Protein-tyrosine Phosphatase 1B as New Activator for Hepatic Lipogenesis via Sterol Regulatory Element-binding Protein-1 Gene Expression*

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Like hyperglycemia, postprandial (diet-induced) hypertriglyceridemia is thought to play crucial roles in the pathogenesis of insulin resistant/metabolic syndrome. Sterol regulatory element-binding protein-1 (SREBP-1) is a key transcription factor to induce postprandial hypertriglyceridemia. We found that insulin-resistant rats fed a diet high in fructose showed an increased protein-tyrosine phosphatase 1B (PTP1B) content with strong expression of SREBP-1 mRNA in the liver. To clarify the association of PTP1B with SREBP-1 gene expression, we overexpressed PTP1B in rat hepatocytes, which led to increased mRNA content and promoter activity of SREBP-1a and -1c, resulting in the increased mRNA expression of fatty-acid synthase, one of the SREBP-1-responsive lipogenic genes. Because PTP1B overexpression increased phosphatase 2A (PP2A) activity, we inhibited PP2A activity by expression of its selective inhibitor, SV40 small t antigen and found that this normalized the PTP1B-enhanced SREBP-1a and -1c mRNA expressions through activation of the Sp1 site. These results indicate that PTP1B may regulate gene expression of SREBP-1 via enhancement of PP2A activity, thus mediating hepatic lipogenesis and postprandial hypertriglyceridemia. We demonstrate here a unique serial activation of the PTP1B-PP2A axis as a novel mechanism for the regulation of gene expression in the biosynthesis of triglyceride.

The insulin resistance/metabolic syndrome is characterized by the variable coexistence of hyperinsulinemia, hyperlipidemia, obesity, and hypertension. Many individuals with obesity and insulin resistance suffer from fatty liver (steatosis). Like hyperglycemia, postprandial (diet-induced) hypertriglyceridemia is thought to play a crucial role in the pathogenesis of the insulin resistance/metabolic syndrome.

Several lines of evidence indicate that fatty liver in insulin-resistant states is caused by activation of the sterol regulatory element-binding protein (SREBP)-1c, which is elevated in response to high insulin levels (1, 2). Although insulin resistance is present in peripheral tissues, compensatory hyperinsulinemia is postulated to persistently activate SREBP-1c transcription and cleavage, which increases lipogenic gene expression, enhances fatty acid synthesis, and accelerates triglyceride accumulation in the liver in obese insulin-resistant mice (3–5). However, the reason that expression of hepatic SREBP-1c mRNA remains high in insulin-resistant states is little understood.

We have reported that high fructose feeding causes insulin resistance with increased hepatic SREBP-1 mRNA content (6), and we recently found that, as reported (7), protein-tyrosine phosphatase 1B (PTP1B) abundance is also increased in the liver. PTP1B is a major regulator of insulin sensitivity and body fat content, and deletion of its gene leads to augmented insulin sensitivity and resistance to obesity induced by high fat feeding (8–11). Moreover, at least one genetic polymorphism of PTP1B is associated with several features of the insulin resistance/metabolic syndrome (12). We reported that overexpression of PTP1B in both liver and muscle cells led to insulin resistance (13). In high fructose-fed rats, overexpression of PTP1B may induce hepatic insulin resistance. However, high mRNA expression of SREBP-1 still continues in these rats. Although the magnitude of hyperinsulinemia is relatively less than that in fatless mice (3), it is not known why overexpression of PTP1B fails to suppress insulin-induced SREBP-1 mRNA expression in high fructose-fed rats. Alternatively, it is possible that there may be another mechanism for the continued strong expression of hepatic SREBP-1c mRNA in insulin-resistant states. The coexistent strong hepatic expression of PTP1B and SREBP-1 in high fructose-fed rats led us to postulate that PTP1B may regulate SREBP-1 gene expression. To clarify this issue, in the present study we examined the effect of PTP1B overexpression on SREBP-1 gene expression in rat hepatocytes. We found that PTP1B may enhance SREBP-1 gene expression by up-regulating Sp1 transcriptional activity via an increase in protein phosphatase 2A (PP2A) activity, as a distinct mechanism of insulin resistance.

