting analysis.

For Western blotting, equal amounts (either 20 or 50 μ g depending on the protein of interest) of protein extract (except immunoprecipitates as indicated above) were subjected to SDS-PAGE using Criterion gels (Bio-Rad Laboratories, Hercules, CA) and then transferred to nitrocellulose (Amersham Pharmacia Biotech, Piscataway, NJ). The nitrocellulose blot was incubated with an antibody to PTP1B (Upstate Biotechnology), phosphotyrosine (PY99; Santa Cruz Biotechnology) phospho-AKT (Ser473), or phospho-GSK-3 α /3 β (Cell Signaling Technology, Beverly, MA). Protein bands were detected using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech), as recommended by the manufacturer. Bands were quantitated on a densitometer (Molecular Dynamics/Amersham Pharmacia Biotech). Phospho-antibody blots were stripped of antibody (according to the manufacturers suggestion for ECL) and reprobed with the corresponding whole-protein antibody,

including AKT, GSK-3 (Santa Cruz Biotechnology), IRS-1, IRS-2 (Upstate Biotechnology), and IR (Pharmingen/BD Biosciences, San Diego, CA).

PI 3-kinase activity assays. PI 3-kinase activity assays were performed on immunoprecipitated samples essentially as previously described (Upstate Biotechnology Catalog, Technical Appendix) with the following exceptions. Frozen mouse liver tissue was suspended at 50 mg/ml in buffer A (137 mmol/l NaCl, 20 mmol/l Tris-HCl, pH 7.4, 1 mmol/l CaCl₂, 1 mmol/l MgCl₂, 0.1 mmol/l sodium orthovanadate, 1% NP-40, and 1 mmol/l phenylmethylsulfonyl fluoride). Samples were sonicated on ice and then centrifuged at 4°C to sediment insoluble material. Supernatant was transferred to new microfuge tubes and standardized to 1 mg protein in 1 ml total volume. IRS-2 was immunoprecipitated from liver lysates as described above; the remainder of the assay was as described.

Statistical analysis. Statistical analysis for all studies was performed with Microsoft Excel analysis tools using a two-tailed Student's *t* test, with $P < 0.05$ considered statistically significant.

RESULTS

PTP1B ASO decreased plasma glucose, plasma insulin, and hepatic PTP1B protein in *ob/ob* diabetic mice. Treatment of *ob/ob* diabetic mice with 25 mg/kg PTP1B ASO twice a week for 6 weeks lowered plasma glucose and insulin levels (Fig. 1A) to 131 ± 3 mg/dl and 6.7 ± 1 ng/ml, respectively, relative to control animals at 255 ± 17 mg/dl and 28.9 ± 4.2 ng/ml, respectively. Normalization of plasma glucose levels occurred within the first 2 weeks of treatment, while the decrease in insulin occurred within the first 4 weeks of treatment. Decreases in glucose and insulin were accompanied by increased sensitivity to insulin and glucose, as demonstrated by improved insulin and glucose tolerance tests (20).

Western blotting of liver extracts revealed that treatment of *ob/ob* mice with PTP1B ASO significantly decreased the basal level of PTP1B protein in liver (Fig. 1B). As expected, insulin stimulation did not affect the protein level of PTP1B in the saline control- or PTP1B ASO-treated animals. In a parallel experiment, PTP1B protein levels were compared in *ob/ob* versus *ob/+* mice (Fig. 1C). PTP1B ASO decreased PTP1B protein levels in *ob/ob* mice to levels equivalent to or lower than *ob/+* levels. In earlier experiments, a universal control oligonucleotide did not lower plasma glucose or insulin and did not affect PTP1B protein in *ob/ob* mice (20).

PTP1B ASO-treated *ob/ob* mice exhibited increased induction in insulin-stimulated tyrosine phosphorylation of the IR and IRS-1 in liver. To better understand the signaling pathways affected by PTP1B ASO treatment, elements of the IR signaling cascade were examined in insulin- and vehicle-stimulated PTP1B ASO and saline control *ob/ob* mice. No statistically significant change in the tyrosine phosphorylation state of the IR was observed in saline control-treated mice upon insulin stimulation (Fig. 2A). In contrast, PTP1B ASO treatment increased tyrosine phosphorylation of the IR in response to insulin more than threefold relative to vehicle-treated PTP1B ASO mice (Fig. 2A). This increased response to insulin was primarily due to a decrease in the basal tyrosine phosphorylation state of the IR in PTP1B ASO mice relative to saline control mice, with an increased ability of the receptor to respond to insulin. IR protein levels remained relatively constant among treatment groups (Fig. 2A, lower panel). Interestingly, treatment of *ob/ob* mice with PTP1B ASO resulted in basal IR tyrosine phosphorylation levels that more closely resembled lean *ob/+* mice than saline-treated *ob/ob* mice (Fig. 2B).

