Essential Role of Protein-tyrosine Phosphatase 1B in the Modulation of Insulin Signaling by Acetaminophen in Hepatocytes*

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Background: PTP1B is a negative modulator of insulin receptor-mediated signaling.
Results: Acetaminophen increased PTP1B in hepatocytes and impaired insulin signaling.
Conclusion: PTP1B plays an essential role in the impairment of insulin signaling mediated by acetaminophen in hepatocytes.
Significance: Chronic acetaminophen administration might be associated with insulin resistance in the liver.

Many drugs are associated with the development of glucose intolerance or deterioration in glycemic control in patients with pre-existing diabetes. We have evaluated the cross-talk between signaling pathways activated by acetaminophen (APAP) and insulin signaling in hepatocytes with or without expression of the protein-tyrosine phosphatase 1B (PTP1B) and in wild-type and PTP1B-deficient mice chronically treated with APAP. Human primary hepatocytes, HuH7 hepatoma cells with silenced PTP1B, mouse hepatocytes from wild-type and PTP1B-deficient mice, and a mouse model of chronic APAP treatment were used to examine the mechanisms involving PTP1B in the effects of APAP on glucose homeostasis and hepatic insulin signaling. In APAP-treated human hepatocytes at concentrations that did not induce death, phosphorylation of JNK and PTP1B expression and enzymatic activity were increased. APAP pretreatment inhibited activation of the early steps of insulin signaling and decreased Akt phosphorylation. The effects of APAP in insulin signaling were prevented by suramin, a PTP1B inhibitor, or rosiglitazone that decreased PTP1B levels. Likewise, PTP1B deficiency in human or mouse hepatocytes protected against APAP-mediated impairment in insulin signaling. These signaling pathways were modulated in mice with chronic APAP treatment, resulting in protection against APAP-mediated hepatic insulin resistance and alterations in islet alpha/beta cell ratio in PTP1B−/− mice. Our results demonstrate negative cross-talk between signaling pathways triggered by APAP and insulin signaling in hepatocytes, which is in part mediated by PTP1B. Moreover, our in vivo data suggest that chronic use of APAP may be associated with insulin resistance in the liver.

Metabolic changes associated with chronic use of drugs develop slowly and are not systematically measured in safety protocols, toxicity, or clinical assays (1). For example, anti-inflammatory doses of corticosteroids not infrequently precipitate diabetes in middle-aged or elderly individuals with no history of glucose intolerance. Other notable examples include oral contraceptives, estrogen replacement therapy, cyclophillin, immunosuppressants, protease inhibitors, antipsychotics, etc. (2, 3).

Acetaminophen (APAP)4 has been used for over 40 years as an analgesic in the treatment of acute and chronic pain and represents a valuable first line agent in the pharmacological management of pain (4). According to many international guideline recommendations, APAP is a first choice drug for relieving mild to moderate pain. It is also used in combination with opioids for the treatment of severe pain. Because of its cardiovascular, renal, and gastrointestinal safety, APAP offers several advantages over non-steroid anti-inflammatory drugs (5).

The liver is the organ primarily responsible for the metabolism of APAP. Glucuronidation and sulfation are the primary routes of metabolism, but a small portion of the drug is

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The abbreviations used are: APAP, acetaminophen; PTP1B, protein-tyrosine phosphatase 1B; IR, insulin receptor; IRS, insulin receptor substrate; NAPQI, N-acetyl-p-aminobenzoquinone imine; LDH, lactate dehydrogenase; AUC, area under the curve; EE, energy expenditure.
metabolized through oxidation, by which APAP is converted to an electrophilic metabolite, N-acetyl-p-aminobenzoquinone imine (NAPQI), by cytochrome P450 2E1 (CYP2E1), CYP3A4, and CYP1A2 (6). At therapeutic doses, NAPQI is detoxified by GSH and eliminated in urine or bile as APAP-cysteine, APAP-N-acetylcysteine, and APAP-APAP-GSH (7).

Following high dose exposures to APAP, these routes of metabolism become saturated, GSH is depleted, and NAPQI binds to cysteine residues on proteins, generating high levels of APAP-protein adducts in the liver.

At the molecular level, it has been extensively reported that APAP triggers signaling pathways in hepatocytes such as the activation of JNK (8). Our laboratory recently demonstrated that APAP increased the expression of protein-tyrosine phosphatase 1B (PTP1B) (9). Both JNK and PTP1B negatively interfere with insulin signaling. JNK phosphorylates the insulin receptor substrate 1 (IRS1) at serine 307 and targets IRS1 for proteosomal degradation (10), whereas PTP1B acts upstream of IRS proteins by dephosphorylating the insulin receptor (IR) (11). However, the specific cross-talk between signaling pathways activated by APAP and insulin signaling in the liver has not been evaluated.

