Protein Tyrosine Phosphatase 1B Reduction Regulates Adiposity and Expression of Genes Involved in Lipogenesis

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Protein tyrosine phosphatase 1B (PTP1B) has been implicated as a negative regulator of insulin action. Overexpression of PTP1B protein has been observed in insulin-resistant states associated with obesity. Mice lacking a functional PTP1B gene exhibit increased insulin sensitivity and are resistant to weight gain. To investigate the role of PTP1B in adipose tissue from obese animals, hyperglycemic obese (ob/ob) mice were treated with PTP1B antisense oligonucleotide (ISIS-113715). A significant reduction in adiposity correlated with a decrease of PTP1B protein levels in fat. Antisense treatment also influenced the triglyceride content in adipocytes, correlating with a downregulation of genes encoding proteins involved in lipogenesis, such as sterol regulatory element-binding protein 1 and their downstream targets spot14 and fatty acid synthase, as well as other adipogenic genes, lipoprotein lipase, and peroxisome proliferator-activated receptor γ. In addition, an increase in insulin receptor substrate-2 protein and a differential regulation of the phosphatidylinositol 3-kinase regulatory subunit (p85α) isoforms expression were found in fat from antisense-treated animals, although increased insulin sensitivity measured by protein kinase B phosphorylation was not observed. These results demonstrate that PTP1B antisense treatment can modulate fat storage and lipogenesis in adipose tissue and might implicate PTP1B in the enlargement of adipocyte energy stores and development of obesity. Diabetes 51:2405–2411, 2002

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2′MOE, 2′-O-(2-methoxy)-ethyl; ASO, antisense oligonucleotide; FAS, fatty acid synthase; FFA, free fatty acid; IR, insulin receptor; IRS, insulin receptor substrate; LPL, lipoprotein lipase; PI, phosphatidylinositol; PKB, protein kinase B; PPAR, peroxisome proliferator-activated receptor; PTP1B, protein tyrosine phosphatase 1B; SREBP, sterol regulatory element-binding protein.

the disruption of the leptin signaling pathway (such as ob/ob and db/db mice and fa/ta rats) exhibit disregulated lipogenesis that cannot be normalized by nutritional or pharmacological treatments (11). One of the transcription factors that has been shown to regulate the expression of several key genes of fatty acid and triglyceride metabolism is sterol regulatory element-binding protein (SREBP)1 (12). The family of SREBP-responsive genes, first limited to the LDL receptor and genes involved in cholesterol synthesis, has been expanded to include several additional genes that control lipid metabolism, such as fatty acid synthase (FAS) (13), acetyl-CoA carboxylase (14), glycerol-3-phosphate acyltransferase (15), leptin (16), and lipoprotein lipase (LPL) (17), which are important for adipose tissue metabolism. SREBPs have also been implicated in the enlargement of adipocyte energy stores and obesity (13).

In this study, we show a decrease in adiposity in animals treated with the PTP1B ASO that correlated with a down-regulation of SREBP1 and other lipogenic genes, suggesting that PTP1B reduction in fat might have a primary role in inhibiting lipogenesis and the development of obesity in these animals.

Interestingly, no increase in insulin-stimulated protein kinase B (PKB) phosphorylation in fat was detected in these animals, although an increase in IRS-2 protein as well as a differential regulation of phosphatidylinositol (PI) 3-kinase splice variants were observed.

**RESEARCH DESIGN AND METHODS**

Identification of ASO inhibitors. Rapid throughput screens for identifying ASO inhibitors selective against PTP1B were performed with 20-base chimeric ASOs in which the first and last five bases had a 2′O-(2-methoxy)ethyl (2′MOE) modification (18). The 2′MOE modification increases binding affinity to complementary RNA sequences and increases resistance to nucleases. These ASOs also have a phosphorothioate backbone and use an RNase H-dependent mechanism for activity. Initial screens were conducted against rat PTP1B, and 10 ASOs were identified as hits, all of which targeted a similar binding site within the coding region of the PTP1B mRNA. Subsequently, a series of in vitro characterization experiments were performed in primary rat and mouse hepatocytes, in which ISIS-113715 was consistently identified to be the most potent and specific oligonucleotide in reducing PTP1B mRNA levels. ISIS-113715 hybridizes to PTP1B mRNA at nucleotides 862–882 in the coding sequence.

