Reduction of Low-Molecular-Weight Protein Tyrosine Phosphatase Expression Improves Hyperglycemia and Insulin Sensitivity in Obese Mice

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To investigate the role of low-molecular-weight protein tyrosine phosphatase (LMW-PTP) in glucose metabolism and insulin action, a specific antisense oligonucleotide (ASO) was used to reduce its expression both in vitro and in vivo. Reduction of LMW-PTP expression with the ASO in cultured mouse hepatocytes and in liver and fat tissues of diet-induced obese (DIO) mice and ob/ob mice led to increased phosphorylation and activity of key insulin signaling intermediates, including insulin receptor-β subunit, PI3-kinase, and Akt in response to insulin stimulation. The ASO-treated DIO and ob/ob animals showed improved insulin sensitivity which was reflected by a lowering of both plasma insulin and glucose levels, and improved glucose and insulin tolerance in DIO mice. The treatment did not decrease body weight or increase metabolic rate. These data demonstrate that LMW-PTP is a key negative regulator of insulin action and a potential novel target for the treatment of insulin resistance and type 2 diabetes.

The incidence of diabetes has been steadily increasing and has become a major public health concern. Over 85% of diabetic patients have type 2 diabetes. Obesity, which can result from a sedentary life style and high calorie diet, is a major risk factor for the development of this disorder (1). A hallmark of type 2 diabetes is insulin resistance, characterized by a decreased insulin response in a variety of tissues (2), including liver, fat, and muscle. Therefore, increasing insulin sensitivity is a practical strategy for the treatment of type 2 diabetes.

Insulin initiates its physiological response by binding to its membrane-bound receptor (IR, a αβ2-heterotetramer protein) which causes autophosphorylation of the β-subunit and receptor activation, resulting in subsequent phosphorylation of its two major down-stream substrates, IRS-1 and IRS-2 (3-5). Phosphorylated IRS-1 and 2 interact with and activate other SH2 domain containing adapter molecules such as NCK2, Grb2, Shc, Syp (4, 5, 6-8) and the regulatory subunit (p85) of phosphatidylinositol 3-kinase (PI3-K) (9, 10). Activated PI3-K stimulates Akt (or PKB) that in turn phosphorylates and inactivates glycogen synthase kinase (GSK)-3 (11) resulting in activation of glycogen synthase (12), thereby increasing the utilization of glucose for glycogen synthesis. In fat and muscle, activation of this pathway...
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also causes transfer of GLUT4 from the cytoplasm to the cell membrane resulting in increased glucose uptake (13). Therefore, IR-IRS-1/2-PI3-K-Akt signaling cascade is a key pathway in mediating the effects of insulin action on blood glucose levels.

A number of studies have established a role for intracellular phosphatases in the negative regulation of insulin signaling (14-16) such as protein tyrosine phosphatase (PTP) 1B that negatively regulates insulin action through dephosphorylating tyrosine-phosphorylated IR (15, 16). Insulin sensitivity was enhanced in PTP1B-knockout mice, in which increased tyrosine phosphorylation of IR was found in liver and muscle but not in adipose tissue. Insulin sensitivity was also increased in ob/ob and db/db mice treated with antisense inhibitors that reduced PTP1B expression in liver and fat (17, 18). Furthermore, in these mice insulin signaling was enhanced in liver but not in fat tissue. Therefore, PTP1B negatively regulates insulin signaling with tissue specificity (15, 16). In addition, PTEN and SHIPs also negatively regulate insulin signaling in vivo via dephosphorylating PI3, 4, 5-P3, which results in decreased phosphorylation and inactivation of Akt. Enhanced insulin sensitivity was observed in ob/ob and db/db mice after antisense reduction of PTEN expression which was accompanied by increased levels of phosphorylated Akt at serine 473 (AktSer473) in liver (14). Increased phosphorylated AktSer473 levels in both liver and muscle were also found in SHIP2-knockout mice (19). Moreover, protection from diet-induced insulin-resistance was observed in these knockout mice when fed a high-fat diet (19). Therefore, intracellular phosphatases play a key role in insulin signaling activity and thus insulin sensitivity.

A distinct PTP called low-molecular-weight PTP (LMW-PTP), also known as acid phosphatase locus 1 (ACP1), is an 18-kDa cytosolic enzyme with two catalytically active isoforms, named A and B, derived from alternate splicing of a single pre-mRNA (20, 21). It is widely expressed in various tissues in mammals (20, 21) and gene expression analysis from adult mouse tissues found that the level of LMW-PTP mRNA is highest in liver and brain and lowest in skeletal muscle (22). Biochemical studies have shown that LMW-PTP can dephosphorylate tyrosine-phosphorylated proteins (23, 24). Limited in vitro studies have suggested a role for LMW-PTP as a negative regulator of insulin-mediated mitogenic and metabolic signaling (23, 25). In addition, epidemiological studies have suggested that LMW-PTP levels are associated with dyslipidemia and hyperglycemia in human subjects (26, 27). However, the precise role of LMW-PTP in regulating insulin action in vivo remains unknown.

Here, we have used a specific antisense oligonucleotide (ASO) to suppress the expression of LMW-PTP in cultured hepatocytes and in liver and fat of diet-induced obese and leptin-deficient obese mice to investigate the mechanism of insulin sensitizing effect of LMW-PTP. Previous pharmacokinetic studies have found that the specific ASO chemistry employed in this study leads to good distribution (and consequent activity) in a variety of tissues in vivo, including liver and adipose tissue (28, 29). Reduction in the levels of both LMW-PTP isoforms in vitro and in vivo increased phosphorylation of IR at tyrosine and AktSer473, and increased IRS-1 and 2-associated PI3-K activities in both liver and fat. The enhanced insulin signaling activity was accompanied by a reduction in blood glucose and insulin levels and improved insulin and glucose tolerance in obese mice. These results demonstrate for the first time a novel role of LMW-PTP as a key negative regulator of insulin signaling in vivo.