The role of PTP1B in modulating hepatic insulin sensitivity has been extensively investigated. Initial studies reported that the tissue specificity responsible for the insulin hypersensitivity of PTP1B-deficient mice was restricted to liver and skeletal muscle (12, 13). The relevance of the liver was demonstrated by attenuation of the enhanced whole body insulin sensitivity by PTP1B re-expression in the liver of PTP1B−/− mice (14). PTP1B expression is elevated in the liver of mice fed with a high fat diet and in aged mice, concomitant with increased hepatic inflammation and steatosis (15, 16). Moreover, liver-specific deletion of PTP1B improves metabolic syndrome and attenuates diet-induced endoplasmic reticulum stress (17, 18).

Based on these data, we hypothesized that APAP-mediated JNK activation and increase of PTP1B elicit negative cross-talk with insulin signaling in hepatocytes. Therefore, the aim of this study was initially to evaluate the impact of APAP in insulin signaling in human and mouse hepatocytes with or without expression of PTP1B. Also, we studied the effect of chronic treatment with APAP on whole body glucose homeostasis and hepatic insulin sensitivity in wild-type and PTP1B-deficient mice.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—FBS and culture media were from Invitrogen. Insulin and APAP were from Sigma (Sigma-Aldrich). Suramin was purchased from Enzo Life Sciences AG (Switzerland). Anti-phospho-JNK (Thr183/Tyr185) (antibody 07-247), anti-phospho-IRS1 (Ser307) (07-248), anti-phospho-IRS1 (Tyr1179) (07-25103-R), anti-IR (sc-711), anti-JNK (sc-571), anti-phospho-IR (Tyr1162/1163) (sc-81500), anti-phospho-IR (Tyr1150/1151) (sc-16646-R), and anti-PTP1B (sc-14021) antibodies were from Santa Cruz (Palo Alto, CA). Anti-IRS-1 (06-248), anti-IRS2 (06-506), anti-p85-PI3K (06-195), anti-PTP1B (07-088), anti-phospho-IRS1 (Ser307) (07-247), anti-phospho-IRS1 (Tyr1179) (07-844), catalase (219010), and anti-phosphotyrosine (clone 4G10; 05-321) antibodies were from Merck Millipore (Merck). Anti-MnSOD antibody (ADI-SOD-111) was from Enzo Life Sciences.

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Cell Culture—Human hepatocytes were isolated by the two-step collagenase procedure from non-tumor areas of liver biopsies from patients submitted to a surgical resection for liver tumors after obtaining patients’ written consent (19). Human Huh7 hepatoma cells were a gift of P. Martin-Sanz (CSIC). The generation of immortalized hepatocyte cell lines from wild-type and PTP1B−/− mice was described previously (20). Cells were grown in DMEM plus 10% heat-inactivated FBS and stimulated with various doses of APAP and insulin.

PTP1B Knockdown in Hepatocytes—Human PTP1B or control (scrambled) shRNA lentiviral particles (Sigma) were used to produce stable knockdown in Huh7 human hepatic cells. Proliferating Huh7 cells were co-incubated with lentiviral transducing particles in culture medium containing Polybrene for 24 h. The cells were cultured with puromycin (5–10 μg/ml), and resistant clones were expanded and examined for PTP1B levels. siRNA oligonucleotide was synthesized by Dharmacon RNAi Technologies for gene silencing of human PTP1B. Huh7 cells were seeded in 6-cm dishes and incubated at 37°C with 5% CO2 overnight. When 40–50% confluence was reached, cells were transfected with 10 nM of PTP1B siRNA or with a scrambled control siRNA following DharmaFECT General Transfection Protocol. After 48 h, cells were used for experiments.

Evaluation of Cellular Viability—Cellular damage was evaluated by measurement of lactate dehydrogenase (LDH) leakage as described (21, 22). Cells were plated in 60-cm-diameter plates at a concentration of 1.5 × 106 per plate. After incubation with APAP, the culture medium (3 ml) was removed and frozen down, and the cells were scraped and collected in 3 ml of PBS. Both culture medium and cells were and frozen down for no longer than 24 h. In the moment of the assay, the medium was thawed, and 10 μl of each sample was deposited in a 96-multiwell plate. The thawed cells were first sonicated to ensure breaking down the cell membrane to release the total amount of LDH. After centrifugation to clear up the samples, 10 μl were placed in a 96-multiwell plate for the assay. To each well containing the sample, 200 μl of a mixture comprising 5 mM pyruvate, 0.35 mM NADH, and 84 mM Tris was added and immediately read at 340 nm for 5 min in a microplate reader (Bio-Tek, Winooski, VT). LDH leakage was estimated from the ratio between the ΔO.D./min in the culture medium and that of the whole cell content. % LDH = ((released LDH/(released LDH + cellular LDH)) × 100.

Determination of GSH—The content of GSH was quantified by the fluorometric assay of Hissin and Hilf (23).

