Basal Activation of p70S6K Results in Adipose-specific Insulin Resistance in Protein-tyrosine Phosphatase 1B−/− Mice*

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Although protein-tyrosine phosphatase 1B (PTP-1B) is a negative regulator of insulin action, adipose tissue from PTP-1B−/− mice does not show enhanced insulin-stimulated insulin receptor phosphorylation. Investigation of glucose uptake in isolated adipocytes revealed that the adipocytes from PTP-1B−/− mice have a significantly attenuated insulin response as compared with PTP-1B+/+ adipocytes. This insulin resistance manifests in PTP-1B−/− animals older than 16 weeks of age and could be partially rescued by adenosinergic expression of PTP-1B in null adipocytes. Examination of adipose signaling pathways found that the basal p70S6K activity was at least 50% higher in adipose from PTP-1B−/− mice compared with wild type animals. The increased basal activity of p70S6K in PTP-1B−/− adipose correlated with decreases in IR substrate-1 protein levels and insulin-stimulated Akt/protein kinase B activity, explaining the decrease in insulin sensitivity even as insulin receptor phosphorylation was unaffected. The insulin resistance of the PTP-1B−/− adipocytes could also be rescued by treatment with rapamycin, suggesting that in adipose the loss of PTP-1B results in basal activation of mTOR (mammalian target of rapamycin) complex 1 leading to a tissue-specific insulin resistance.

The binding of insulin to the insulin receptor (IR) induces autophosphorylation of the IR on several key tyrosine residues. Tyrosine phosphorylation of the receptor leads to the recruitment of adaptor proteins and further propagation of the insulin signal in the cell. Protein-tyrosine phosphatase 1B (PTP-1B) has been shown to be a negative regulator of insulin signaling by interacting with (1, 2) and dephosphorylating (3, 4) the activated IR. Furthermore, disruption of PTP-1B in mice results in improved glucose tolerance and insulin sensitivity, attributable to increased insulin signaling in the muscle and liver of the null animals (5). The metabolic rate and glucose disposal in PTP-1B−/− animals have also been shown to be improved, mainly through increased glucose uptake in skeletal muscle (6). In addition, liver-specific overexpression of PTP-1B in null animals greatly reduces insulin signaling in the liver, resulting in reduced insulin sensitivity in the whole animal (7), whereas transgenic overexpression of PTP-1B in the muscle of wild type mice results in decreased insulin signaling both in the muscle and the liver (8).

Although the negative regulatory effects of PTP-1B activity on insulin action have been well documented in the muscle and liver, studies on the role of PTP-1B in adipose tissue have yielded some contrary results. Elchebly et al. (5) found that insulin-induced IR phosphorylation was unchanged in the adipose of the PTP-1B−/− mice; these mice were also found to be resistant to age and diet-induced obesity. Both results counter what would be expected if the adipose of the PTP-1B−/− animals were insulin hypersensitive. Typically, insulin promotes glucose uptake and lipid storage while inhibiting lipolysis; if PTP-1B deficiency enhanced insulin signaling in the adipose, one would expect that this should result in increased lipid storage. Furthermore, when PTP-1B is overexpressed in cultured adipocytes, insulin-induced IR and insulin receptor substrate-1 (IRS-1) phosphorylation and insulin-induced phosphatidylinositol 3′-kinase activity are disrupted (9). However, none of these effects disrupted insulin-induced Akt activation or insulin-stimulated glucose uptake (9), suggesting that perhaps PTP-1B has a different role in insulin signaling in adipose.

A number of reports have recently shown that p70S6K, when activated, can down-regulate the insulin signaling pathway through serine phosphorylation of IRS-1 (10, 11). Multiple pathways can lead to the activation of p70S6K including the insulin signaling pathway, and therefore, it has been implicated as a negative feedback regulator of this pathway (12). One direct activator of p70S6K is the rapamycin-sensitive Raptor-mTOR complex, known as mTOR complex 1 (TORC1). TORC1 is activated by insulin and other cytokines as well as excess amino acids and is inhibited by low cellular energy states through the activation of AMP-activated protein kinase (13).

To understand this adipose-specific lack of insulin sensitivity, various components of the insulin signaling pathway as well as other mechanisms involved in regulating insulin signaling...
were examined in the adipose of PTP-1B−/− mice. The data presented here indicate that adipocytes from PTP-1B−/− mice display an age-dependent decrease in insulin responsiveness that is attributable to a reduction in IRS-1 protein levels. The reduced IRS-1 protein levels are the result of an insulin-independent increase in the activity of p70S6K in PTP-1B−/− adipocytes. This suggests that PTP-1B deficiency in adipose causes dysregulation of p70S6K resulting in tissue-specific insulin resistance.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies used were as follows: p70S6K (H-160) antibody from Santa Cruz Biotechnologies; IRS-1 antibody from Upstate Cell Signaling Solutions; Akt, phospho-Akt (Ser-473), phospho-Akt (Thr-380), CREB, phospho-CREB (Ser-133), phospho-glycogen synthase kinase-3 (Ser-478), phospho-Akt (Thr-380), CREB, phospho-CREB (Ser-133), phospho-Akt (Ser-21/9), phospho-mTOR (Ser2448), mTOR and phospho-p70S6K (Thr-389) (1A5) antibodies from Cell Signaling Technology; β-actin antibody (clone AC-74) from Sigma; PTEN and Akt2 antibodies from R&D Systems. Peptides were obtained from California Peptide Research Inc. Radiolabeled glucose and ATP were purchased from Amersham Biosciences. siRNA SMARTools® directed to murine Akt2, PTP-1B and PTEN were obtained from Dharmacon RNA Technologies. Adenoviruses expressing LacZ and PTP-1B were graciously provided by Dr. Barry Goldstein. All statistical analysis was done with a standard one-way analysis of variance and has been adjusted for multiple testing where applicable.