EXPERIMENTAL PROCEDURES

Selection of LMW-PTP ASOs-Rapid throughput screens of about 80 ASOs against LMW-PTP were performed in A549 cells and the reduction of target gene expression was analyzed with real-time quantitative RT-PCR after transfection of the cells with different concentrations of the ASOs for 24 h. Based on IC50 values, 3-4 potent ASOs were selected and their in vivo activity was confirmed in lean C57BL/6J-Lepob/+ mice. The final
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selection of the ASOs was based upon the maximal reduction of the hepatic LMW-PTP mRNA levels in lean mice without any evidence of overt toxicity. The selected LMW-PTP ASO (ASO #1), ISIS 288267, was then used for the current study. Another LMW-PTP ASO (ASO #2) ISIS 282291, was also used in some in vitro experiments for comparison. All ASOs have a uniform phosphorothioate backbone with 20-base chimeric design with 2'-O-(methoxy)-ethyl (2’-MOE) modification in the first 5 and last 5 bases. The modification enhances their binding affinity to complimentary sequences and their resistance to the action of nucleases. A negative control ASO (ISIS141923), which has the same chemical composition as ISIS 288267 but no complementarities to any known gene sequence, was also included in the study.

Isolation and transfection of primary hepatocytes-Mouse primary hepatocytes were prepared according to method as described earlier (29). Briefly, mice were anesthetized by Avertin and the livers were perfused through portal vein with a buffer containing Ca\(^{2+}\)/Mg\(^{2+}\)-free Hank’s balanced salt solution (Invitrogen, Carlsbad, CA), 10 mM Heps, and 0.5 mM EGTA (pH7.4) for 4 min. Then it was perfused with digestion buffer (William’s E medium, 10 mM Heps, 2 mM glutamic acid, 0.63 mg/ml collagenase B, and 0.01 mg/ml gentamycin) for 6-8 min. Perfused livers were detached from the animal and the liver cells were gently dissociated and dispersed in a wash buffer (the digestion buffer without collagenase but with 10% FBS). The cell suspension was then centrifuged at 450-500 rpm in a CR412 centrifuge (Jouan, Winchester, VA) to separate parenchymal cells from non-parenchymal cells and the former were washed twice with cold PBS. The parenchymal cells were then plated and maintained in William’s E medium supplemented with 10% FBS and 1 nM insulin at 37°C under 95% O\(_2\) / 5% CO\(_2\). After overnight culture the cells were washed with PBS and incubated with 1-2 ml of transfection mixture containing 150 nM ASO (control or LMW-PTP ASO), 4.5 μg lipofectin (Invitrogen, CA) in William’s E medium for 4-6 h. Then the cells were switched to a complete medium containing William’s E and 10% FBS for addition 16-18 h for mRNA analysis or 48-55 h for protein analysis. For insulin signaling-related protein analysis, the hepatocytes were incubated in William’s E medium containing only 0.1% BSA overnight prior to challenge with 10 nm insulin.

Mice care and treatments-Animal care and other procedures were the same as described previously (29). In brief, male C57BL/6J mice and C57BL/6J-Lep\(^{ob/ob}\) (ob/ob) mice were purchased from the Jackson Laboratory at 6-7 weeks of age and maintained at 12-h light/dark cycle with free access to food and water. C57BL/6J mice were fed a diet containing 60kcal% fat (Research diet D12492; Research Diets, New Brunswick, NJ) for twelve weeks to induce obesity and insulin resistance. The animals were then divided into different groups based on BW, blood glucose and insulin concentrations and treated (s.c. injection) with LMW-PTP ASO or control ASO (dissolved in saline) at a dose of 25 mg/kg BW, or with a similar volume of saline, twice a week for 6 weeks. A group of lean C57BL/6J mice fed normal rodent chow and injected with saline served as normal controls. At the end of the study, the mice were sacrificed; blood was collected through cardiac puncture, and different tissues were dissected and quickly frozen in liquid N\(_2\) until further analysis.

For the ob/ob mice study, after acclimation to the animal care facility for 4-5 days, the mice were grouped based on BW and blood glucose levels and injected with LMW-PTP ASO at 25 mg/kg BW or a similar volume of saline twice a week for 4 weeks. Since the negative control ASO treatment did not exhibit specific metabolic effects as compared to saline treatment in above DIO mice study, it was not included in the ob/ob mice study.

For the insulin challenge studies, DIO mice were treated with LMW-PTP ASO or control ASO at a dose of 50 mg/kg BW, or with a similar volume of saline, twice a week
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for 2 weeks, and ob/ob mice were treated with LMW-PTP ASO at 25 mg/kg BW or saline twice a week for 4 weeks. The animals were then fasted overnight and given a bolus i.p. injection of insulin at 2U/kg BW or vehicle. Ten minutes later, the animals were sacrificed, and liver and epididymal fat were collected and quickly frozen in liquid N₂ for further analysis. All above experiments were performed in accordance with Institutional Animal Care and Use Committee guidelines.

Metabolic Rate Measurement—After 3.5 weeks of treatment, metabolic rate in ob/ob mice was measured using indirect calorimetry (Oxymax System, Columbus Instruments, Columbus, OH). Animals were acclimated to metabolic chambers for 24 h before initiation of the measurement. For each treatment group, 5 animals were measured for a 24-h period.