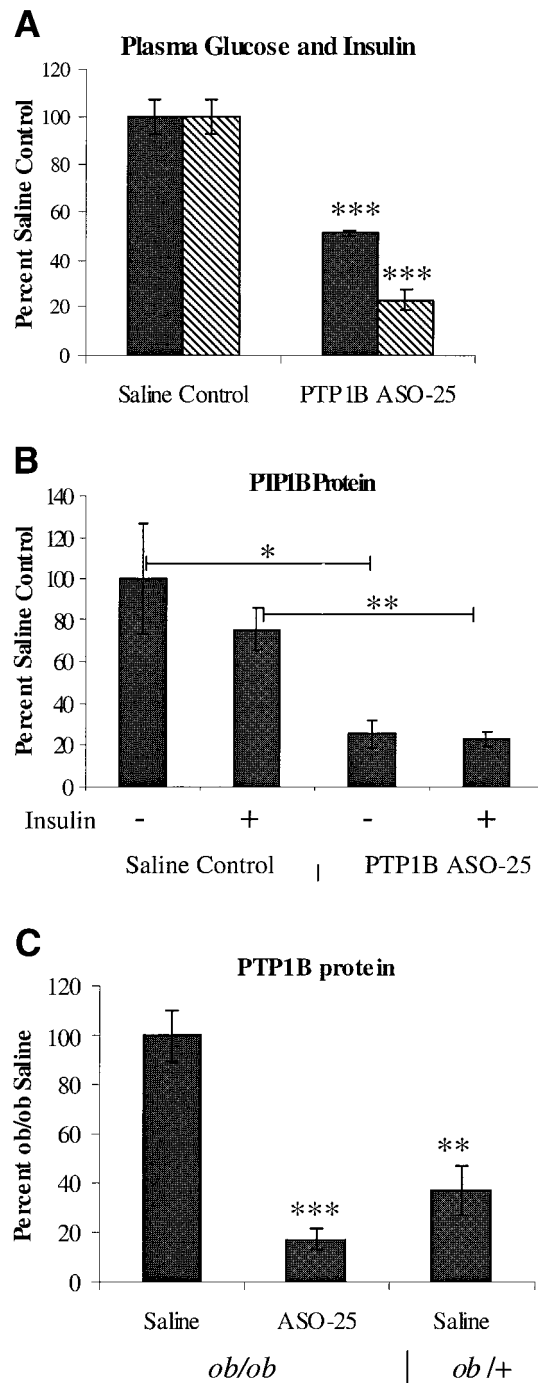


FIG. 1. A: Plasma glucose (■) and insulin (▨) levels at the end of 6 weeks of treatment for 25 mg/kg PTP1B ASO-treated *ob/ob* mice relative to saline control-treated mice. Data shown are the means \pm SE of 10 mice. B and C: Western blotting for PTP1B protein levels in liver is shown for saline control- and 25 mg/kg PTP1B ASO-treated *ob/ob* mice in the presence and absence of insulin stimulation for 1 min (B) and for saline control- and 25 mg/kg PTP1B ASO-treated *ob/ob* mice compared with *ob/+* lean mice (C). Quantitation is shown as percent PTP1B protein relative to vehicle-treated saline control (B) or saline control *ob/ob* mice (C). Experiments were repeated two or three times. Data are the means \pm SE of three or four mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The basal and insulin-stimulated response of IRS-1 showed a similar pattern to that observed for the IR. No statistically significant change in the phosphorylation state of IRS-1 was observed upon insulin stimulation in saline control-treated *ob/ob* mice (Fig. 2C) paralleling the effects

observed with the IR (Fig. 2A) and validating the insulin-resistant state of these mice. However, insulin-treated PTP1B ASO mice produced a statistically significant increase in the tyrosine phosphorylation of IRS-1 relative to vehicle-treated PTP1B ASO mice (Fig. 2C). As with the phosphorylation state of the IR, this increased response to insulin was primarily due to a decrease in the basal phosphorylation state of IRS-1 in PTP1B ASO mice relative to saline control mice. IRS-1 protein levels were not affected by treatment (Fig. 2C, lower panel).