**DNA Content**—Overnight-fasted mice were euthanized, and the inguinal white adipose tissue (IWAT) and interscapular brown adipose tissue (IBAT) were removed. The fat pads were weighed, placed in 2 µl of solubilization buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 7.4, 0.5% Triton X-100) per mg of tissue and homogenized on ice 3 × 10 s. Homogenates were warmed to 37 °C to dissolve the fat. 4 volumes of 0.625 mM NH₄OH were added, and samples were incubated at 37 °C for 10 min. 50 µl of sample were added to 1 ml of dilution buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 7.4). To 50 µl of diluted sample 150 µl of diluted Hoechst 33258 was added (stock: 750 µg/ml water diluted 1:7500 in assay buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 7.4, 0.025 mM NH₄OH, 0.005% Triton X-100)), and fluorescence of the entire samples were read at 30 °C.
centrifuged through 100 μL of dinonyl phthalate (Sigma) in 400 μL of soft microcentrifuge tubes (30 s at 300 × g), and cells above the oil were counted.

3T3-L1 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. The cells were differentiated as described in Frost and Lane (14). Briefly, cells were cultured until 2 days post-confluency (day 0) at which time they were treated in growth media supplemented with isobutylmethylxanthine (0.5 mM), dexamethasone (0.25 μM), and insulin (0.85 μM). At day 2 the isobutylmethylxanthine and dexamethasone were removed, and at day 4 the insulin was removed. On days 6 and 7 cells were trypsinized and seeded into 24-well plates (2–2.5 × 10⁵ cells/well) for transfection. siRNAs were transfected using the TransIT-TKO system (Mirus) as per the manufacturer’s protocol, and the final concentration of siRNA used was 100 nM per well. 2 days post-transfection, cells were washed 3× with Dulbecco’s modified Eagle’s medium (no supplements) and incubated at 37 °C for 3 h. After incubation cells were washed 2× with PBS and preincubated in PBS for 25 min. Then cells were incubated in 1 ml of PBS plus 200 mM insulin at 37 °C for 30 min, then washed once with PBS and incubated in 1 ml of PBS plus 0.1 mM 2-deoxyglucose/2-deoxy-D-[2,6-³H]glucose at 2 μCi/ml for 5 min. Reactions were terminated by rapidly washing cells 3× with ice-cold PBS, cells were lysed with 0.4 ml of 1% SDS, and lysates were counted. Nonspecific deoxyglucose uptake was measured in the presence of 20 μM cytochalasin B and subtracted from each determination to obtain specific uptake.

Akt Activity Assay—Akt activity in adipocyte cell lysates was assayed using the nonradioactive Akt kinase assay kit from Cell Signaling Technologies. After incubation with or without insulin, adipocytes were re-isolated and washed once with KRBH with no BSA. Cells were lysed with an equal volume of 1 × lysis buffer (provided) including Complete™ EDTA-free protease inhibitor mixture (Roche Diagnostics), and lysates were assayed as per the manufacturer’s protocol.

p70S6K Activity Assay—After incubation with or without insulin, adipocytes were re-isolated and washed once with KRBH with no BSA. Cells were lysed with an equal volume of radiolabeled phosphorylation assay buffer (10 mM sodium phosphate, pH 7, 150 mM NaCl, 1% Nonident P-40, 0.1% SDS, 1 mM sodium pyrophosphate, 100 μM pervanadate, 1 tablet of Complete™ EDTA-free protease inhibitor mixture (Roche Diagnostics)) and incubated with anti-p70S6K antibody cross-linked to Protein G-agarose beads (Upstate) with dimethyl pimelimidate (Pierce) (50 μL of beads per 1 ml of lysate) overnight at 4 °C end-over-end. Beads were washed 3× with assay dilution buffer (ADB) (20 mM MOPS, pH 7.2, 1 mM diethiothreitol, 1 mM vanadate, 5 mM EGTA, 20 mM β-glycerol phosphate) plus 150 mM NaCl and once with ADB, then resuspended in equal volume of ADB. 10 μl of immune complex was incubated with 30 μl of assay dilution buffer containing 80 μM peptide substrate plus 10 μl of diluted [γ-³²P]ATP ([γ-³²P]ATP diluted 1:20 in ATP dilution buffer (75 mM MgCl₂, 20 μM cold ATP)) at 37 °C for 60 min. The reaction was stopped by adding 200 μl of 0.75% H₃PO₄, applied to a phosphocellulose filter (96-well Multiscreen, Milipore), and washed 3× with H₂PO₄ and twice with acetone. Filters were dried and counted. The remaining immunocomplex was assayed for p70S6K phosphorylation by Western blot.