Biochemical analysis—Plasma insulin levels were measured with a commercial Elisa kit (ALPCO diagnostics, Manufactured by Merckodia, Sweden) according to manufacturer’s instructions. Plasma glucose, triglycerides, cholesterol, AST and ALT levels were measured with an Olympus Analyzer AU400 (Melville, NY).

Gene expression analysis—For gene expression analysis, total RNA from hepatocytes was isolated by using Qiagen RNA easy kit and total RNA from animal tissues was isolated by homogenizing tissues in RLT buffer (Qiagen) followed by centrifugation with cesium chloride gradient. Real-time quantitative RT-PCR was performed with custom-made RT-PCR enzymes and reagents kit (Invitrogen Life Technology Inc., Carlsbad, CA), primer & probe sets designed with Primer Express Software (PE Applied Bioscience, Foster city, CA) and ABI prism 7700 Sequence Detector (PE Applied Biosciences, Foster City, CA). For the analysis, 100 ng total RNA was used for each reaction. Each sample was run in duplicate or triplicate and the mean values were used to calculate the mRNA levels and gene expression. The expression was normalized with the amount of total RNA loaded that was determined with a ribogreen assay.

Insulin and glucose tolerance tests (ITT and GTT)—ITT and GTT in DIO mice were conducted after 4.5 and 5.5 weeks of treatment respectively. The mice were fasted for 4 hours prior to ITT and overnight prior to GTT. Blood glucose levels were measured before insulin or glucose injection (0 min, baseline values), and the animals were then administered (i.p) insulin (R-insulin, Lilly Research Laboratories, Indianapolis, IN) at a dose of 0.5 U/kg BW for ITT or glucose at 1.0 g/kg BW. Blood glucose was then measured at 30, 60, 90 and 120 min after insulin or glucose injection using a Glucometer (Abbott Laboratories, Bedford, MA).

Histological analysis—Liver samples were fixed in 10% buffered formalin and embedded in paraffin wax for staining with hematoxylin and eosin (H&E staining), or directly embedded in an optimal temperature embedding medium followed by immediate snap-freezing in liquid N₂ for oil-red O staining. Multiple adjacent 6-μm sections were cut and mounted on glass slides, and then stained. Images of the histological sections were analyzed.

Western blot analysis—Cells were lysed or tissues were homogenized in a lysis buffer (150mM NaCl, 50 mmol/l Tris, pH 7.5, 1% Triton X-100, 0.5% NP-40, 0.25% sodium deoxycholate, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mM NaOV, 1 mM NaF) containing protease inhibitor cocktail I (Calbiochem). The lysates were cleared by centrifugation for 15 min at 12,000 g. Equal amount of total protein for different samples was separated on a 10% or 16% SDS-PAGE gel under reduced conditions and then transferred onto a PVDF membrane. The blots were then incubated with antibody against IR-β subunit (C–19; Santa Cruz, CA), tyrosine-phosphorylated proteins (4G10, Upstate, Lake Placid, NY) or serine473-phosphorylated Akt (Cell Signaling, Danvers, MA). The blots were also incubated with antibody against PTP1B, SHP2/SHPPTP2 (from Upstate, Lake Placid, NY), PTEN (Cell Signaling, Danvers,
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MA and Upstate, Lake Placid, NY) or LMW-PTP antibody (kindly provided by Dr. Tom Mustelin of Burnham Institute, La Jolla, CA). The signals were detected by using HRP-conjugated goat anti-rabbit IgG antibody and ECL detection reagents.

**Immunoprecipitation**—The clarified lysates were first incubated with protein agarose A/G beads (1:1 ratio) for 3-4 h at 4°C followed by incubation with anti-phosphotyrosine antibody for another 3-4 h at 4°C. The immunocomplex was washed with lysis buffer 3x and then boiled in Laemmli’s sample buffer and Western blotted with anti-IR-β subunit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described earlier.

**PI-3 kinase activity assay**—The PI3-kinase activity was measured as described previously (30). Briefly, the clarified lysates (500 μg protein) were subjected to immunoprecipitation with 1 μg of IRS-1, or IRS-2 antibody for 2 h at 4°C, followed by incubation with protein A/G sepharose for an additional 2 h. The immunocomplexes were then washed and used for PI3-K activity assay using L-α-phosphatidylinositol and [γ-32P] ATP as substrates. The reaction was stopped by addition of a mix of CHCl₃:CH₃OH:HCl (100:200:2). The produced phosphorylated lipid was extracted with CHCl₃ and separated on a TLC plate. The plate was then exposed to a Kodak film. The radioactivity associated with PIP₃ spots was quantified by Image Quant or the PIP₃ spots were scratched off the plates and counted in scintillation counter.

**Statistical analysis**—Values are expressed as mean ± SEM of at least three to five in vitro or five in vivo independent measurements per treatment. Statistical difference across treatment groups was determined using student t-test or one-way ANOVA with Tukey HSD multiple comparisons. Differences were considered significant at P < 0.05.

**RESULTS**

**Reduction of LMW-PTP expression enhances insulin signaling in mouse hepatocytes**—To investigate the role of LMW-PTP in insulin signaling, mouse primary hepatocytes were transfected with LMW-PTP ASO or vehicle. LMW-PTP ASO treatment reduced LMW-PTP mRNA levels by approximately 90% (Fig. 1A), which was associated with dramatic reduction in the protein levels for both A and B isoforms (Fig. 1B). Re-probing the membrane with anti-PTP1B antibody revealed no compensatory increase in PTP1B protein levels in the ASO-treated cells (Fig. 1B). We next examined the effect of LMW-PTP reduction on the insulin activation of IR and Akt, two critical members of the insulin signaling cascade. LMW-PTP ASO or control ASO transfected mouse primary hepatocytes were incubated with insulin (10 nM) for 10 minutes and cell lysates were prepared for Western blot analysis. Tyrosine phosphorylation levels of IRβ subunit (pY-IRβ; Fig. 1C) and serine⁴⁷³ phosphorylation levels of Akt (pAkt⁴⁷³; Fig. 1D) were higher in LMW-PTP ASO-treated cells than in control ASO-treated groups after insulin stimulation. Under these conditions there was no change in the total protein levels of either IRβ subunit (Fig. 1C) or Akt (Fig. 1D).