PTP1B ASO-treated mice exhibited increased tyrosine phosphorylation of IRS-2 and increased IRS-2-associated PI 3-kinase activity in liver in response to insulin treatment. IRS-2 is another downstream substrate of the IR and is the predominant functional IRS in liver with regards to the metabolic effects of insulin (21,22). There was a trend toward an increase in basal IRS-2 tyrosine phosphorylation in PTP1B ASO mice relative to similarly treated saline control mice (Fig. 3A). In addition, a statistically significant increase in tyrosine phosphorylation of IRS-2 in insulin-stimulated PTP1B ASO mice was observed relative to vehicle-treated PTP1B ASO mice or insulin-stimulated saline control mice (Fig. 3A). At least part of the increase in the basal phosphorylation of IRS-2 was due to an increase in IRS-2 protein levels (Fig. 3A, lower blot) in PTP1B ASO-treated mice. Previous reports have indicated that IRS-2 protein levels range from low to undetectable in liver of *ob/ob* mice (16) relative to lean littermates. Treatment of *ob/ob* mice with PTP1B ASO reversed this, increasing IRS-2 protein levels approximately twofold ($P < 0.001$) relative to saline control-treated mice (18 and Fig. 3A, lower blot), thus contributing to an increase in tyrosine phosphorylation of IRS-2 (Fig. 3A).

In addition, IRS-2-associated PI 3-kinase activity was increased approximately sixfold in insulin-stimulated PTP1B ASO mice relative to insulin-stimulated saline control mice (Fig. 3B). PI 3-kinase activity was not increased in insulin-stimulated saline control mice relative to vehicle-treated saline control mice (Fig. 3B). The increase in PI 3-kinase activity of PTP1B ASO mice relative to saline control mice (Fig. 3B) showed a pattern very similar to that observed for tyrosine phosphorylation of IRS-2 (Fig. 3A).

PTP1B ASO treatment increased insulin-stimulated levels of serine phosphorylation of PKB in liver of *ob/ob* mice. An element downstream of IRS-2 and PI 3-kinase in the IR cascade is PKB, a key kinase for transmitting the metabolic effects of insulin via the IR. PKB is activated by phosphorylation on serine and threonine residues. Basal serine 473 phosphorylation of PKB, as detected by Western blotting, was not significantly changed, using a two-tailed Student's *t* test ($P = 0.051$), in vehicle-treated PTP1B ASO *ob/ob* mice relative to vehicle-treated saline control mice (Fig. 4). However, as with both IRS-2 tyrosine phosphorylation (Fig. 3A) and IRS-2-associated PI 3-kinase activity (Fig. 3B), insulin stimulation significantly increased serine phosphorylation of PKB in the PTP1B ASO mice relative to insulin-stimulated saline control mice and relative to vehicle-treated PTP1B ASO mice (Fig. 4). PKB protein levels were not significantly changed by treatment with PTP1B ASO (Fig. 4, lower panel).