Animal care and treatments. Six- to seven-week-old ob/ob mice (Jackson Laboratories, Bar Harbor, ME) were acclimated to the animal research facilities for 5 days. The following investigations were conducted in accordance within Institutional Animal Care and Use Committee guidelines. Animals were housed and maintained on mouse diet (ob/ob Labdiets, St. Louis, MO) ad libitum.

After acclimation, the ob/ob mice were weighed and tail-snip glucose levels were determined by the glucose oxidase method (Precision G glucose meter; Abbott Laboratories, North Chicago, IL). The animals were randomized to the various treatment groups based on plasma glucose levels and body weight. Baseline plasma insulin samples were taken from a subset of the animals representing each treatment group once randomized (n = 10 ob/ob and n = 10 lean littermates) (ELISA [enzyme-linked immunosorbent assay]; ALPCO Diagnostics, Windham, NH). Six-week treatment groups (n = 10/treatment) were 25, 2.5, and 0.25 mg/kg ob/ob PTP1B ASO and saline. All mice were dosed twice per week intraperitoneally. At the end of each week, tail bleed glucose and insulin (ob/ob only) levels as well as body weight were determined under nonfasting conditions by 10:00 a.m. (as described above). At the end of the studies, epididymal fat pads were frozen immediately in liquid nitrogen for further analysis.

Insulin challenge. Insulin (2 units/kg in 0.1% BSA) or saline control was given intraperitoneally after an overnight fast. Tissue samples from fat (0, 1, and 5 min) were taken under both saline and insulin-stimulated conditions (n = 4/treatment/time point). Subgroups of saline- or antisense-treated (25 mg/kg) mice were within each challenge (saline and insulin).

**Fat triglyceride content.** The triglyceride content of fat pads was measured as previously described (19).

Tissue extract preparation and immunoblotting techniques. Tissues were sonicated (using a Branson 450 Sonifier) in lysis buffer containing 20 mmol/l Tris - HCl (pH 7.4), 1% Triton X-100, 10% glycerol, 150 mmol/l NaCl, 2 mmol/l EDTA, 25 mmol/l β-glycerophosphate, 20 mmol/l sodium fluoride, 1 mmol/l sodium orthovanadate, 5 mmol/l sodium pyrophosphate, 10 μg/ml leupeptin, 1 mmol/l benzamidine, 1 mmol/l 4-(2-amoethoxyl) benzenesulfonfluoride hydrochloride, and 1 mmol/l microcystin and rocked for 40 min at 4°C. Detergent-insoluble material was sedimented by centrifugation at 12,000g for 10 min at 4°C. Cell lysates proteins (50 μg protein) were separated by SDS/PAGE on 10 and 7.5% gels. Proteins were transferred from the gel to nitrocellulose sheets and blocked in 5% milk. The blots were probed with various primary antibodies as follows: anti-PTP1B, anti-IRS-1 (PH domain), anti-IRS-2, anti-phospho-AKT (Ser473), anti-phospho-tyrosine antibodies (Upstate Biotechnology, Lake Placid, NY), anti-IRβ antibody (Transduction Laboratories, San Diego, CA), and phospho-PKB antibody (New England Biolabs, Beverly, MA) according to the recommendations of the manufacturer.

The proteins were detected by enhanced chemiluminescence with horseradish peroxidase–labeled secondary antibodies (Amersham, Piscataway, NJ). The intensity of the bands was quantified with a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

**RNA preparation.** RNA was prepared by grinding ~100 mg fat tissue in 1 ml TRIzol reagent, and analysis was done according to the Affymetrix (Santa Clara, CA) protocol. Briefly, the RNA from four mice in PTP1B ASO–treated or control groups was pooled using equal amounts, making a total of 20 μg RNA. cRNA was prepared using the Superscript Choice protocol from Gibco BRL Life Technologies (Rockville, MD) (cat. no. 18090-019). The protocol was followed with the exception that the primer used for the RT reaction was a modified T7 primer with 24 thymidines at the 5′ end. The sequence was 5′GGCCAGCTAATGTTACAGCTATATAAGGAGCCTGG(dT)24 3′. After this, labeled cRNA was synthesized according to the manufacturer’s instructions from the cDNA using the Enzo (New York, NY) RNA Transcript Labeling Kit (cat. no. N9010S). Approximately 20 μg cRNA was then fragmented in a solution of 40 mmol/l Tris-acetate, pH 8.1, 100 mmol/l KOac, and 30 mmol/l MgOac at 94°C for 35 min. Labeled cRNA was hybridized to the Affymetrix GeneChip Test2 Array to verify the quality of labeled cRNA. After this, cRNA was hybridized to the Affymetrix MU11K A and B chip. The cRNA was hybridized overnight at 45°C. The data were analyzed using Affymetrix GeneChip version 3.2 software and Spotfire.Net version 5.0. The results are an average of the two experiments.