To further confirm that these insulin enhancing effects were secondary to a specific antisense reduction of LMW-PTP, we evaluated the effect of second ASO (#2) that was targeted to a different region of the LMW-PTP mRNA. ASO #2 reduced LMW-PTP expression to a similar extent as compared to ASO #1 and also enhanced the phosphorylation levels of Akt⁴⁷³ to a similar extent upon insulin stimulation (Fig 1D, middle panel). These data suggest that reduction of LMW-PTP expression sensitized the response of the hepatocytes to insulin stimulation without any compensation by PTP1B.

**Suppression of LMW-PTP improves insulin sensitivity in DIO mice with no change in body weight**—To ascertain the role of LMW-PTP in insulin action in vivo, we suppressed its expression with the ASO in high-fat (HF) diet-induced obese (DIO) mice. After being fed a 60kcal% fat diet for 12 weeks, mice were treated with saline or a control ASO or LMW-
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PTP ASO (dissolved in saline) at a dose of 25 mg/kg body weight (BW) twice a week for 6 weeks. LMW-PTP ASO treatment reduced LMW-PTP mRNA levels by about 90% in liver and 75% in fat as compared to controls (Fig. 2A), which resulted in a significant reduction in both tissues of LMW-PTP protein levels (Fig. 2B). As was observed in hepatocytes, ASO-mediated reduction of LMW-PTP did not cause a compensatory increase in PTP1B protein levels in either tissue (Fig. 2B). Furthermore, we examined the expression of SHP2/SHPTP2 and PTEN; two well know phosphatases known to play an important role in glucose homeostasis (14, 19). Western blot analysis revealed that there was no significant difference in the protein levels of both these phosphatases between LMW-PTP ASO treatment group and saline or control ASO treatment group in liver or fat tissues of DIO mice (Fig. 2B, upper and middle panels). Note that we observed two strong bands for SHP2/SHPTP2 in the liver tissues from both DIO and ob/ob mice (Fig. 2B, upper panels respectively) but one band for SHP2/SHPTP2 from the fat tissues of either animal model (Fig. 2B & 3B, lower panels respectively).

As expected, high fat feeding resulted in increased plasma insulin and glucose levels in the mice. Treatment with LMW-PTP ASO lowered both insulin and glucose levels as compared to the controls (Fig. 2C). In fact, both insulin and glucose levels in LMW-PTP ASO-treated mice were similar to those seen in chow-fed mice after 3.5 and 4.5 weeks of treatment, respectively (Fig. 2C). These results suggest that reduced LMW-PTP expression following ASO treatment increased insulin sensitivity. This was further confirmed by GTT and ITT conducted in these mice, in which glucose excursions after glucose or insulin challenge were significantly lower in LMW-PTP ASO-treated animals relative to saline or control ASO treated-mice (Fig. 2D).

To further address the mechanisms underlying the effects of LMW-PTP inhibition in DIO mice, mRNA levels of various genes involved in glucose and lipid metabolism were determined. The analysis demonstrated that LMW-PTP treatment caused a significant reduction in the levels of glucose-6-phosphatase (G6Pase) mRNA, which could in part contribute to the positive effects on glucose metabolism (Fig. 2E). Taken together, these data indicate that suppression of LMW-PTP with a specific ASO improved insulin sensitivity in DIO mice.

In addition, treatment with LMW-PTP ASO resulted in significant lowering of plasma cholesterol levels (see Table 1) and liver triglyceride content (34.5 ± 4.5 mg/g vs. 55.6 ± 8.0 mg/g in control ASO group vs. 66.3 ± 8.4 mg/g tissue in saline group; P < 0.01 vs. either group). However, ASO treatment did not affect BW (see Table 1). Gene expression analysis found that LMW-PTP ASO treatment did not cause changes in the expression of hepatic lipogenic genes, including fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1), diacylglycerol acyltransferase (DGAT)2, or the genes related to lipid homeostasis in fat, including hormone-sensitive lipase (HSL), FAS, DGAT1 and DGAT2 (Fig. 2E). Furthermore, ASO treatment was well tolerated and no evidence of liver toxicity as determined by plasma AST and ALT activities (see Table 1) was observed.

Suppression of LMW-PTP also improves insulin sensitivity in ob/ob mice with no change in body weight. To extend our investigation on the role of LMW-PTP in insulin action and glucose metabolism, LMW-PTP ASO was used to suppress its expression in a genetically obese mouse model and the resultant metabolic effects were studied. For this purpose, leptin deficient ob/ob mice were treated with LMW-PTP ASO at a dose of 25 mg/kg or a similar volume of saline twice a week for 4 weeks. ASO treatment resulted in a reduction of LMW-PTP mRNA levels by approximately 90% in liver and 60% in fat (Fig. 3A). Western blot analysis of liver samples demonstrated that both isoforms of LMW-PTP protein were reduced by more than 75% versus controls (Fig. 3B). A significant reduction in LMW-PTP protein levels of both the isoforms were also found in the fat samples from LMW-PTP ASO treated-mice (Fig. 3B). Again, no significant compensatory

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increase in PTP1B, SHP2/SHPTP2 or PTEN (data not shown) protein levels were observed (Fig. 3B).