* The abbreviations used are: SREBP-1, sterol regulatory element-binding protein-1; O/S, Cys(32)°Ser(35); EMSA, electrophoretic mobility shift assay; Erk, extracellular signal-regulated kinase; IRS, insulin receptor substrate; PP2A, protein phosphatase 2A; PTP1B, protein-tyrosine phosphatase 1B; ST, SV40 small t antigen; WT, wild type.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Porcine insulin was kindly provided by Eli Lilly, Inc. Polyclonal anti-PTP1B antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and monoclonal PTP1B antibody was obtained from Oncogene Research Products (San Diego). Anti-IRS-1 antibody and anti-IRS-2 antibody were purchased from Upstate Biotechnology, Inc. Anti-phospho-Akt antibody and anti-phospho-MAP (mitogen-activated protein) kinase antibody were from New England Biolabs (Beverly, MA). Horseradish peroxidase-conjugated phosphotyrosine antibody, insulin receptor antibody, and anti-mouse antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). William’s E medium was obtained from Sigma. Dulbecco’s modified Eagle’s medium and fetal calf serum were obtained from invitrogen. All radioisotopes were obtained from PerkinElmer Life Science. XAR-5 film was obtained from Eastman Kodak Co. All other reagents and chemicals were purchased from Sigma.

**Preparation of Recombinant Adenovirus**—The recombinant adenovirus containing the cDNA encoding the rat PTP1B wild-type (WT) and the Cys319/Ser315 mutant (CS1) were prepared as described (13, 14). Plasmid encoding small t antigen (pCMV5-small t) was a gift from Dr. Marc C. Mumby (University of Texas Southwestern Medical Center, Dallas). (15). PP2A exists primarily as a heterotrimer consisting of a catalytic subunit (C), a scaffolding subunit (A), and one of a variety of regulatory subunits (B). The C subunit is always associated with the A subunit. A number of variable B subunits can bind to this dimeric core, and these B subunits affect enzymatic activity and substrate specificity of PP2A. Small t antigen is known to specifically inhibit PP2A by binding to the regulatory subunit, interfering with the ability of PP2A to bind to B subunits that determine its cellular substrates (15). Adeno vector encoding small t antigen was generated using the Adeno-X Expression System (Clontech, Palo Alto, CA). Cells were infected at 5–10 multiplicities of infection for 1 h unless otherwise indicated and incubated for 56 h at 37°C in 5% CO2 and the appropriate medium with 2% heat-inactivated serum followed by incubation in the starvation medium required for the assay (13).

**High Fructose Feeding**—Six-week-old male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) were housed in an environmentally controlled room with a 12-h light/dark cycle. The animals were divided into a normal diet (control) and a diet high in fructose (oriental Japan) consisting of 58% carbohydrate (no fructose), 12% fat, and 30% protein (energy percent of diet). The diet high in fructose (oriental Japan SLC, Shizuoka, Japan) were housed in an environmentally controlled room with a 12-h light/dark cycle. The animals were divided (6). Cells were plated on 100-mm rat collagen dishes (Asahi Techno Glass, Chiba, Japan) and cultured in William’s E medium supplemented with 10% fetal calf serum, 100 μM penicillin, 100 μM streptomycin (6). Fao hepatoma cells, kindly provided by Dr. C. R. Kahn (Joslin Diabetes Center, Boston), were cultured in Dulbecco’s modified Eagle’s medium.

**Western Blotting**—Each liver was homogenized in a solubilizing buffer containing 20 mM Tris, 1 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 50 mM NaF (pH 7.5) for 30 min at 4°C. The tissue lysates were centrifuged to remove insoluble materials. Each sample (20 μg protein/lane) was denatured by boiling in Laemmli sample buffer containing 100 μM dithiothreitol and resolved by SDS-PAGE. The gels were transferred to nitrocellulose by electroblotting in Towbin buffer containing 0.02% SDS and 20% methanol. Membranes were blocked and probed with specific antibodies. Blots were then incubated with horseradish peroxidase-linked second antibody followed by chemiluminescence detection as described (13). Northern Blot Analysis—Total RNA was isolated with Trizol reagent (Invitrogen), and 20-μg RNA samples were run on a 1% agarose gel containing formaldehyde and transferred to a nylon membrane (Nytran N, Schleicher & Schuell). The cDNA probes were prepared by reverse transcriptase-PCR amplification from total RNA isolated from the rat liver as reported (6). Each cDNA probe labeled with [α-32P]dCTP (PerkinElmer Life Sciences) with a labeling kit (Takara, Shiga, Japan) was hybridized to UV cross-linked blots overnight at 88°C in the hybridization buffer Perfecthyb (Toyobo, Tokyo) and washed at 68°C for 40 min with 1× SSC, 0.1% SDS. The blots were exposed to Kodak Biomax MR film at ~80°C. The signal was quantified with a densitometer, and loading differences were normalized to the signal generated with a probe for 18S ribosomal RNA.