For microarray experiments on cDNA-based chips (Incyte Genomics, Palo Alto, CA), total RNA was isolated and amplified as described. In vitro transcription was performed using a MEGAScript T7 Kit (Ambion, Austin, TX) according to the manufacturer’s protocol. Amplified antisense RNA was purified with an RNaseasy mini-kit and protocol (Qiagen, Valencia, CA) and then eluted in a volume of 60 μl RNase-free water. The RNA was quantified by fluoroscence using Ribogreen dye (Molecular Probes, Eugene, OR), and the integrity of the sample was assessed by separation on an Agilent 2100 Bioanalyzer, a high-resolution electrophoresis system (Agilent Technologies, Palo Alto, CA). RNA was submitted to Incyte Genomics (Palo Alto, CA) for fluorescent labeling and hybridization to the Mouse Gen 2 microarray. After scanning and data extraction, the results were exported to the GEMTools database for further gene expression analysis.

**Real-time PCR analysis.** Real-time PCR was performed using the Taqman EZ RT-PCR Core Reagents kit (Perkin Elmer part no. N808-0236). For the analysis, 100 ng total RNA was used. The reactions were done in triplicate. The probe sequences for FAS mRNA were TGCATGACAGATATTGCAGACAAGAGA-forward probe; CTCTTCCTTACAGATTGGTCACCA-reverse probe; and AGCT GCGCAGCTCACGTTGCA Taqman probe. The probe sequences for spot14 mRNA were CCCGGTTTACCTGCTGACTT-forward probe; CTCTGG TGTGCTTCCGACTT-reverse probe; and CAGGCTCTTACATCTCATTACCCCCCA-Taqman probe.

**Statistical analysis.** Statistical evaluation was performed via one-way ANOVA and t tests where appropriate using InStat (GraphPad Software, San Diego, CA). The level of significance was P < 0.05 (two-sided test).

**RESULTS**

**Effect of PTP1B reduction on body weights and epididymal fat pads.** The anti-PTP1B 2′MOE–modified phosphorothioate oligonucleotides (ASOs) were characterized and selected for follow-up studies as previously described. Insulin-resistant hyperglycemic obese (ob/ob) mice were treated with 25, 2.5, or 0.25 mg/kg PTP1B ASO two times
per week for 6 weeks. Plasma glucose levels were reduced by 25% (192 ± 21 mg/dl) and 49% (131 ± 3) with 2.5 and 25 mg/kg PTP1B ASO, respectively (saline control = 255 ± 17 mg/dl), whereas insulin levels were reduced by 77% (6.7 ± 1 ng/ml) with the 25-mg/kg PTP1B ASO dose only (saline control = 28.9 ± 4.2 ng/dl). Overall, there was no significant effect of PTP1B ASO treatment on triglycerides and free fatty acid (FFA) plasma levels. At the completion of the study, epididymal fat pads were obtained, homogenized, and analyzed by immunoblotting. PTP1B protein expression in epididymal fat pads was decreased by 33, 46, and 61% by treatment with 0.25, 2.5, and 25 mg/kg PTP1B ASO, respectively.

No significant difference in body weight was observed between animals treated with saline or those treated with 0.25 or 2.5 mg/kg PTP1B ASO over the duration of the study. However, the 25-mg/kg group gained 15% less weight at 5 weeks of treatment (Fig. 1A) without significant effect in the estimated food intake. Growth rate per week was not different between treatments at lower doses, but a small but significant difference was found with the highest dose of antisense treatment (25 mg/kg) at 5 weeks (Fig. 1B).