LMW-PTP ASO treatment improved insulin sensitivity in ob/ob mice. After 4 weeks of treatment both fed and fasted plasma glucose levels were markedly lowered in ASO-treated mice than in the controls (Fig. 3C and 3D). Furthermore, LMW-PTP ASO treatment resulted in approximately a 50% reduction in plasma insulin levels versus controls (Fig. 3E). These data demonstrate that suppression of LMW-PTP with ASO also improved insulin sensitivity in ob/ob mice.

LMW-PTP ASO treatment also improved hepatic steatosis in ob/ob mice. Histological examination of H&E stained liver sections showed that there was a large number of multilobular and unilobular cytoplasmic lipid droplets in control animals. In contrast, the lipid droplets were greatly reduced in both size and number as a result of LMW-PTP ASO treatment (Fig. 3F). This finding was further confirmed with oil-red O staining in which the intense oil-red O stained lipid spots found in saline-treated mice were markedly reduced in both size and number as a result of LMW-PTP ASO treatment (Fig. 3F). This improvement in liver steatosis was accompanied by an improvement in liver function, as demonstrated by a decrease in plasma AST and ALT levels in these mice (see Table 1). The treatment lowered plasma triglyceride levels but not cholesterol levels (see Table 1). However, the ASO treatment did not cause changes in either BW or metabolic rate as determined by a lack of effect on both VO₂ (see Table 1) and respiratory quotient (data not shown).

**Suppression of LMW-PTP ASO improves insulin action in both liver and fat-** To investigate the possible mechanisms underlying the improved insulin sensitivity after LMW-PTP ASO treatment, both DIO and ob/ob mice treated with LMW-PTP ASO were challenged with insulin and the activities of the key insulin signaling enzymes were examined in both liver and fat. Western blot analysis found that treatment with LMW-PTP ASO caused a greater increase in pY-IRβ levels versus controls upon insulin stimulation in liver in both DIO (Fig. 4A) and ob/ob (Fig. 5A) mice, and in fat in ob/ob mice (data not shown) without affecting total protein levels of IRβ subunit. LMW-PTP ASO treatment significantly reduced the basal levels of pY-IRβ versus controls (Fig. 4C). Injection of insulin increased pY-IRβ levels by over 5-fold versus the basal levels in ASO-treated mice (Fig. 4C; P < 0.001) whereas it only increased pY-IRβ by about 1.2-fold in control mice (Fig. 4C; P < 0.05). A decreased basal pY-IRβ level in ASO-treated ob/ob mice versus controls was also observed (Fig. 5A). The decreased basal pY-IRβ levels probably were due to ASO-caused reduction of plasma insulin levels as shown in Fig. 2C and 3E. Taken together, these data demonstrate increased response of the IR to insulin after LMW-PTP ASO treatment.

The activity of PI3-K, a key intermediate component of insulin signaling pathway, was also measured in ob/ob mice treated with LMW-PTP ASO. In liver, IRS-1 associated PI3-K activity was found to be increased by approximately 2.5-fold in the ASO treated-mice upon insulin administration (Fig. 5B), which was in sharp contrast to saline-treated animals in which insulin stimulation did not cause any increase in activity (Fig. 5B). IRS-2 associated PI3-K activity was enhanced by over 55% in the liver of the ASO-treated animals but not in controls upon insulin stimulation (Fig. 5C). Increased IRS-1 associated PI3-K activity (approximately 60%) was also observed in fat from LMW-PTP ASO-treated ob/ob mice, but not in fat from saline-treated mice upon insulin stimulation (Fig. 5D). Insulin challenge also caused a greater increase in pAktSer473 levels, a downstream signaling molecule of PI3-K, in both liver (~5-fold vs. ~3-fold; Fig. 4B) and fat (~6-fold vs. ~3-fold; Fig. 4D) in ASO-treated DIO mice versus controls. Similar observations were made in insulin challenged ob/ob mice liver (Fig. 5E). These data strongly suggest that increased insulin sensitivity after suppression of LMW-PTP expression by ASO was through alteration of the phosphorylation status and.

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activities of the key insulin signaling members in both liver and fat.

DISCUSSION

The results presented in this study demonstrate for the first time that LMW-PTP is a key negative regulator of insulin signaling in vivo and reduction of its expression can improve hyperglycemia and insulin sensitivity in insulin-resistant DIO and ob/ob mice. Reduction of LMW-PTP expression with an antisense inhibitor lowered both plasma glucose and insulin levels in both models versus their saline or control ASO treated groups, suggesting improved insulin sensitivity. This was further supported by improved glucose and insulin tolerance in LMW-PTP ASO-treated DIO mice.

LMW-PTP appears to regulate hepatic insulin signaling sensitivity in a manner similar to other phosphatases such as PTP1B (15-18), SHIP2 (19) and PTEN (14) by changing the phosphorylation status of the key members of the signaling pathway. Tyrosine phosphorylation of IRβ subunit and serine473 phosphorylation of Akt in both liver and fat in response to insulin stimulation were more markedly increased in LMW-PTP ASO-treated ob/ob mice relative to controls. In addition, both IRS-1 and 2 associated PI3-K activity was significantly enhanced upon insulin stimulation in LMW-PTP ASO-treated ob/ob mice versus controls. Interestingly, LMW-PTP ASO treatment lowered the basal phosphorylation levels of Aktser473 in liver and tyrosine of IRβ subunit in fat, which is in agreement with the previous findings with other phosphatases (17) and could be due to a reduction in hyperinsulinemia in the basal state. Furthermore, Chiarugi et al. have reported that dominant negative LMW-PTP interacts with the catalytic site of the IRβ subunit, resulting in enhanced insulin-stimulated tyrosine phosphorylation of the subunit in NIH-3T3 IR cells (23). In agreement with their finding, we observed that suppression of LMW-PTP expression enhanced tyrosine phosphorylation of the IRβ subunit in response to insulin stimulation in cultured primary hepatocytes and in obese animals. Therefore, LMW-PTP, like PTP1B, regulates insulin signaling primarily by a direct action on IR, thus indirectly affecting the down-stream components of the insulin signaling pathway.