RESULTS

Altered Adipose Morphology in PTP-1B Null Mice—We have previously shown that deletion of PTP-1B in mice led to an increase in the insulin sensitivity of these animals, partially attributable to increased insulin-stimulated tyrosine phosphorylation of the IR in muscle and liver (5). Somewhat surprisingly, loss of PTP-1B had no effect on insulin-stimulated IR phosphorylation in the adipose tissue of the null animals. This suggested that unlike the muscle and liver, PTP-1B in the adipose tissue may not be involved in the dephosphorylation of the activated IR or there was compensation by some other PTP. Even though the phosphorylation of the IR in the adipose of PTP-1B−/- animals was unaffected, there was nevertheless a profound effect on the adipose morphology (Fig. 1A). In the PTP-1B−/- mice there was a clear shift toward smaller adipocytes that was maintained whether the animals were on a high fat or chow diets. This shift toward smaller adipocytes in the absence of PTP-1B was also described by Klaman et al. (6).

To estimate the number of cells per fat depot, the DNA content of the inguinal white (IWAT) and the interscapular brown (IBAT) adipose depots was measured. For the IWAT, although there was not a significant difference (p = 0.08) between wild type and PTP-1B−/- mice on chow diet, it was typically higher in the PTP-1B-deficient mice. However, on high fat diet there was about a 3-fold increase in the DNA content of the PTP-1B−/- fat depot compared with wild type depot (Fig. 1B). Furthermore, in the IBAT from the PTP-1B-deficient mice on either diet there was at least a 3-fold increase in DNA content. This would indicate that there was a significant increase in adipocyte proliferation in the fat depots of the PTP-1B−/- animals compared with PTP-1B+/- animals. Consistent with this increased adipocyte proliferation, peroxisome proliferator-activated receptor-γ mRNA levels in IWAT from PTP-1B−/- animals maintained on high fat diets (Fig. 1C) were ~4 times higher compared with PTP-1B+/- animals. In contrast, measuring isolated adipocytes recovered per epididymal fat pads, Klaman et al. (6) reported that there was no significant difference in cell number between PTP-1B−/- and wild type animals. This discrepancy may be due to the different fat depot used for the studies (epididymal versus inguinal) or perhaps reflects a difference in the method used to determine cell number. Alternatively, if these results were for chow fed animals, then there is no discrepancy.

Insulin Resistance in Adipose of PTP-1B−/- Mice—To investigate the physiological consequences of PTP-1B deficiency in the adipose of null animals, various downstream effects of insulin signaling were analyzed. Under normal conditions, insulin has an anti-lipolytic effect on the adipose through inactivation of PKA. However, in 5–6-month-old PTP-1B−/- animals fed ad libitum, there was a 3-fold increase in PKA activity in the white adipose that was not observed in any of the other insulin-sensitive tissues tested (Fig. 2A). The increased PKA phosphorylation in the adipose tissue of the PTP-1B−/- animals, however, does not affect the plasma free fatty acid levels in these...
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To test the insulin responsiveness of the PTP-1B\(^{-/-}\) adipose, insulin-stimulated glucose uptake experiments were performed on adipocytes isolated from PTP-1B\(^{-/-}\) mice. Isolated adipocytes from 16-week-old wild type mice were insulin-responsive and took up glucose in a dose-dependent manner, reaching 4–5-fold over background (Fig. 3A). In contrast, insulin-dependent glucose uptake in adipocytes isolated from 16-week-old PTP-1B\(^{-/-}\) mice was significantly blunted. These adipocytes required 10-times higher concentrations of insulin to achieve any significant glucose uptake and only reached a maximum of 2-fold over background (40% of wild type levels, Fig. 3A). In fact, this result was also observed for all PTP-1B\(^{-/-}\) animals older than 16 weeks to at least 28 weeks, the oldest mouse that was tested. However, PTP-1B\(^{-/-}\) adipocytes from 9-week-old mice displayed similar insulin responsiveness as the PTP-1B\(^{+/+}\) mice in that there was significant glucose uptake in response to treatment with low insulin concentrations, and uptake plateaued at ~4-fold over base line (Fig. 3A). Previously, Klaman et al. (6) reported no difference in insulin-stimulated glucose uptake between adipocytes isolated from PTP-1B\(^{+/+}\) and PTP-1B\(^{-/-}\) animals at 12 weeks of age. Therefore, the results of Klaman et al. (6) and the results presented here both indicate that the age of the PTP-1B\(^{-/-}\) animal has a profound effect on the insulin responsiveness of the adipose. This could explain why there was no significant weight difference between the PTP-1B null and wild type animals in the first few months (6).