To investigate whether the decrease in body mass in the group of animals treated with the higher concentration of antisense could be the consequence of reduced fat stores, epididymal fat pads were obtained and weighed. Epididymal fat weight was unchanged with the lower dose treatment (0.25 mg/kg), and a minimal effect was detected with 2.5 mg/kg ASO (Fig. 2). In contrast, fat weight was significantly reduced by 42% in animals treated with 25 mg/kg ASO (P < 0.05, normalized to brain weight) at 6 weeks of treatment.

Expression of SREBP1 and downstream target genes are downregulated in fat from PTP1B antisense-treated ob/ob mice. Because PTP1B ASO-treated mice showed a decrease of adiposity, and in order to investigate whether the expression of genes coding proteins involved in lipid metabolism were altered by PTP1B ASO treatment, an integrated gene expression study using fat samples was performed. Total RNA was isolated from epididymal fat from ob/ob mice treated with or without 25 mg/kg PTP1B ASO and analyzed by oligonucleotide or cDNA-based microarray, as described in RESEARCH DESIGN AND METHODS. PTP1B gene expression was reduced 2.7-fold by ASO treatment. PTP1B antisense resulted in a coordinate decrease in the expression of genes involved in fatty acid homeostasis (Table 1). Interestingly, PTP1B ASO treatment decreased expression of the transcription factor SREBP1, which regulates several genes involved in lipo-
genesis in mature adipocytes (12). We also found down-regulation of expression of several SREBP1 target genes involved in fatty acid synthesis, including spot14, ATP citrate-lyase, FAS, and steroylCoA desaturases as well as LPL and peroxisome proliferator-activated receptor (PPARγ), a member of the nuclear hormone receptor family of ligand-activated transcription factors that plays a pivotal role in fat cell differentiation (Table 1). The changes in gene expression of some of these genes were confirmed by hybridization to the Mouse Gem 2 microarray at Incyte Genomics (Table 1) and using quantitative PCR.

### TABLE 1
Differentially expressed genes in fat from ob/ob mice treated with 25 mg/kg PTP1B antisense oligonucleotide

<table>
<thead>
<tr>
<th>Gene lipid metabolism</th>
<th>Affymetrix</th>
<th>Incyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroyl CoA desaturase (scd1)</td>
<td>−1.7</td>
<td>−8.65</td>
</tr>
<tr>
<td>Steroyl CoA desaturase (scd2)</td>
<td>−3.4</td>
<td>−2.5</td>
</tr>
<tr>
<td>Glutathione-S transferase</td>
<td>−3.2</td>
<td>−3.6</td>
</tr>
<tr>
<td>FAS</td>
<td>−2.5</td>
<td>−3.9</td>
</tr>
<tr>
<td>Glycerol 3-P acyltransferase</td>
<td>−2.1</td>
<td>−2.1</td>
</tr>
<tr>
<td>LPL</td>
<td>−2.3</td>
<td>ND</td>
</tr>
<tr>
<td>ATP citrate-lyase</td>
<td>−1.8</td>
<td>ND</td>
</tr>
<tr>
<td>Pyruvate decarboxylase</td>
<td>−2.1</td>
<td>−3.1</td>
</tr>
<tr>
<td>Spot14</td>
<td>−8.2</td>
<td>−6.6</td>
</tr>
<tr>
<td>Hormone-sensitive lipase</td>
<td>−2.2</td>
<td>−3.65</td>
</tr>
<tr>
<td>Transcription factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADD1/SREBP</td>
<td>−2.7</td>
<td>ND</td>
</tr>
<tr>
<td>Adipocyte-specific genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPARγ</td>
<td>−2.5</td>
<td>−2.1</td>
</tr>
</tbody>
</table>

The fold changes were determined by microarray hybridization pools (n=3) from four mice. ND, nondetectable or low signal-to-noise ratio.

FIG. 3. Reduction of SREBP1 target genes, spot14, and FAS by PTP1B ASO treatment. Total RNA isolated from epididymal fat pads of ob/ob mice treated with different concentrations of PTP1B ASO was analyzed by murine 11K oligonucleotide microarray (Affymetrix) (A). The expression level of FAS and spot14 in ob/ob mice treated with 2.5 or 25 mg/kg PTP1B antisense was compared with that in ob/ob mice treated with saline, and fold change of expression in antisense- versus saline-treated mice was calculated using Affymetrix Gene Chip 3.2 software (B) or by qPCR as described in Research Design and Methods. Values are expressed as the means ± SE (n = 4). **P < 0.01 and ***P < 0.001 versus saline-treated animals.

of spot14 and FAS, as measured by qPCR analysis (Fig. 3B). Spot14 and FAS are critical genes involved in fatty acid synthesis from glucose.