The results from the current study show some similarities in LMW-PTP suppressed mice, such as lowering of plasma glucose and insulin, to those reported in PTP1B-knockout mice (15, 16) and the mice treated with a PTP1B antisense inhibitor (17, 18). However, although both PTPs regulates insulin signaling by a direct action on IR, there are major differences in the phenotype that results after reduction of the two phosphatases. First, studies from different laboratories with two different PTP1B knockout mouse models found that a deficiency in PTP-1B resulted in increased insulin signaling in liver and muscle, but not in fat upon exposure to insulin stimulation (15). Similarly, ob/ob and db/db mice treated with PTP1B ASO demonstrated increased insulin signaling activity in liver but not in fat although the expression of PTP1B was dramatically suppressed in both tissues (17, 18). In contrast, the present study found that knockdown of LMW-PTP levels with specific ASO increased insulin signaling activity in both liver and fat in both obese models. These results indicate that LMW-PTP has unique tissue specificity in the regulation of insulin signaling relative to PTP1B. Second, PTP1B-knockout mice (15, 16) and PTP1B ASO-treated mice (17, 18) showed decreased body weight and adiposity. The knockout mice were resistant to BW gain on a HF diet and displayed an increased metabolic rate (15, 16). The PTP1B ASO-treated ob/ob mice had lowered lipogenesis with decreased lipogenic gene expression in fat (17). SHIP2-knockout mice also showed a similar phenotype to that seen in PTP1B-knockout mice in this regard (19). However, in this study both LMW-PTP ASO-treated DIO and ob/ob mice showed no significant difference in BW than the controls. Metabolic rate measurement in ob/ob mice did not reveal any difference in either O2 consumption rate or respiratory quotient between treatment groups. Gene expression
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analysis also failed to identify differences in the expression of key lipogenic genes in liver or the genes related to lipid homeostasis in fat among the DIO groups. Therefore, these two intracellular phosphatases play distinct roles in regulating metabolism in the body.

Non-alcoholic steatohepatitis (NASH) can result from chronic accumulation of fat in the liver. Epidemiological studies suggest that increased LMW-PTP activity is associated with high levels of serum triglycerides in obese and diabetic subjects where incidence of fatty liver and NASH is a common occurrence (26). In this study, a significant decrease in liver triglyceride levels in DIO mice and an improvement in liver steatosis in ob/ob mice following ASO treatment was observed. This observation, coupled with unchanged expression of the key hepatic lipogenic genes, suggests increased hepatic fatty acid oxidation in these ASO-treated mice. This is in the light of our recent observation where suppression of DGAT2 expression with an antisense inhibitor improved liver steatosis in both DIO and ob/ob mice due to decreased hepatic lipogenesis and increased fat oxidation (29). In addition, decreased plasma cholesterol levels in DIO mice and decreased plasma triglyceride levels in ob/ob mice were also observed in this study. These data suggest that, in addition to a role in regulation of insulin action, LMW-PTP may also be involved in regulation of lipid metabolism. Further studies are needed to delineate the mechanism of lipid lowering effects of LMW-PTP ASO in these mice.

Several phosphatases, including PTP1B, LAR, SHIP2, and PTEN, have been reported to exert negative regulation on insulin action through their dephosphorylation action on different components of the insulin signaling pathway (14-16, 19, 31, 32). Here, using an antisense approach, we demonstrate that LMW-PTP also plays a key role in modulating insulin signaling and insulin sensitivity in vivo. This effect of LMW-PTP was distinct and independent of PTP1B, SHP2/SHPTP2 or PTEN. Whether additional PTPs or other phosphatases exist that are involved in regulation of several components of insulin signaling activities under normal and diabetic settings remains to be determined.

In conclusion, the present study demonstrates for the first time that reduction of LMW-PTP protein levels in insulin-resistant obese animals improves insulin action and glucose metabolism through positively regulating insulin signaling pathway in liver and adipose tissues. Thus, LMW-PTP could be a novel drug target for the treatment of type 2 diabetes.

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FOOTNOTES

The abbreviations used are: LMW, low molecular weight; PTP, protein tyrosine phosphatase; IR, insulin receptor; IRS, insulin receptor substrate; PI3-k, phosphatidylinositol 3-kinase; ASO,
Reduction of LMW-PTP improves insulin sensitivity

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FIGURE LEGENDS

Fig. 1. Suppression of LMW-PTP expression by LMW-PTP ASO leads to increased insulin signaling activity in mouse primary hepatocytes. Transfection with LMW-PTP ASO suppressed LMW-PTP mRNA levels by more than 90% in mouse primary hepatocytes (A), which caused marked reduction in the protein levels of LMW-PTP but not PTP-1B (B). Challenge with insulin demonstrated that the increase in both tyrosine phosphorylation of IRβ subunits (pY-IRβ; C) and serine473 phosphorylation of Akt (AktSer473; D) was much greater in the cells treated with the LMW-PTP ASO vs. those treated with control ASO. Data and representative blots presented were from 3 to 5 experiments with duplicate or triplicate for each treatment. *P<0.05, **P < 0.01.