To further confirm that a decrease in PTP-1B levels can blunt glucose uptake in adipocytes, insulin-stimulated glucose uptake was measured in differentiated 3T3-L1 cells in which PTP-1B was knocked down using RNA-mediated interference. Cells transfected with a non-targeting control siRNA demonstrated a 10-fold increase in glucose uptake in response to insulin. After siRNA knockdown of PTP-1B, which resulted, on average, in a 60% decrease in mRNA levels and a 50% reduction in protein levels (Fig. 3C), there was a 20% decrease in insulin-stimulated glucose uptake. Similarly, knockdown of Akt2 (protein level knockdown on average 60%, Fig. 3C), which has been shown to be required for proper insulin signaling and glucose uptake, showed a 10% reduction in insulin-stimulated glucose uptake. These results suggest that PTP-1B is not required for glucose uptake, but rather, plays a role in insulin signaling and glucose uptake.

One possible explanation for this tissue-specific increase in PKA activity is that the adipose is not as insulin responsive, and therefore, the basal activation of PKA is due to the low circulating insulin levels.
uptake in the differentiated 3T3-L1 cells (15, 16), resulted in a 30% decrease in insulin-stimulated glucose uptake (Fig. 3B). In contrast, decreasing levels of PTEN (protein level knockdown on average 60%, Fig. 3C), a phosphatase involved in shutting down the insulin signaling pathway, resulted in a 25% increase in glucose uptake, which is consistent with previously published data (17). Taken together, these results suggest that, in contrast to its insulin-sensitizing effects in liver and muscle, loss of PTP-1B has the opposite effect in adipocytes.

Expression of PTP-1B Partially Rescues Insulin-stimulated Glucose Uptake—The reduction in glucose uptake due to PTP-1B knockdown in differentiated 3T3-L1 cells suggested that the reduced insulin responsiveness of PTP-1B−/− adipose may be the result of a direct effect of PTP-1B on glucose uptake rather than an alteration of endocrine signals caused by the global loss of PTP-1B. To verify that this was indeed the case, PTP-1B was reintroduced into isolated adipocytes from 16-week-old null mice in an effort to rescue insulin-stimulated glucose uptake. Adenoviral infection was implemented for the re-introduction of PTP-1B into primary adipocytes since this technique has been described previously to achieve greater than 75% transfection efficiency in these cells (18).

Adipocytes isolated from 16-week-old PTP-1B−/− animals were infected with a PTP-1B-expressing adenovirus. The optimal time for PTP-1B expression and maintenance of glucose uptake was found to be 2 h post-infection, which resulted in PTP-1B levels that were ~5% of wild type levels (Fig. 4, inset). Although longer times of infection resulted in greater PTP-1B expression, these longer incubations of isolated adipocytes resulted in a significant loss of insulin-responsive glucose uptake. Even though only 5% of wild type levels were achieved in the PTP-1B−/− adipocytes, this small amount of expression resulted in a significant increase in insulin-stimulated glucose uptake compared with PTP-1B−/− adipocytes infected with an...
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Reduced IRS-1 Proteins Levels and Akt Activity in PTP-1B<sup>-/-</sup> Adipose—To determine how the loss of PTP-1B results in insulin resistance in adipocytes, pathways downstream of the IR were analyzed. IRSs are substrates of the IR which, under normal conditions, are recruited to and phosphorylated by the insulin-activated receptor. In adipocytes, the major IRS is IRS-1, and it has been demonstrated that disruptions in IRS-1 levels either by knock-out or RNA-mediated interference are detrimental to insulin sensitivity (19, 20). IRS-1 protein levels in the PTP-1B<sup>-/-</sup> adipocytes were assessed and found to be ~40% that of those seen in wild type lysates (Fig. 5A).

To determine the effect of the loss of IRS-1 has on downstream effectors of the insulin signaling pathway, insulin-induced Akt activity was assessed in adipocytes from 16-week-old PTP-1B<sup>+/+</sup> and PTP-1B<sup>-/-</sup> animals. In wild type adipocytes there was a 50% increase in Akt activity after incubation with insulin. However, in PTP-1B<sup>-/-</sup> adipocytes, insulin was unable to stimulate Akt activity, indicating that loss of IRS-1 in the PTP-1B<sup>-/-</sup> adipocytes significantly affected downstream insulin signaling (Fig. 5B, graph). Because fully activated Akt requires phosphorylation at both Thr-308 and Ser-473, insulin-induced phosphorylation at these sites was investigated in wild type and PTP-1B<sup>-/-</sup> adipocytes. In PTP-1B<sup>+/+</sup> and PTP-1B<sup>-/-</sup> adipocytes, insulin-stimulated phosphorylation of Akt on Ser-473 was robust, indicating that it was unaffected by the reduced protein levels of IRS-1; however, there was a slight increase in basal Akt phosphorylation at this site in the PTP-1B<sup>-/-</sup> adipocytes (Fig. 5B, top panel). On the other hand phosphorylation of Akt at Thr-308 is exclusively the result of phosphoinositol-dependent protein kinase-1 activity that is mediated through phosphatidylinositol 3′-kinase activation (for review, see Ref. 21). In this case insulin effectively induced phosphorylation of Akt at Thr-308 in adipocytes isolated from PTP-1B<sup>-/-</sup> animals, whereas the phosphorylation of Akt at Thr-308 in PTP-1B<sup>-/-</sup> adipocytes was severely blunted (Fig. 5B, middle panel). Therefore, the loss of IRS-1 protein in the PTP-1B<sup>-/-</sup> adipose results in a significant attenuation of the insulin signal preventing downstream activation of Akt and resulting in a blunting of glucose uptake.