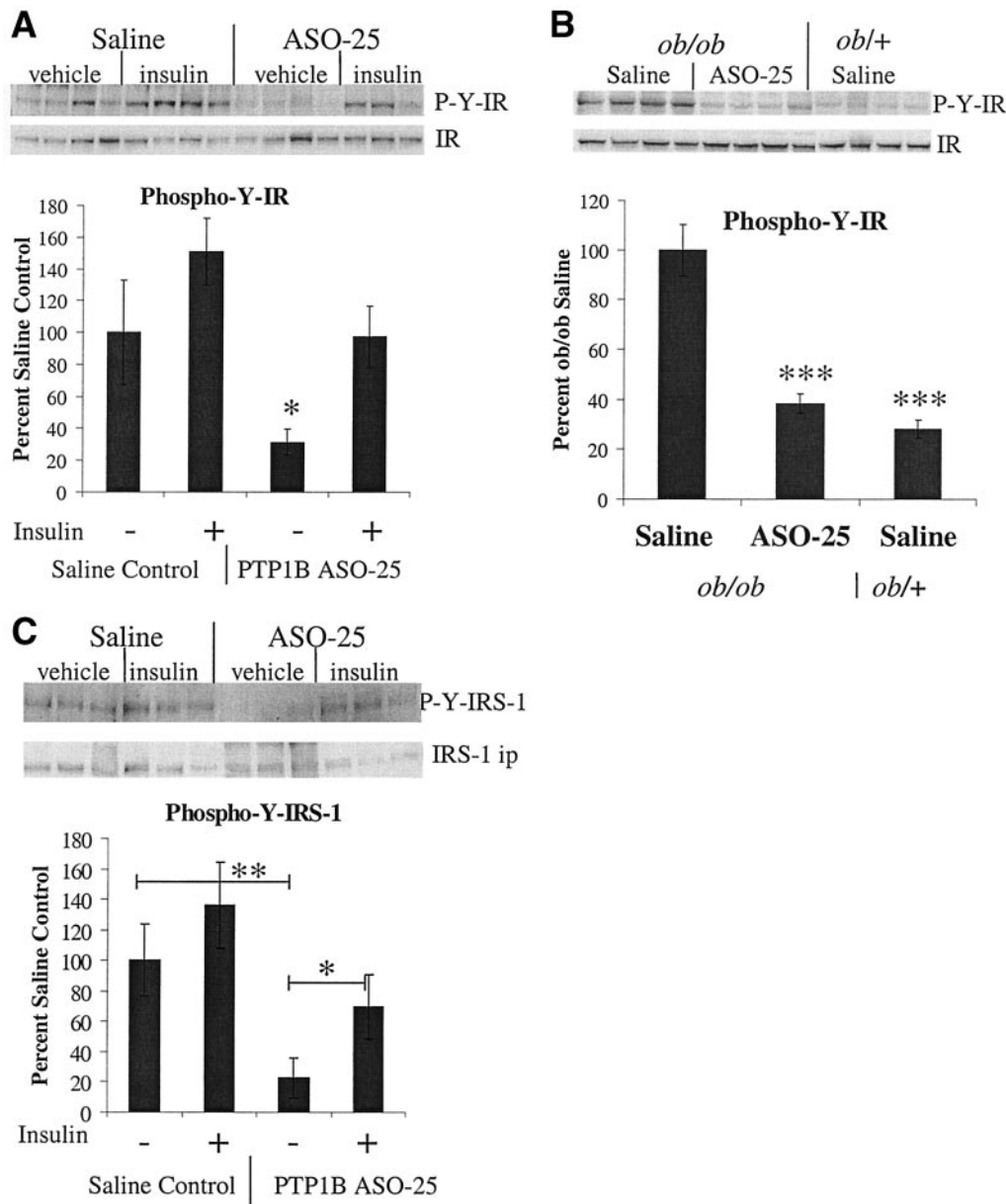


FIG. 2. Western blots of tyrosine phosphorylation of the IR (A, upper blot and B, upper blot) and of immunoprecipitated IRS-1 (C, upper blot) in liver of saline control- or 25 mg/kg PTP1B ASO-treated *ob/ob* mice stimulated with vehicle or insulin for 1 min or of *ob/ob* versus lean mice. Protein levels of IR (A and B, lower blot) and immunoprecipitated IRS-1 (C, lower blot) are shown below their respective phospho-blots. Quantitation below the blots shows changes in IR tyrosine phosphorylation (A and B) and tyrosine phosphorylation of immunoprecipitated IRS-1 (C) in insulin-stimulated saline control and PTP1B ASO mice relative to vehicle-treated saline controls (A and C) or in lean mice and PTP1B ASO-treated *ob/ob* mice relative to saline control-treated *ob/ob* mice (B). The significance of unstimulated PTP1B ASO-treated animals in A is relative to insulin-stimulated and -unstimulated saline control and insulin-stimulated PTP1B ASO mice (the latter three were not statistically different). Experiments were repeated two to four times using two or three independent sets of extracts. Data are the means \pm SE of three or four mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Insulin stimulation enhanced phosphorylation of GSK-3 α and -3 β in liver of PTP1B ASO-treated *ob/ob* mice. GSK-3 α and -3 β are downstream substrates of PKB. There was no significant difference in the basal phosphorylation state of GSK-3 α or -3 β (Fig. 5A and B, respectively) in liver of PTP1B ASO-treated mice relative to saline control-treated mice, nor did insulin treatment affect phosphorylation of GSKs in saline control mice. In contrast, insulin treatment significantly increased GSK-3 α and -3 β phosphorylation in PTP1B ASO-treated animals (Fig. 5A and B, respectively).

Increased phosphorylation of PKB was observed in skeletal muscle of PTP1B ASO-treated *ob/ob* mice

despite no measurable effect on PTP1B protein levels in muscle. Previous reports have shown that PKB activation is critical for insulin sensitivity in muscle (23). To investigate the effects of PTP1B ASO on peripheral insulin resistance, and specifically on insulin sensitivity in muscle, the phosphorylation state of PKB was analyzed in skeletal muscle extracts from saline control and PTP1B ASO *ob/ob* mice in the presence and absence of insulin stimulation. ASOs do not usually accumulate in skeletal muscle as they do in liver (24), so a decrease in PTP1B protein was not expected in muscle in response to PTP1B ASO. Indeed, no change in PTP1B protein levels was detected in muscle (Fig. 6A), as compared with a 60% decrease in liver (Fig.