Fig. 2. Reduction of LMW-PTP expression by LMW-PTP ASO improved insulin sensitivity in DIO mice. Treatment with LWP-PTP ASO reduced LMW-PTP mRNA levels by about 90% and 75% in liver and fat, respectively (A), which resulted in dramatic reduction in LMW-PTP protein levels (B), but no effect on PTP1B, SHP2 and PTEN protein levels in both tissues as compared to the controls (B). The ASO treatment reduced fed plasma insulin and glucose levels (C), improved glucose (IPGTT) and insulin tolerance (ITT; D), decreased hepatic G6Pase gene expression, but did not affect the expression of the key lipogenic genes (FAS, SCD1, and DGAT2) in liver or the genes (HSL, FAS, DGAT1 and DGAT2) related to lipid homeostasis in fat (E). Data and representative blots presented were from 4-6 mice for each treatment group. *P < 0.05, **P < 0.01 vs. either DIO control group.
Fig. 3. Suppression of LMW-PTP expression by LMW-PTP ASO improved insulin sensitivity in ob/ob mice. Treatment with LMW-PTP ASO reduced LMW-PTP mRNA levels by about 90% in liver and 60% in fat (A), which resulted in similar degrees of reduction in the LMW-PTP protein levels (both A and B isoforms) in liver and fat respectively without any compensatory changes in PTP1B, SHP2 and PTEN expression in these tissues (B). ASO treatment caused reduced fed (C) and fasted (D) plasma glucose levels as well as insulin levels (D). The ASO treatment improved liver steatosis, as demonstrated by H&E staining and oil-red O staining (F). Data and representative blots presented were from at least 5 mice for each treatment group. *P < 0.05, **P < 0.01 vs. controls.

Fig. 4. Reduction of LMW-PTP expression with ASO leads to augmented insulin signaling in liver and fat in DIO mice. DIO mice were fasted over night and then challenged with an i.p. bolus injection of saline or insulin after treatment with control ASO or LMW-PTP ASO for 2 weeks. LMW-PTP ASO treatment enhanced insulin stimulated tyrosine phosphorylation levels of IR-β subunits in liver (pY-IRβ; A) and serine^{473} phosphorylation levels of Akt in both liver and fat (pAkt^{Ser473}; B & D), and lowered basal levels of pY-IRβ in fat (C). Data and representative blots presented were from samples of at least 3 mice for each treatment with repeats of analysis. *P < 0.05, **P < 0.01.

Fig 5. Suppression of LMW-PTP expression with ASO leads to augmented insulin signaling in liver and fat in ob/ob mice. After treatment with LMW-PTP ASO or saline in ob/ob mice for 4 weeks, mice were fasted overnight and then challenged with an i.p. bolus injection of saline or insulin. ASO treatment enhanced the levels of tyrosine-phosphorylated IRβ subunits (pY-IRβ) but not the total levels of IRβ subunits in liver (A; each lane represents 3 pooled samples) and fat (data not shown), which resulted in increased IRS-1 and IRS-2 associated PI3-kinase activities in liver (B & C) and fat (D), and increased serine^{473} phosphorylation levels of Akt (pAkt^{Ser473}) in liver (E) and fat (data not shown). Data and representative figures for b through e were from samples of at least 3 mice for each treatment with repeats of analysis. *P < 0.05, **P < 0.01.
Reduction of LMW-PTP improves insulin sensitivity

Table 1  Body weight (BW), plasma cholesterol (Chol), triglyceride (TG) levels and transaminases (AST and ALT) activities, and metabolic rate (VO₂) in mice

|                      | Saline  | Control ASO | LMW-PTP ASO |
|----------------------|---------|-------------|-------------|
| **DIO mice (n = 6)** |         |             |             |
| BW (g), 0 weeks      | 39.8 ± 1.3 | 39.4 ± 0.6  | 39.8 ± 0.7  |
| BW (g), 5 weeks      | 39.9 ± 1.4 | 39.2 ± 1.0  | 38.4 ± 0.4  |
| Chol (mg/dl), 6 weeks| 183.7 ± 6.7 | 181.4 ± 7.3 | 135.8 ± 9.2*|
| AST (IU/L)           | 40.7 ± 5.6 | 39.4 ± 4.9  | 36.3 ± 2.1  |
| ALT (IU/L)           | 62.4 ± 7.3 | 64.1 ± 4.3  | 63.9 ± 3.3  |
| **ob/ob mice (n = 5)** |       |             |             |
| BW (g), 0 weeks      | 36.3 ± 0.4 |             | 38.4 ± 0.8  |
| BW (g), 5 weeks      | 54.5 ± 0.8 |             | 55.9 ± 0.9  |
| Chol (mg/dl), 4 weeks| 182.9 ± 61.0 |           | 189.7 ± 47.3|
| TG (mg/dl), 4 weeks  | 168.0 ± 3.1 |             | 75.0 ± 5.4**|
| AST (IU/L)           | 167.0 ± 9.0 |             | 111.0 ± 7.0 |
| ALT (IU/L)           | 264.0 ± 20.0|             | 198.0 ± 16.0|
| VO₂ (ml/kg₀.⁷⁵/h):   |         |             |             |
| In dark              | 921.9 ± 25.5 |             | 906.2 ± 25.0|
| In light             | 875.7 ± 52.0 |             | 838.5 ± 22.2|

Data are expressed as the mean ± SEM. *P < 0.05; **P < 0.01 vs. controls.
Figure 1

Reduction of LMW-PTP improves insulin sensitivity

A

B

C

LMW-PTP mRNA (%)

LMW-PTP Protein (%)

MW (kDa)

Vehicle

LMW-PTP ASO

Insulin

- +

- +

Cont ASO

LMW-PTP ASO

pY-IRβ (arbitrary unit)

Vehicle

LMW-PTP ASO

pY-IRβ (arbitrary unit)