p70S6K Is Activated in Adipocytes of PTP-1B Null Mice—Although IRS-1 is normally phosphorylated on various tyrosine residues by the activated IR, much recent work has shown that IRS-1 can also be phosphorylated on serine and threonine residues, which can inhibit its adaptor function and consequently inhibit insulin signaling as well (for review, see Ref. 12). One mechanism by which serine/threonine phosphorylation can adversely affect IRS-1 is by targeting it for degradation (22), which could explain the reduction in IRS-1 levels observed in the PTP-1B<sup>-/-</sup> adipocytes.

The activation of p70S6K, a kinase shown to directly affect serine/threonine phosphorylation of IRS-1 and resulting in its degradation (10, 11), was explored as a possible contributing factor in the loss of IRS-1 in the PTP-1B<sup>-/-</sup> adipose. Although p70S6K activity is modulated by many phosphorylation events and through many pathways (23), phosphorylation at Thr-389 is required for kinase activity (24, 25). Therefore, the phosphorylation of p70S6K at this site was examined in adipocytes isolated from PTP-1B<sup>+/+</sup> and PTP-1B<sup>-/-</sup> mice. Isolated adipocytes were treated with or without insulin, after which they were solubilized, and p70S6K was immunoprecipitated. A comparison of untreated PTP-1B<sup>+/+</sup> and PTP-1B<sup>-/-</sup> adipocytes revealed a striking 4-fold increase in phosphorylation of p70S6K in the absence of PTP-1B, suggesting an enhanced basal p70S6K activation. Treatment with insulin resulted in slight increases in phosphorylation of p70S6K in both PTP-1B<sup>+/+</sup> and PTP-1B<sup>-/-</sup> adipocytes (Fig. 6, A and B).

To confirm that the phosphorylation results indicated increased activity, adipocytes isolated from wild type and PTP-1B<sup>-/-</sup> animals were treated with insulin and solubilized to assay for p70S6K activity. In PTP-1B<sup>+/+</sup> adipocytes, p70S6K activity was stimulated by insulin to a maximum of 50% over basal levels, which is consistent with previously reported data (26); however, there was no significant stimulation of p70S6K activity in PTP-1B<sup>-/-</sup> adipocytes (Fig. 6C). However, when the basal p70S6K activities were compared, there was 60% higher activity in the PTP-1B<sup>-/-</sup> adipocytes as compare with PTP-1B<sup>-/-</sup> (Fig. 6D), consistent with the p70S6K phosphorylation data.

p70S6K phosphorylation was also examined in other tissues that are more insulin-sensitive in the PTP-1B<sup>-/-</sup> animals. Although basal p70S6K phosphorylation levels in the liver of PTP-1B<sup>-/-</sup> animals were unaffected, the phosphorylation of p70S6K in the muscle of the PTP-1B<sup>-/-</sup> animals was slightly elevated as compared with wild type. However, this increase in p70S6K phosphorylation in PTP-1B<sup>-/-</sup> muscle was much less

adenoviral control. Glucose uptake in adenovirus PTP-1B-infected adipocytes reached a maximum at more than 3-fold over background, amounting to a 50% increase over adenovirus-control-infected PTP-1B<sup>-/-</sup> adipocytes (Fig. 4).

FIGURE 4. Re-introduction of PTP-1B into PTP-1B<sup>-/-</sup> adipocytes rescues glucose uptake. Adipocytes isolated from PTP-1B<sup>-/-</sup> (WT, ◦) and PTP-1B<sup>-/-</sup> (KO, □ and ◇) mice (>16 weeks old) were infected with adenoviruses expressing either LacZ (Ad-LacZ, ◦ and ◇) or PTP-1B (Ad-PTP-1B, △) for 2 h, and glucose uptake in response to insulin was analyzed as described under “Experimental Procedures.” Inset, whole cell lysates from Ad-LacZ-infected (□) and Ad-PTP-1B-infected (△) PTP-1B<sup>-/-</sup> adipocytes were analyzed for PTP-1B expression by Western blot. A much longer exposure was required for detection of PTP-1B in the adenovirus (Ad) PTP-1B-infected cells; therefore, the section of the blot representing PTP-1B levels from WT cells were excluded due to the overwhelming intensity of the signal. Data are expressed as a percent of the base line (glucose uptake at 0 nm insulin) and are represented as the mean of three independent experiments using pooled isolated primary adipocytes (3–4 animals each) and performed, at minimum, in triplicate. The error bars represent the S.E. where single and double asterisks are p values versus KO + adenovirus-LacZ.
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pronounced relative to that observed in the adipose (Fig. 6, E and F).