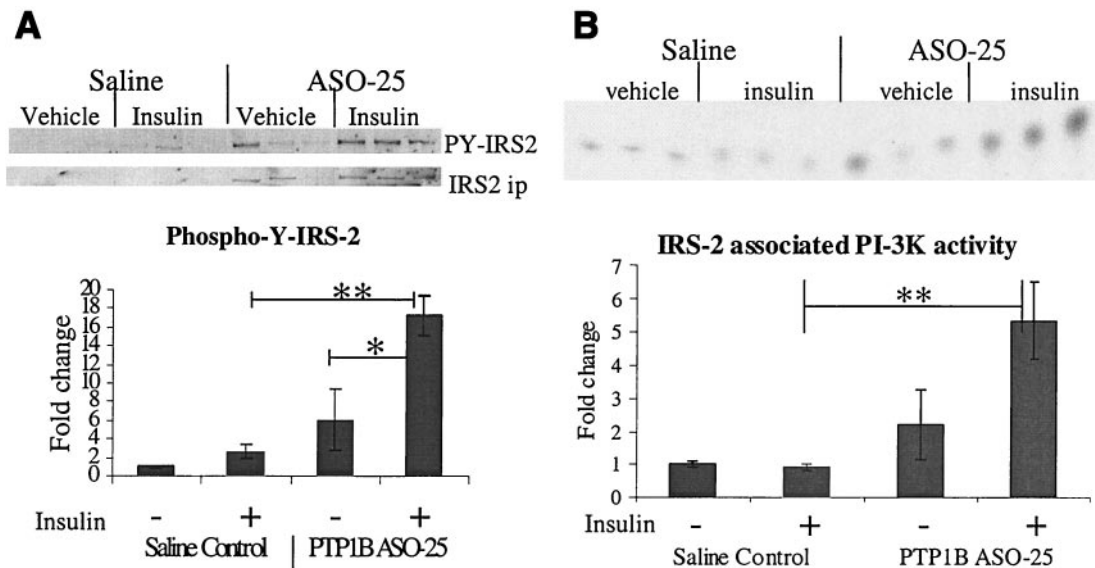


FIG. 3. Western blots of tyrosine phosphorylation and IRS-2 protein levels of immunoprecipitated IRS-2 (A, upper and lower blots, respectively) and IRS-2-associated PI 3-kinase activity (B) in liver extracts from saline control- or 25 mg/kg PTP1B ASO-treated *ob/ob* mice stimulated with vehicle or insulin for 1 min are pictured above graphs showing quantitation of the phosphotyrosine blot of IRS-2 (A) and PI 3-kinase activity (B). Quantitation for A and B is shown relative to vehicle-treated saline control. Data are the means \pm SE of three mice. * $P < 0.05$ and ** $P < 0.01$.

1B) and fat (18). Insulin stimulation did, however, have an effect on signaling in muscle. PKB (Ser473) phosphorylation was significantly increased in skeletal muscle of insulin-stimulated PTP1B ASO mice relative to both vehicle-treated PTP1B ASO mice and insulin-stimulated saline control mice (Fig. 6B).

DISCUSSION

The signaling pathways affected by inhibition of PTP1B, particularly in a diabetic model, have not been well characterized. In this study, we have demonstrated specific effects on metabolic signaling elements in the IR signaling cascade. Reduction of PTP1B in *ob/ob* mice leads to increased insulin-dependent signaling through the IR cascade, particularly via IRS-2, PI 3-kinase, and PKB, resulting in inactivation of GSK-3. Thus, a possible mechanism for decreased glucose levels in these mice may be through increased activity of glycogen synthase via PKB and GSK-3, as well as through decreased transcription of gluconeogenic enzymes via PI 3-kinase and PKB.

Our initial hypothesis was that reducing PTP1B protein levels would enhance insulin signaling and perhaps lead to increased activity of the insulin pathway in the basal nonstimulated state. The decrease in basal IR and IRS-1 tyrosine phosphorylation in liver of ASO-treated mice was therefore unexpected although not unexplainable. Earlier reports showed increased basal tyrosine phosphorylation of the IR in obese and hyperinsulinemic animals (25–27) in agreement with our own results, indicating that *ob/ob* mice had increased basal IR tyrosine phosphorylation in liver relative to lean *ob/+* mice, probably due to hyperinsulinemia. Thus, reduction of insulin levels with PTP1B ASO treatment may increase the IR's responsiveness to insulin stimulation, at least in part, by decreasing basal tyrosine phosphorylation of the IR. In contrast, this was not observed in liver from PTP1B^{-/-} mice, where there was no difference in tyrosine phosphorylation of the IR relative to PTP1B^{+/+} mice in the basal state and 1 min after insulin

stimulation; differences occurred only in the length of time that the receptor of the PTP1B^{-/-} mice remained phosphorylated (14). One important point to be considered is the background of the animals. PTP1B^{-/-} mice were derived from normal, nonhyperglycemic, nonhyperinsulinemic mice, while the *ob/ob* mice that were used in our studies were hyperinsulinemic and hyperglycemic. Since PTP1B ASO mice had reduced but not absent PTP1B levels, this may also have contributed to the difference relative to PTP1B^{-/-} mice. Obviously, the effects of PTP1B ASO on IR in diabetic mice are more complex than originally anticipated and may involve both direct and indirect effects of PTP1B. We propose that hyperinsulin-