**RNase Protection Assay**—SREBP-1 mRNA is produced from a single gene through the use of alternative transcription start sites that produce alternate forms of exon L, designated 1a and 1c (1). Thus, the amount of SREBP-1a and SREBP-1c mRNA was assessed by RNase protection assay according to the method of Shimomura et al. (16). Aliquots of total RNA (10 μg) from each sample were incubated with the SREBP-1 cRNA probe or with a cDNA probe for the mRNA of β-actin. After digestion with RNase A/T1, protected fragments corresponding to SREBP-1a and SREBP-1c were quantified.

**Measurement of Luciferase Reporter Gene Activity**—The 5'-flanking region of rat Srebpf-1c was amplified by PCR using rat genomic DNA (17). The resultant PCR product was inserted upstream of the luciferase gene in the pGL3-basic reporter vector (Promega, Madison, WI) to form pGL3-SREBP-1c (0.4 kb). A fragment composed of ~0.9 kb of a human SREBP-1c gene promoter and ~0.5 kb of a human PPARγ promoter was inserted upstream of the luciferase gene in the pGL3-basic reporter vector to form pGL3-SREBP-1a (0.9 kb).

For the luciferase reporter gene assay, transient transfection into Fao hepatoma cells was performed with SuperFect in accordance with the manufacturer’s instructions. Expression plasmids for WT, mutated PTP1B, small t antigen, or control vector without insert were cotransfected with the reporter plasmid and pRL-TK. Luciferase activity was assayed using the Dual-Luciferase Reporter Assay System as described (19).

**PP2A Phosphatase Activity**—Phosphatase activity was measured using nitro-para-nitrophenyl phosphate as a substrate as described (20). Briefly, starved rat hepatocytes were stimulated with insulin for 30 min at 37°C and lysed. Clarified supernatants were incubated with anti-PP2A antibody and protein G-agarose for 2 h at 4°C. The immunoprecipitates were washed and incubated with 900 μg/ml para-nitrophenyl phosphate for 30 min at 30°C. The amount of para-nitrophenol produced was determined by measuring the absorbance at 405 nm.

**Electrophoretic Mobility Shift Assay (EMSA)**—EMS was performed using radiolabeling double-stranded oligonucleotide corresponding to the Sp1 consensus sequence (Promega). The protein-DNA binding reaction was performed at room temperature for 20 min in a volume of 20 μl. The reaction mixture contained 5 μg of nuclear protein extract, 1 mg of poly(dI-dC) (10 μM Hepes [pH 7.9], 60 mM KCl, 1 mM EDTA, 7% glycerol, and 100,000 cpm labeled probe. After the incubation, samples were loaded onto 5% polyacrylamide gels in 0.25 mM Tris borate-EDTA buffer and run at 150 V for 1.5 h. The gels were dried, and the bands were visualized by autoradiography. For competition assay, nonlabeled oligonucleotides were added at a 50-fold molar excess to the reaction mixture before the addition of nuclear extract protein as described (21).

**Generation of Mutant SREBP-1a and SREBP-1c Promoter**—The putative Sp1 binding site in the proximal promoter region of human SREBP-1a gene was mutated using the oligonucleotides 5′-CTCTCT-CAAGGTTGCGCGCCGTACCTATCG-3′ and 5′-GTUTATCTGGCCCG-GGCCTGTTGGAACCTCTTC-3′ as primers in the in vitro mutagenesis reaction. The putative Sp1 binding site in the proximal promoter region of rat Srebpf-1c gene was also mutated using the oligonucleotides 5′-CACCAGGACCGGGGttACGCGGGGCGGCCCTTC-3′ and 5′-CTTCCGGGG-GGCAacct GCGGGGGAGGCCAC-3′ as primers. Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions.

**Statistical Analysis**—Data are expressed as means ± S.E. of four separate experiments unless stated otherwise. Scheffe’s multiple comparison test was used to determine the significance of any differences among more than three groups. p < 0.05 was considered significant.

**RESULTS**

**Coexpression of High Expression of PTP1B and SREBP-1 mRNA in the Liver of High Fructose-fed Rats**—High fructose feeding for 8 weeks induced postprandial hyperinsulinemia
and hypertriglyceridemia in rats as reported previously (6). These rats also revealed several characteristic features of the insulin resistance/metabolic syndrome. Moreover, we found the coexistence of high expression of PTP1B and SREBP-1 mRNA in the liver (Fig. 1).