Rapamycin Treatment Partially Rescues Insulin-stimulated Glucose Uptake—Phosphorylation of p70S6K at Thr-389 has been shown to be rapamycin-sensitive in vivo (24, 25) and directly mediated by the Raptor-mTOR complex (TORC1) (27). To determine that the basal activation of p70S6K was due to activated TORC1 in the PTP-1B<sup>−/−</sup> adipose, glucose uptake in isolated adipocytes was determined after rapamycin treatment. Isolated adipocytes from both PTP-1B<sup>−/+</sup> and PTP-1B<sup>−/−</sup> mice were treated with 1 h with 25 nM rapamycin or vehicle control (0.01% Me<sub>2</sub>SO) after which the cells were used in a standard glucose uptake experiment. Under these conditions, treatment of wild type adipocytes with rapamycin resulted in a significant attenuation of glucose uptake (Fig. 7A), whereas rapamycin treatment of adipocytes from the PTP-1B<sup>−/−</sup> animals resulted in a 50% increase in the insulin-stimulated glucose uptake over PTP-1B<sup>−/−</sup> adipocytes treated with vehicle alone (Fig. 7A). This increase in insulin-stimulated glucose uptake corresponded with a 30–40% decrease in p70S6K phosphorylation at Thr-389 in the adipocytes from the PTP-1B<sup>−/−</sup> animals after rapamycin treatment (Fig. 7B). This partial rescue of insulin-stimulated glucose uptake is very similar to what was observed when PTP-1B was re-expressed in adipocytes isolated from PTP-1B<sup>−/−</sup> animals and confirms that there is a rapamycin-sensitive component to the loss of insulin signaling in the adipose of the PTP-1B<sup>−/−</sup> animals.

**DISCUSSION**

Under normal conditions insulin action on the adipose promotes glucose uptake through glucose transporter 4 translocation to the membrane and fatty acid uptake through the activity of lipoprotein lipase. Insulin also stimulates lipogenesis in adipocytes, which is mediated by sterol regulatory element-binding protein-1 and results in increased fatty acid synthase expression and activity. In addition, insulin signaling inhibits lipolysis through activation of phosphodiesterase 3b, thus reducing cAMP levels and inactivating PKA (for review, see Ref. 28). Therefore, an insulin-sensitive adipose would be expected to readily accumulate triglycerides, and in fact, one of the side effects of insulin therapy is weight gain (29). In contrast, PTP-1B-deficient mice have enhanced insulin sensitivity but at the same time are also resistant to weight gain (5, 6).

One of the factors that has been suggested to contribute to the obesity resistance of PTP-1B<sup>−/−</sup> mice is that these animals have been shown to have an increased leptin sensitivity (30, 31). This is thought to be due to the negative action of PTP-1B on the leptin signaling pathway in the brain, which has been further supported by recent data showing mice that have a neuronal-specific deletion of PTP-1B are also resistant to diet-induced obesity and show improved insulin sensitivity (32) similar to the whole body PTP-1B knock out. This effect has been partly attributed to an increase in leptin signaling in the
brain but is also, in part, due to a 2-fold increase in leptin levels in these animals (32). However, in the absence of leptin (ob/ob mice), loss of PTP-1B has a weight-reducing effect, suggesting that PTP-1B can regulate obesity in a leptin-independent manner (30, 31). This other factor in prevention of weight gain in PTP-1B-deficient mice is most likely the reduction in insulin sensitivity of the adipose tissue. Support for this is the observation that mice with an adipose-specific deletion of the insulin receptor (FIRKO mice) (33) have a surprisingly similar phenotype to the PTP1B−/− mice. FIRKO mice have a significant reduction in insulin-stimulated glucose uptake in isolated adipocytes and a low fat mass and are protected against age-related and hypothalamic lesion-induced obesity (33). The FIRKO mice results clearly demonstrate that proper insulin signaling in adipose is critical for the development of obesity. The data presented herein show that the PTP-1B−/− mice have a significant reduction in insulin signaling in adipose tissue that is very likely a contributing factor to their obesity resistance.

There is evidence suggesting that the role of PTP-1B on insulin signaling in adipose is different than it is in muscle and liver. Venable et al. (9) have investigated the role of PTP-1B over-expression on insulin signaling in differentiated adipocyte cell lines and have shown that high level PTP-1B overexpression had minimal effects on insulin signaling in adipose cells. In contrast, PTP-1B overexpression in FAO (liver) and L6 (muscle) cell lines, resulted in significantly impaired insulin signaling and glucose metabolism (34). In concurrence with that data, the muscle and liver of the PTP-1B−/− mice show enhanced insulin signaling as opposed to what has been shown in the adipose of these animals (5, 6). Furthermore, with the data presented here we have shown that, in fact, loss of PTP-1B in the adipose is having a negative effect on insulin signaling. The observed tissue specificity could be the result of compensatory mechanisms due to the developmental loss of PTP-1B activity. For example, it could be possible that in muscle and liver there is no replacement for PTP-1B deficiency, whereas in adipose PTP-1B activity could be compensated by another PTP, or alternatively, physiological changes may have occurred in the adipose due to the insulin-sensitive phenotype of the PTP-1B−/− mice. However, the fact that the PTP-1B−/− adipocyte phenotype (i.e. blunted insulin-stimulated glucose uptake) can be acutely mimicked by PTP-1B RNA-mediated interference knockdown in differentiated adipocytes provides support for this hypothesis.
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negatively regulates insulin signaling by dephosphorylation of the activated insulin receptor, in adipose PTP-1B activity seems to positively affect insulin signaling.