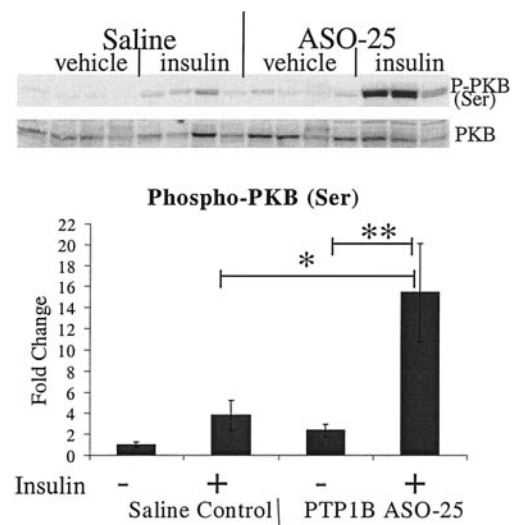


FIG. 4. Western blots of phosphorylated PKB (Ser473) (upper blot) and total PKB protein levels (lower blot) in liver of saline control- or PTP1B ASO-treated *ob/ob* mice stimulated with vehicle or insulin for 1 min. Quantitation below the blots shows changes in phosphorylated PKB relative to the vehicle-treated saline control group. Experiments were repeated four times with three independent sets of extracts. Data are the means \pm SE of three or four mice. * $P < 0.05$ and ** $P < 0.01$.

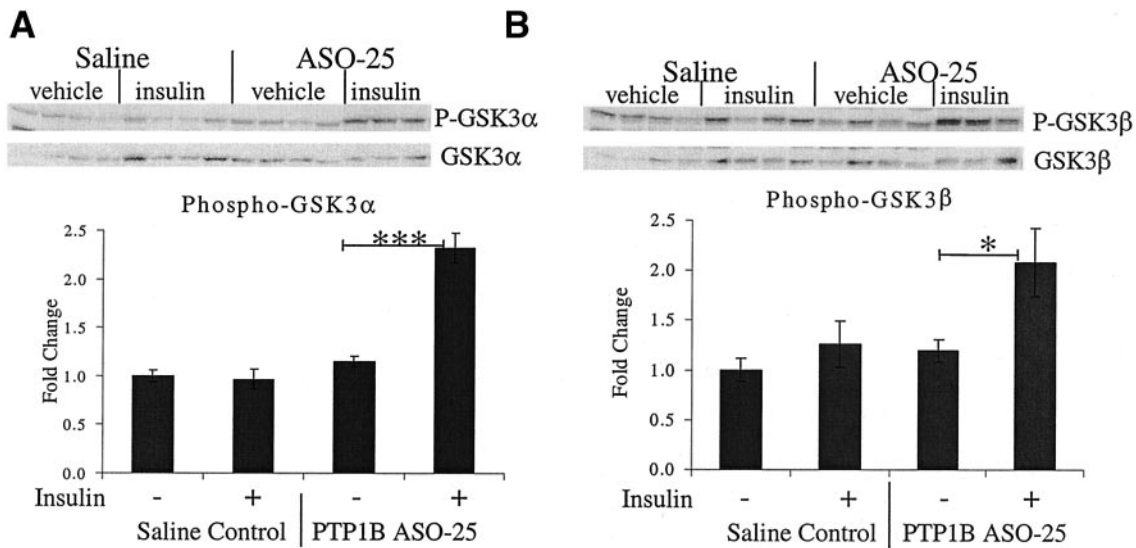


FIG. 5. Western blot of phosphorylated GSK-3 α (A) and phosphorylated GSK-3 β (B) in liver of saline control- or PTP1B ASO-treated *ob/ob* mice stimulated with saline or vehicle for 1 min. The upper blot in each panel is the phospho-specific blot, whereas the lower blot is the whole-protein blot of the same protein. Quantitation below the blots shows changes relative to the vehicle-treated saline control group for the phospho-specific blot. Experiments were repeated three times. Data are the means \pm SE of three or four mice. ** $P < 0.01$ and *** $P < 0.001$.

emia caused increased basal activation of the IR in *ob/ob* mice relative to lean littermates, as reported previously for other obese hyperinsulinemic animals (25–27) and in *in vitro* studies using HEPG2 cells chronically exposed to insulin (28). This hypertyrosine phosphorylation of the IR leads to decreased insulin sensitivity, increased insulin resistance, and decreased insulin-dependent signaling. The basal state is increased, but the insulin response is blunted. Over time, PTP1B ASO treatment resulted in decreased basal tyrosine phosphorylation of the receptor, restoring its ability to respond to insulin and to phosphorylate its substrates and allowing the mice to transition to a more normal phenotype.