Overexpression of PTP1B Enhances SREBP-1 mRNA but Impairs Insulin Signaling in Isolated Rat Hepatocytes—We next examined the effect of PTP1B overexpression on both SREBP-1 mRNA expression and insulin signaling in isolated hepatocytes by the adenovirus-mediated gene transfer technique. PTP1B overexpression markedly enhanced SREBP-1 mRNA expression to a level comparable with that seen after insulin stimulation alone. We did not observe any synergic effect of PTP1B on insulin-stimulated SREBP-1 mRNA expression. Furthermore, mRNA expression of fatty-acid synthase, one of the SREBP-1-responsive lipogenic genes, was also increased (Fig. 2a). On the other hand, overexpression of PTP1B led to decreased insulin-induced tyrosine phosphorylation of the insulin receptor and insulin receptor substrates 1 and 2 (IRS-1 and IRS-2), as shown in Fig. 2b. The insulin-stimulated phosphorylation of Akt and Erk was also attenuated in cells overexpressing PTP1B (Fig. 2b). Thus, PTP1B regulates "off"-signaling of insulin action in hepatocytes, as is also reported to occur in Fao hepatoma cells, L6 myocytes, and 3T3-L1 adipocytes (13, 21).

Effect of PTP1B Overexpression on SREBP-1 Gene Expression in Rat Hepatocytes—Although insulin signaling was inhibited by overexpression of PTP1B, its overexpression markedly enhanced SREBP-1 mRNA expression. This PTP1B effect was mediated by tyrosine-phosphatase activity, because a phosphatase-defective PTP1B C/S mutant had no such effect (Fig. 3a). PTP1B overexpression enhanced SREBP-1 mRNA expression in a time- and dose-dependent manner (data not shown). Although insulin selectively increases SREBP-1 mRNA expression (16), RNase protection assay revealed that PTP1B increased the mRNA contents of both SREBP-1a and -1c in rat hepatocytes (Fig. 3b). Consistently, PTP1B overexpression increased transcriptional activities of both SREBP-1a and SREBP-1c promoter-luciferase constructs, but a phosphatase-defective PTP1B C/S mutant was without effect (Fig. 3, c and d). These results indicate that tyrosine phosphatase activity is required for up-regulation of SREBP-1 expression. Identical findings were also observed in Fao hepatoma cells.

PTP1B and Activation of SREBP-1 Expression in Rat Hepatocytes—To clarify the molecular mechanism for PTP1B-induced SREBP-1 gene expression, we selected as a candidate molecule, protein phosphatase 2A (PP2A), which is activated by dephosphorylation of the tyrosine residue and might regulate SREBP-1 gene expression, because catalytic A subunit of PP2A is inactivated by in vitro phosphorylation of Tyr

New Role of PTP1B in SREBP-1 Gene Expression

PTP1B/PP2A Signaling Is Required for Activation of Sp1 Promoter Activities of SREBP-1 Gene—In EMSA, we found that PTP1B overexpression markedly increased nuclear Sp1 binding in both the basal and insulin-stimulated states (Fig. 5a). When the Sp1 binding sites in the SREBP-1a and -1c promoters were mutated from GCGGGG to GCGGTT by PCR mutagenesis, we were unable to detect any stimulation of SREBP-1a and -1c promoter activities by PTP1B overexpression in hepatocytes (Fig. 5b). It is clear that these sites are important for the PTP1B effect on the regulation of the activities of SREBP-1a and -1c promoters (2, 17, 18, 23). Thus, we conclude that activated PP2A may enhance Sp1 transcriptional activity when PTP1B is overexpressed. These results indicate that PTP1B enhances the activity of the SREBP-1 promoter by modulating Sp1 transcriptional activity through increased PP2A activity.

DISCUSSION

PTP1B is a major negative regulator of insulin signal transduction and is associated with the development of insulin resistance (13, 21, 24). Furthermore, we and others have observed that hyperinsulinemia as well as increased carbohydrate intake stimulate its gene expression (6, 24–26).
Moreover, we observed that hepatic PTP1B content was increased by 3-fold in high fructose-fed rats and that exposure of isolated hepatocytes to 25 mM fructose for 8 h led to doubled PTP1B mRNA content (data not shown). Thus, a vicious metabolic cycle may be initiated, in which PTP1B plays a key role. A mouse model lacking PTP1B consistently exhibited augmented insulin sensitivity and was resistant to the development of obesity induced by high fat feeding (10, 11). Therefore, PTP1B is postulated to be an important therapeutic target to reduce insulin resistance (9) as well as resistance to diet-induced obesity.

Postprandial (diet-induced) hypertriglyceridemia is thought to be a central feature of the insulin resistance/metabolic syndrome, and enhanced expression of SREBP-1c mRNA is responsible in part for these abnormalities (1, 2). In insulin-resistant fatless mice, IRS-2 protein has been shown to be diminished, but SREBP-1 expression is continuously activated (4). Thus, despite the presence of insulin resistance in both the peripheral tissues and liver in these mice, the compensatory hyperinsulinemia maintains a high level of SREBP-1c gene transcription (3–5). Therefore, there may be another mechanism for the continued strong expression of hepatic SREBP-1c mRNA in insulin-resistant mice.