Consistent with this finding, the triglyceride content of fat from PTP1B antisense-treated mice was significantly decreased, in correlation with the decreased SREBP1 expression in these mice (Fig. 4).

**PTP1B reduction regulates IRS-2 and PI 3-kinase isoforms without modifying insulin sensitivity in fat.** Although PTP1B has been implicated as a tissue-specific regulator of insulin sensitivity in knock out animals (9) and in the dephosphorylation of IR and IRS-1 after acute

FIG. 4. PTP1B reduction lowers triglyceride levels in epididymal fat. Epididymal fat pads from ob/ob mice treated with saline or with 25 mg/kg PTP1B ASO for 6 weeks were subjected to triglyceride content analysis as described. Values are expressed as the means ± SE (n = 4) *P < 0.01 versus saline-treated animals.
insulin stimulation in vivo (8), it is not clear whether a decrease in PTP1B levels can alter the expression of proteins known to be involved in the insulin signaling pathway in fat. Therefore, the protein expression of IR, IRS-1, IRS-2, and PI 3-kinase isoforms was measured in fat by immunoblotting with specific antibodies. No significant differences in IR or IRS-1 expression were detected in fat from obese animals treated with PTP1B ASO (data not shown). In contrast IRS-2 levels were significantly increased in a dose-dependent manner in fat from ob/ob animals treated with the PTP1B ASO (Fig. 5). In addition, a change in the expression level of splice variants of p85α in fat (Fig. 5) was observed in PTP1B ASO–treated animals. This change was characterized by a reduction in p85α and an upregulation of the p50α splice variant.

To investigate whether an increase in IRS-2 expression and/or differential expression of PI 3-kinase regulatory subunits observed in fat from the PTP1B ASO–treated ob/ob mice could increase insulin sensitivity, an intraperitoneal insulin challenge (2 units insulin/kg) was performed in ob/ob mice previously treated for 6 weeks with saline or the PTP1B ASO (25 mg/kg i.p. twice per week). Fat was extracted at 1 min postinsulin challenge, and general tyrosine phosphorylation of proteins and PKB phosphorylation at Ser-473 was measured. Treatment with PTP1B ASO had no effect on tyrosine phosphorylation of the IR (data not shown) or on basal or insulin-induced PKB phosphorylation (Fig. 5), suggesting that changes in the levels of PTP1B, IRS-2, or p85 splice variants might not be implicated in the insulin sensitization of downstream pathways like PKB phosphorylation in adipose tissue.

**DISCUSSION**

In this study, we have demonstrated that reduction of PTP1B levels induced a decrease in adiposity in association with a decrease in fat triglycerides and a downregulation of the main genes that encode proteins involved in lipogenesis.

Adipose tissue is the primary site of energy storage, building triglycerides in response to nutritional excess and releasing this energy in the form of FFAs and glycerol in times of fasting. The ability to carry out these functions efficiently is dependent on changes in the expression of genes that carry out the lipogenesis and the lipolytic program. Among these are many genes whose products are required for lipogenesis, including FAS, ATP citrate lyase, stearoyl CoA desaturase, and proteins involved in triglyceride biosynthesis (PEPCK and pyruvate carboxylase). SREBP1 belongs to a family of transcription factors involved in tryglyceride and fatty acid metabolism (15,17,20), and a number of these genes have been shown to be regulated at the transcriptional level by SREBP1 (12,16). Interestingly, PTP1B reduction in fat downregulated both SREBP1 and PPARγ gene expression, resulting in a reduction of lipogenic gene expression that should contribute to adipose-
cyte hypertrophy. This result is in agreement with other studies showing that moderate reduction of PPARγ activity observed in heterozygous PPARγ-deficient mice decreases triglyceride content in fat, and direct antagonism of PPARγ reduces lipogenesis in fat and prevents adipocyte hypertrophy (26). In addition, the reduced rate of weight gain and reduced adiposity observed with PTP1B ASO treatment is consistent with previous reports in knock out mice where whole-body fat content reduction was also observed (8,9), and a reduction in obesity might lead to improved insulin action. Similar results have been obtained in normal mice on a high-fat diet treated with the PTP1B ASO (M.B. and B.P.M., unpublished observations). Furthermore, results of magnetic resonance image analysis have demonstrated that abdominal fat and subcutaneous fat storage were reduced by 27 and 16%, respectively, with PTP1B ASO treatment in Zucker fatty rats (27).