The critical role of IRS-2 in metabolic insulin signaling was demonstrated in mice lacking the IRS-2 gene (IRS-2^{-/-}) (29,30), which, unlike their normal littermates, do not show increased glucose disposal or suppression of hepatic glucose production in response to insulin. *ob/ob* mice are insulin resistant, have a reduction in IRS-2 protein levels, and show little induction in IRS-2 phosphorylation or PI 3-kinase activity in response to insulin (16). Tyrosine phosphorylation of IRS-2 and IRS-2-associated PI 3-kinase activity was significantly increased, approximately threefold, in response to insulin in liver of PTP1B ASO-treated mice, similar to that of lean *ob/+* mice (16). Since IRS-2 is the principle docking protein for PI 3-kinase in liver (29,31), increased IRS-2 protein and phosphorylation levels in response to insulin may be of critical importance in increasing insulin sensitivity in this tissue. Indeed, decreased or absent protein levels of IRS-2 are associated with decreased PI 3-kinase activity and decreased glucose homeostasis because of insulin resistance in liver and muscle (29). Activation of PI 3-kinase is important for a number of insulin-regulated metabolic functions, including glucose uptake, antilipolysis, suppression of hepatic gluconeogenesis through transcriptional regulation of key proteins including PEPCK (7), and increased glycogen synthesis via PKB and GSK-3 (16,29). Furthermore, the IRS-2/PI 3-kinase complex mediates the

metabolic actions of insulin in liver (30,31). Thus, abnormalities in PI 3-kinase activation, and particularly IRS-2-associated PI 3-kinase activation, may disrupt glucose homeostasis, and reversing the resistance of PI 3-kinase to insulin in PTP1B ASO mice may have been instrumental in reducing hyperglycemia and reversing the diabetic phenotype.

PKB is a downstream substrate of PI 3-kinase and, when activated, is involved in both inhibition of gluconeogenesis through its effects on cAMP response element binding protein and PEPCK and increased glycogen synthesis through its effects on GSK-3. Increased phosphorylation of PKB is a downstream indicator of increased PI 3-kinase activity in response to insulin (32). Indeed, like PI 3-kinase activation, PKB phosphorylation and phosphorylation of its substrates, GSK-3 α and -3 β , were increased in liver in response to insulin in PTP1B ASO-treated *ob/ob* mice. Phosphorylation of GSKs render them inactive, preventing phosphorylation of their substrate, glycogen synthase, which is active in the unphosphorylated state. Glycogen synthase can then synthesize glycogen from glucose, thus decreasing glucose levels in plasma via the liver. Increased hepatic glucose production, including increased gluconeogenesis and reduced glycogen synthesis and storage, is the predominant means by which plasma glucose concentrations are increased in type 2 diabetes (33,34). Therefore, a possible mechanism for decreased plasma glucose levels in these mice may be through increased activity of glycogen synthase via PKB and GSK-3, as well as decreased transcription of gluconeogenic enzymes such as PEPCK via PI 3-kinase, PKB, and GSK-3. Indeed, PEPCK gene transcription was decreased approximately twofold in liver of PTP1B ASO-treated mice relative to saline control-treated mice (35).

ASO have been shown to preferentially accumulate in liver, kidney, and (to a lesser degree) fat, but not in muscle (24). PTP1B protein levels were decreased in liver and fat but not in muscle (our data and 18). Despite decreased PTP1B protein levels in fat, no effect on insulin stimulation