LMW-PTP Protein (%)

pY-IRβ (arbitrary unit)

Vehicle

LMW-PTP ASO

pY-IRβ (arbitrary unit)

**

*
Reduction of LMW-PTP improves insulin sensitivity

Figure 1

D

| Insulin | Cont ASO | LMW-PTP ASO #1 | LMW-PTP ASO #2 |
|---------|----------|---------------|---------------|
| -       | +        | +             | +             |
| +       | +        | +             | +             |

**LMW-PTP**

**pAkt<sup>Ser473</sup>**

**Akt**

Insulin-stimulated pAkt (arbitrary unit)

- Cont ASO +Ins
- LMW-PTP ASO #1+Ins
- LMW-PTP ASO #2+Ins
Reduction of LMW-PTP improves insulin sensitivity

Figure 2

A

Liver
WAT

Saline
Cont ASO
LMW-PTP ASO
Reduction of LMW-PTP improves insulin sensitivity

Figure 2

Reduction of LMW-PTP in liver and WAT tissues:

- **Liver**
  - Saline
  - Cont ASO
  - LMW-PTP ASO

- **WAT**
  - Saline
  - Cont ASO
  - LMW-PTP ASO

Bar graph showing LMW-PTP protein levels (%):

- **Liver**
  - Saline
  - Cont ASO
  - LMW-PTP ASO

- **WAT**
  - Saline
  - Cont ASO
  - LMW-PTP ASO

Graph indicates a significant decrease in LMW-PTP expression in LMW-PTP ASO-treated tissues compared to control groups, suggesting improved insulin sensitivity.
Reduction of LMW-PTP improves insulin sensitivity

**Figure 2**

**C**
- Fed plasma insulin (ng/ml)
- Treatment time (weeks)
- Saline
- Cont ASO
- LMW-PTP ASO
- Lean-saline

**D**
- IPGTT
- Blood glucose (mg/dl)
- Treatment time (weeks)
- Saline
- Cont ASO
- LMW-PTP ASO
- Lean-saline

**E**
- Liver
  - Relative abundance of mRNA (%)
  - G6Pase
  - FAS
  - SCD1
  - DGAT2

- Fat
  - Relative abundance of mRNA (%)
  - HSL
  - FAS
  - DGAT1
  - DGAT2
Reduction of LMW-PTP improves insulin sensitivity

Figure 3

A

B

Saline  LMW-PTP ASO

Liver

WAT

PTP1B

SHP2/SHPTP2

LMW-PTP

LMW-PTP Protein (%)
Reduction of LMW-PTP improves insulin sensitivity

Figure 3

C

Fed plasma glucose (mg/dl)

- - Saline
- - LMW-PTP ASO

Treatment time (days)

Saline
LMW-PTP ASO

D

Fasted plasma glucose (mg/dl)

E

Plasma insulin (ng/ml)

* * *

F

H & E Staining
oil-red O Staining

Saline
LMW-PTP ASO
Figure 4

Reduction of LMW-PTP improves insulin sensitivity

A

| Insulin   | Cont ASO | LMW-PTP ASO |
|-----------|----------|-------------|
| Liver     |          |             |
| pY-IRβ    | ![Image](image1.png) | ![Image](image2.png) |
| Total IRβ | ![Image](image3.png) | ![Image](image4.png) |

B

| Insulin   | Cont ASO | LMW-PTP ASO |
|-----------|----------|-------------|
| Liver     |          |             |
| pAktSer473| ![Image](image1.png) | ![Image](image2.png) |
| Akt       | ![Image](image3.png) | ![Image](image4.png) |

C

| Insulin   | Cont ASO | LMW-PTP ASO |
|-----------|----------|-------------|
| Fat       |          |             |
| pY-IRβ    | ![Image](image1.png) | ![Image](image2.png) |
| Total IRβ | ![Image](image3.png) | ![Image](image4.png) |

D

| Insulin   | Cont ASO | LMW-PTP ASO |
|-----------|----------|-------------|
| Fat       |          |             |
| pAktSer473| ![Image](image1.png) | ![Image](image2.png) |
| Akt       | ![Image](image3.png) | ![Image](image4.png) |
Reduction of LMW-PTP improves insulin sensitivity

**Figure 5**

**A**

|        | Saline | LMW-PTP ASO |
|--------|--------|-------------|
| Insulin| -      | +           |

![Bar graph showing Liver pY-IRβ activity](https://via.placeholder.com/150)

![Western blot showing pY-IRβ and Total IRβ](https://via.placeholder.com/150)

**B**

|        | Saline | LMW-PTP ASO |
|--------|--------|-------------|
| Insulin| -      | +           |

![Western blot showing PIP3](https://via.placeholder.com/150)

![Bar graph showing Liver IRS-2 associated PI3-K activity](https://via.placeholder.com/150)

**C**

|        | Saline | LMW-PTP ASO |
|--------|--------|-------------|
| Insulin| -      | +           |

![Western blot showing PIP3](https://via.placeholder.com/150)

![Bar graph showing Liver pAkt/Akt activity](https://via.placeholder.com/150)

**D**

|        | Saline | LMW-PTP ASO |
|--------|--------|-------------|
| Insulin| -      | +           |

![Western blot showing PIP3](https://via.placeholder.com/150)

![Bar graph showing Fat IRS-1 associated PI3-K activity](https://via.placeholder.com/150)

**E**

|        | Saline | LMW-PTP ASO |
|--------|--------|-------------|
| Insulin| -      | +           |

![Western blot showing pAktSer473 and Total Akt](https://via.placeholder.com/150)

![Bar graph showing Liver pAkt/Akt activity](https://via.placeholder.com/150)
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