We had previously shown that insulin-stimulated phosphorylation of the IR in muscle and liver of PTP-1B$^{-/-}$ mice was significantly enhanced compared with wild type controls, whereas in adipose there was no difference between PTP-1B$^{-/-}$ and PTP-1B$^{+/+}$ mice (5). Similarly, Klaman et al. (6), using hyperinsulinemic-euglycemic clamp studies, demonstrated that the muscle of PTP-1B$^{-/-}$ mice had 75% higher glucose uptake than that of wild type controls, whereas glucose uptake into adipose was no different between the two genotypes. They also showed that insulin-stimulated glucose uptake into isolated adipocytes from 12-week-old mice was no different between PTP-1B$^{-/-}$ and PTP-1B$^{+/+}$ animals. These results demonstrated that the adipose from PTP-1B$^{-/-}$ mice did not have enhanced insulin sensitivity. However, we have now extended these studies and shown that the adipose of PTP-1B$^{-/-}$ mice becomes insulin-resistant in animals older than 16 weeks. This insulin resistance is most likely due to the reduced level of IRS-1 in the PTP-1B$^{-/-}$ adipose which is the result of a basal hyperactivation of p70S6K. Similarly, a number of recent reports have shown that constitutive activation of p70S6K as well as one of its activators, the Raport-mTOR complex, in cells either through continued amino acid stimulation (35) or TSC protein deficiency (36) and in animal models of insulin resistance (11) there is impaired insulin signaling due to increased IRS-1 Ser/Thr phosphorylation and degradation.

Treating the PTP-1B$^{-/-}$ adipocytes with rapamycin was able to partially rescue insulin-stimulated glucose uptake in these cells; however, we also observed a negative effect of rapamycin on insulin-stimulated glucose uptake in isolated adipocytes from PTP-1B$^{+/+}$ animals. In contrast, recent evidence from Tremblay et al. (35) has suggested that rapamycin can potentiate insulin-stimulated glucose uptake in differentiated 3T3-L1 cells by inhibiting the mTOR-dependent negative feedback loop. This may, however, represent a difference between cell lines and freshly isolated primary adipocytes, in which discrepancies in mTOR signaling have previously been reported (37, 38). The slight decrease in insulin response in the presence of rapamycin may also support the hypothesis that some mTOR activity is required for proper insulin signaling through IRS-1 (39).

It is likely that the increase in p70S6K activity in the adipose of the PTP-1B$^{-/-}$ animals is due to increased TORC1 activity.

3T3-L1 adipocytes or that it can be reversed by adenoviral reintroduction of PTP-1B in isolated PTP-1B$^{-/-}$ adipocytes suggests that the adipose phenotype of the PTP-1B$^{-/-}$ mouse is a result of PTP-1B deficiency and not some compensatory mechanism. In fact, unlike muscle and liver, where PTP-1B activity

![FIGURE 6. Basal activation of p70S6K in PTP-1B$^{-/-}$ adipocytes.](Image)

A representative blot of p70S6K immunoprecipitated from lysates of isolated adipocytes from PTP-1B$^{-/-}$ (WT) and PTP-1B$^{-/-}$ (KO) mice that had been treated (+) or without (−) 100 nM insulin before lysis as analyzed by Western blot. The blot was probed for phospho-Thr-389-p70S6K (top panel), and pulldown efficiency was determined by probing for total p70S6K (bottom panel). B, blots were quantified by MultiGauge Version 2.3 (FujiFilm), and phosphorylation was normalized versus total p70S6K. Results are expressed as the mean of four independent experiments using pooled isolated primary adipocytes (three-four animals each), and error bars represent the S.E. Single and double asterisks are p values versus WT at corresponding insulin levels; † are p values versus WT without insulin stimulation. D, same as in C, only basal p70S6K activity is expressed as a percent of WT levels. Results are expressed as the mean and error bars represent the S.E. E, whole tissue lysates were prepared from the livers of four PTP-1B$^{-/-}$ and four PTP-1B$^{+/+}$ mice fed ad libitum on a normal chow diet, p70S6K was then immunoprecipitated from the lysates as described under “Experimental Procedures” and subsequently analyzed by Western blot. The blot was probed for phospho-Thr-389-p70S6K (top panel), and pulldown efficiency was determined by probing for total p70S6K (bottom panel). F, same as in E, except using quadriceps muscles.