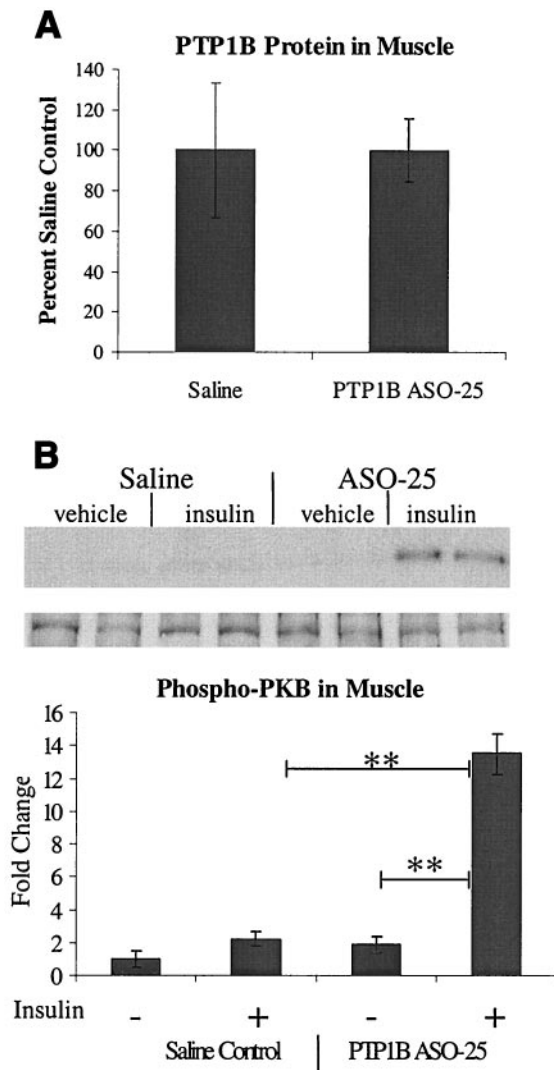


FIG. 6. Western blots of PTP1B protein (A) and phosphorylated PKB (Ser473) (B) levels in muscle of saline control- or PTP1B ASO-treated *ob/ob* mice stimulated with vehicle or insulin for 1 min. The upper blot in B is the phospho-specific blot, whereas the lower blot is the whole-protein blot of the same protein. Quantitation shows changes relative to the saline control group (A) or vehicle-treated saline control group (B) and is expressed as the means \pm SE of four mice (A; vehicle- and insulin-treated mice are combined in each group since insulin had no effect on protein levels) or two mice (B). $^{**}P < 0.01$.

of the insulin signaling cascade was observed in this tissue, including IR and PKB phosphorylation (18), consistent with the effects observed in fat of PTP1B^{-/-} mice (14,15). However, PKB phosphorylation was increased in response to insulin stimulation in muscle of PTP1B ASO-treated mice, despite the lack of an effect on PTP1B protein levels. There are several possible explanations for this result. Studies have shown that insulin sensitivity is increased upon correction of hyperglycemia or the diabetic state (36,37). In muscle, hyperglycemia impairs insulin signaling between PI 3-kinase and PKB, and the defect in PKB activation underlies the insulin resistance in muscle caused by hyperglycemia (23). Since plasma glucose and insulin were normalized several weeks before the end of the study, all tissues in the body may have been rendered more insulin sensitive due to the lower glucose and insulin levels, leading to restoration of signaling between PI 3-kinase and PKB in muscle. Alternatively,

sensitized liver or fat may secrete factors that can sensitize muscle to insulin and alter utilization of glucose (as proposed for the mechanism of increased peripheral insulin sensitivity by TZDs) (38,39). This would mean that decreased glucose production by the liver and increased insulin sensitization in liver and/or fat would be sufficient to render muscle more insulin sensitive, implying that liver and fat could be more critical target tissues for initial sensitization than muscle. A third possible explanation for sensitization of muscle to insulin in ASO-treated mice is the effect of PTP1B ASO on fat stores. PTP1B ASO-treated mice exhibited reduced weight gain and decreased adiposity relative to control *ob/ob* mice (18), consistent with the effect observed in PTP1B^{-/-} mice (15). In addition, Zucker fatty rats treated with PTP1B ASO exhibited reduced and redistributed fat stores, the latter of which was detected by magnetic resonance image analysis (40), indicating that PTP1B ASO-treated animals show changes in both amount and localization of fat stores. A number of genes involved in lipogenesis and adipose differentiation were downregulated in fat of PTP1B ASO-treated mice relative to control *ob/ob* mice (18,35), which may have contributed to the observed decreased fat mass. Further reflecting the decreased and redistributed fat stores, liver was less fatty in PTP1B ASO-treated mice both visually and by histological examination (35). Based on these findings, it is conceivable that less fat may also have been present in or near the muscle tissue in PTP1B ASO-treated mice relative to saline control-treated mice, which might be sufficient to render the muscle more insulin sensitive since fatty muscle is more insulin resistant (4,41,42). By whichever mechanism, decreasing PTP1B in fat and liver appears to result in increased insulin sensitization and signaling in muscle.

In conclusion, we have found that reduction of PTP1B protein in *ob/ob* mice using a PTP1B ASO resulted in increased insulin sensitivity and signaling in liver and muscle, as well as in conversion of the PTP1B ASO-treated mice to a more normal, nondiabetic phenotype. These results support PTP1B as a plausible target for treatment of type 2 diabetes and suggest that reduction of PTP1B in liver and fat is sufficient for restoration of insulin sensitivity and normalization of glucose and insulin levels in a diabetic animal model.

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