We found coexistent strong expression of PTP1B and...
SREBP-1 in rats that were insulin-resistant by a high fructose diet. Overexpression of PTP1B protein has been also observed in insulin-resistant db/db mice, and treatment with antisense PTP1B in db/db mice has been reported to improve hyperglycemia and hyperinsulinemia (27). Furthermore, this antisense treatment in ob/ob mice also ameliorates adiposity and attenuates metabolic syndrome.

**FIG. 4.** Enhanced activities of PP2A and SREBP-1 promoter in hepatocytes overexpressing PTP1B. The effect of PTP1B overexpression on PP2A activity in rat hepatocytes was analyzed (a). Phosphatase activity was measured using para-nitrophenyl phosphate. 5 and 10 multiplicities of infection of adenovirus containing PTP1B (WT5 and WT10) were used. *, p < 0.05, **, p < 0.01 versus controls. The effects of ST overexpression on SREBP-1 mRNA expression were analyzed in rat hepatocytes. Co-expression of ST with PTP1B inhibited SREBP-1 mRNA expression in hepatocytes (b). **, p < 0.01 versus controls. Effects of expression of ST on PTP1B-enhanced promoter activities of both SREBP-1a and -1c were analyzed in Fao hepatoma cells (c and d). **, p < 0.01 versus controls.

**FIG. 5.** Effect of PTP1B overexpression on nuclear Sp1 binding. Using nuclear protein extract from rat hepatocytes overexpressing PTP1B, EMSA was performed using radiolabeling double-stranded oligonucleotides corresponding to the Sp1 consensus sequence (a). For competition, a 50-fold molar excess of unlabeled probe (cold) or anti-Sp1 antibody (Ab) was added to nuclear extracts. The effects of mutation of Sp1 binding site on SREBP-1a and -1c promoter activities were measured in Fao hepatoma cells (b). **, p < 0.01 versus controls.

SREBP-1 in rats that were insulin-resistant by a high fructose diet. Overexpression of PTP1B protein has been also observed in insulin-resistant db/db mice, and treatment with antisense PTP1B in db/db mice has been reported to improve hyperglycemia and hyperinsulinemia (27). Furthermore, this antisense treatment in ob/ob mice also ameliorates adiposity and attend-
transcriptional activity (29, 30), increased Sp1 glycosylation is responsible for increased gene expression in hyperglycemic condition by an enhanced hexosamine pathway (31). However, we did not observe increased glycosylation of Sp1 protein in PTP1B-overexpressed cells (data not shown). PP2A is known to activate Sp1 transcriptional activity in nonhepatic cultured cells (32). SREBP-1 promoter activities were well correlated with PP2A activities. Thus, it is possible that PTP1B may activate SREBP-1 gene expression by enhancing Sp1 transcriptional activity via increased PP2A activity. It has been reported that PP2A dephosphorylates Sp1 protein and increases DNA binding. However, the dephosphorylation site(s) of Sp1 by PP2A has not been determined (29, 30). In the current study, we did not detect any significant changes in phosphorylation levels of Sp1 protein in cells overexpressing PTP1B (data not shown), although subtle change might not have been detectable by the methods used. Thus, the dephosphorylation site(s) by PP2A needs to be determined in further studies.

We propose that the molecular mechanism for the activation of SREBP-1 gene expression by activation of Sp1 transcription factor is through activation of PP2A. We demonstrate here a unique serial activation of the PTP1B-PP2A axis as a novel mechanism for the regulation of gene expression in the biosynthesis of triglyceride. Therefore, PTP1B is a crucial molecule in the pathogenesis of postprandial hypertriglyceridemia and the insulin resistance/metabolic syndrome (Fig. 6). Further in vivo study is necessary to confirm the in vitro findings and to clarify the clinical significance of PTP1B roles in insulin-resistant states.

In summary, we demonstrate here that another important action of PTP1B stimulates SREBP-1 gene expression, hepatic triglyceride synthesis, and postprandial hypertriglyceridemia, even though PTP1B is postulated to be an important target to reduce resistance to insulin and leptin in obesity and other insulin-resistant states (9). We therefore propose that PTP1B represents a novel therapeutic target for the amelioration of postprandial (diet-induced) hypertriglyceridemia through modulation of SREBP-1a and -1c expression in the liver by activation of the PTP1B-PP2A axis.

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