Obesity generally involves an increase in both cell size and cell number of adipocytes. SREBP1 may represent a mechanism unifying both (16). It can increase the percentage of cells that differentiate into adipocytes (17) by increasing the transcriptional activity of PPARγ. Hence, chronic overfeeding and its attending elevation of insulin would be expected to result in increased expression and activity of SREBP1. This is likely to affect not only lipid accumulation in fat cells through increased expression of FAS, LPL, and other lipogenic genes but also increased differentiation of adipocytes from precursor cells (16). In the current study, we have shown that treatment with PTP1B antisense reduces the expression of these lipogenic genes and at the same time reduces adipose tissue mass. Although SREBP1 gene expression is upregulated by insulin and glucose in vitro (16,28), the in vivo significance of these factors in SREBP1 regulation is a matter of controversy. It is very likely, however, that in the intact animal, hyperinsulinemia and altered nutritional conditions characteristic of the obese state might induce SREBP1, additionally contributing to altered adipocyte metabolism so that a decrease in plasma insulin levels might lead to a reduction in SREBP1 expression. Yet, PPARγ activators induce an increase in the expression of lipogenic genes and fat mass, while simultaneously acting as insulin sensitizers, decreasing insulin and glucose levels in the animals (29). This is supported by clinical data showing that patients who take synthetic PPARγ activators frequently gain weight (30). Furthermore, heterozygous PPARγ mutant mice exhibit smaller fat stores on a high-fat diet (23,31).

Our results are similar to a study of leptin treatment in which a reduction was observed in the expression of genes encoding lipogenic genes such as SREBP1, spot14, and FAS (32). Thus, mRNA levels of genes involved in fatty acid and cholesterol synthesis are rapidly repressed by leptin administration, in association with an acute decrease in plasma insulin levels and decreased SREBP1 expression (33).

One interesting observation reported in this study was the increase in IRS-2 levels and the differential regulation of p85α splice variants observed in fat from animals treated with PTP1B ASO, whereas no increases in PKB activation in response to insulin were observed. However, improved insulin-stimulated PKB activation in liver obtained from the same animals was detected (data not shown), suggesting that the increase in insulin sensitivity by PTP1B ASO treatment is tissue specific. These results are in agreement with previous studies using PTP1B KO animals in which no effect on glucose transport in fat was found (9) and with other reports showing that overexpression of PTP1B in adipocytes inhibited insulin-stimulated phosphoinositide 3-kinase activity without altering glucose transport or PKB activation (34). However, we cannot discard the possibility that changes in these proteins might affect other important functions in fat other than glucose transport, such as lipolysis or lipogenesis or regulation of genes involved in these functions. It is known that the lack of IRS-2 has little effect on insulin-stimulated glucose uptake in isolated adipocytes of IRS2+/− mice, so IRS-2 probably does not have an essential role in the insulin pathway resulting in glucose transport in adipose tissue (35). Interestingly, a recent study showed that induction of SREBP1 gene expression was found in liver of diabetic insulin-resistant IRS-2−/− mice (35). This observation raises the possibility that IRS-2 expression could be linked to SREBP1 expression. However, it was not clear in that study whether IRS-2 had a direct role in the regulation of lipogenesis.

In summary, our results demonstrate that PTP1B reduction decreased fat depots and triglyceride levels in fat by downregulating important genes known to be involved in lipogenesis. Our observation provides the first evidence of a PTP1B effect in adipose tissue, with a possible implication of PTP1B in the enlargement of adipocyte energy stores and obesity.

**References**

tyrosine phosphatase 1B (PTP1B) antisense treatment in a rodent model of diabetes (Abstract). *Diabetes* 50 (Suppl. 2):A332, 2001