![FIGURE 7. Rapamycin partially rescues insulin resistance of the PTP-1B$^{-/-}$ adipocytes.](Image)

A, adipocytes isolated from PTP-1B$^{-/-}$ (WT; • and X) and PTP-1B$^{+/+}$ (KO; ■ and △) mice (>16 weeks old) were treated with either vehicle control (0.01% Me2SO; • and ■) or 25 nM rapamycin (X and △) for 1 h, and glucose uptake in response to insulin was analyzed as described under “Experimental Procedures.” Data are expressed as a percent of the base line (glucose uptake at 0 nM insulin) and are represented as the mean of three independent experiments using pooled isolated primary adipocytes (3–4 animals each) and performed, at minimum, in triplicate. The error bars represent the S.E., where the asterisks are p values for KO + 25 nM rapamycin versus KO + 0 nM rapamycin. B, rapamycin treatment as in A followed by lysis of the adipocytes and immunoprecipitation of p70S6K from those lysates. The immunoprecipitated proteins were analyzed by Western blot for phospho-Thr-389-p70S6K, and pulldown efficiency was determined by probing for total p70S6K. Results are expressed as the mean of three separate experiments using pooled isolated primary adipocytes (three-four animals each), and error bars represent the S.E. The asterisk is the p value for KO + 25 nM rapamycin versus KO + 0 nM rapamycin.

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which may also explain a number of other observations we have described in this issue. For example, the increased adipocyte cell number in the adipose of the PTP-1B−/− mice could be related to the proliferation effect of TORC1 activation and its effect on adipogenesis (13, 40).

Currently, it is unclear how the loss of PTP-1B activity causes increased basal activation of p70S6K. Normally, p70S6K is activated through the TORC1 pathway by various signals including growth factors (i.e. insulin), nutrients, and high cellular energy levels, as measured by ATP/AMP ratios (for review, see Ref. 13). The down-regulation of insulin signaling by p70S6K activation has been suggested to be a negative feedback mechanism (for review, see Ref. 12). However, the adipose-specific p70S6K activation is unlikely due to sustained activation of the insulin receptor in adipose since plasma insulin levels in these animals maintained on normal chow or high fat diet are less than 50% that of the levels in wild type mice (5, 6). Furthermore, when AMP-activated protein kinase phosphorylation was analyzed in adipocytes from PTP-1B+/+ and PTP-1B−/− mice, which is an indication of ATP/AMP ratios, there was no detectable differences observed between the two phenotypes. There were also no discernable differences in basal insulin receptor phosphorylation in the adipose of PTP-1B+/+ and PTP-1B−/− mice during early development of the animals.4

One common checkpoint in modulating TORC1 activity is the TSC1-TSC2 (hamartin-tuberin) complex. TSC2 is a GTPase-activating protein that, when bound to TSC1, will activate the intrinsic GTPase of the small G-protein Rheb, changing it from an active GTP-bound state to an inactive GDP bound state. When GTP-bound, Rheb will bind to and activate mTOR. TSC2 is differentially regulated through serine/threonine phosphorylation by AMP-activated protein kinase, Akt, and through Ras/mitogen-activated protein kinase signaling. The former stimulates the GTPase-activating protein activity of TSC2, whereas phosphorylation by the latter inhibits it (for review, see Ref. 13). TSC2 has also been shown to be phosphorylated on tyrosine (41), although it is unclear whether the tyrosine phosphorylation is required for interaction with TSC1 or if TSC2 interaction with TSC1 is required for tyrosine phosphorylation of the former. The effect of this phosphorylation on TSC2 function is also unclear. Therefore, TSC1-TSC2 may be a possible direct link between PTP-1B and p70S6K, although there is no evidence yet to support p-Tyr TSC2 as a substrate of PTP-1B. Current studies are under way to understand the mechanism involved in the adipose-specific hyperactivation of p70S6K due to the loss of PTP-1B.

Bence et al. (32) also showed that mice with an adipose-specific knockdown of PTP-1B had an apparent increase in diet-induced weight gain, which is somewhat in contrast to what is reported here for whole body PTP-1B deficiency. The reason for this discrepancy is not clear; it could be, as mentioned, a difference between an adipose-targeted knockdown and a complete PTP-1B deficiency. However, it is of interest to note that as described above, a small amount of PTP-1B re-expression into PTP-1B-deficient adipocytes can significantly improve adipocyte insulin sensitivity. In fact, in the mice with the adipose-specific deficiency, there was at most a 50% reduction in the PTP-1B protein levels, which could be sufficient to maintain normal adipose function. Because very little information other than weight gain has been reported for the PTP-1B adipose-specific knockdown mouse, additional studies are necessary to understand how this observation relates to whole body PTP-1B deficiency.

Here we have shown that the loss of PTP-1B results in adipose-specific activation of PKA while leading to a reduction in insulin-induced Akt activation and insulin-stimulated glucose uptake. These results point to an adipose-specific insulin resistance in the PTP-1B−/− animals attributable to basal hyperactivation of p70S6K resulting in a reduction of IRS-1 levels. The reduced insulin signaling in the adipose of the PTP-1B−/− animals is very likely one of the factors contributing to their lean phenotype.

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