Animal Models—12–14-week-old male PTP1B+/+ (wild type) and PTP1B−/− mice maintained on a C57Bl/6J × 129Sv/J genetic background (24) were treated with APAP dissolved in the drinking water for 6 months. APAP dose was 1.5 mg/day (equivalent to 4 g/day in adult humans). The time of 6 months for the study in mice corresponds to approximately 13 years in humans considering that, according to the United States Life Tables of 2009 (25), life expectancy at birth is 78.5 years. Alanine amino transaminase activity was determined in serum by
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direct measurement with Reflotron test (Roche Diagnostics) every 2 weeks. Body weight and food intake were measured weekly through the study. Animal experimentation was approved by the ethics committee at CSIC.

Glucose, Pyruvate, and Insulin Tolerance Tests—At the end of the APAP treatment, mice were fasted overnight and given an intraperitoneal injection of D-glucose (2 g/kg) for a glucose tolerance test or sodium pyruvate (1.5 g/kg) for a pyruvate tolerance test. Blood glucose was measured before and at 15, 30, 60, 90, 120, and 150 min after glucose injection with an Accu-Check Aviva glucometer (Roche Diagnostics), and the area under the curve (AUC) was calculated (26). Insulin tolerance tests were performed on 4-h fasted mice injected with 0.75 unit/kg of human regular insulin. Blood glucose was measured before and at 15, 30, 60, 90, and 120 min after injection.

Analysis of Energy Expenditure (EE) and Locomotor Activity—EE and home cage activity were assessed using LabMaster metabolic cages from TSE Systems (Bad Homburg, Germany). Mice were individually housed in chambers maintained at 24 °C with free access to chow diet and water with or without APAP. Before the experiment, mice were allowed to adapt to the new environment for 24 h. Measurements were taken every 10 min during the following 24 h. Home cage locomotor activity was determined using a multidimensional infrared light beam system expressed as beam breaks. Fine activity (XF, breaks X-beam, fine movement) measures two consecutive breaks of a single beam of light at the x axis. The counter records the interruption of a light barrier that has been interrupted for the second time in succession without a different light barrier having first been interrupted. The data were analyzed with the Phenomaster software (TSE Systems).

Insulin Signaling Studies—At the end of APAP treatment, 4-h fasted mice were intraperitoneally injected with PBS or 0.75 unit/kg of human regular insulin and sacrificed 15 min later. Livers and skeletal muscle were removed, and total protein extracts were prepared.

Homogenization and Preparation of Tissue Extracts—Frozen tissues were homogenized in 16 volumes (w/v) of ice-cold lysis buffer containing 50 mM Tris-HCl, 1% Triton X-100, 2 mM EGTA, 10 mM EDTA acid, 100 mM NaF, 1 mM Na4P2O7, 2 mM Na3VO4, 100 µg/ml phenylmethylsulfon fluoride, 1 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin. Samples were homogenized in the same lysis buffer using the Brinkman PT 10/35 Polytron. Extracts were kept ice-cold at all times. Tissue extracts were cleared by microcentrifugation at 40,000 × g for 20 min at 4 °C. The supernatant was aliquoted and stored at −70 °C. Protein content was determined by the Bradford method, using the Bio-Rad reagent and BSA as the standard. Protocols for immunoprecipitations and Western blot have been described previously (20).

Liver Histology—Histological analysis was performed by two blinded observers using hematoxilin and eosin-stained sections.

PTP1B Phosphatase Activity—PTP1B phosphatase activity was determined in cellular or liver extracts by measuring phosphatase release using a synthetic monophosphoryoseryl-containing peptide and the malachite green assay (Millipore) as described previously (24).

Quantitative Real Time PCR Analysis and Primer Sequence—Total RNA extraction was performed with TRIzol (Invitrogen). Total RNA was reverse transcribed using a SuperScriptTM III first strand synthesis system for quantitative PCR following the manufacturer’s indications (Invitrogen). Quantitative PCR was performed with an ABI 7900 sequence detector using the SyBr Green method and d(N)6 random hexamer with the primers indicated. The primers used in the real time RT-PCR were the following: PTP1B, forward, 5'-CACCTGCTGGAACACACACTT-3', and reverse, 5'-AAGGTGAACCGGGACAGAGA-3'; and 18s, forward, 5'-TCTGGACACACCT-3', and reverse, 5'-CTTCGGAAAAACGGTGAAG-3'.

Islet Studies—Pancreas sections were immunostained for insulin (Dako, Carpinteria, CA), glucagon (Merck Millipore), and IL6 (sc-1265; Santa Cruz), using the indirect peroxidase method (27). The relative volume of beta and alpha cells was determined as the percentage of pancreatic area occupied by insulin and glucagon immunoreactive cells, respectively, and

FIGURE 1. Effect of APAP in JNK activation, IRS1 Ser307 phosphorylation, and PTP1B expression and activity in human primary hepatocytes. A, human primary hepatocytes were treated with APAP (10 mM) for various time periods. GSH content was determined at 8 and 12 h. The results are expressed as percentage of untreated cells (100%). Released LDH activity was analyzed at 24 h as described under “Experimental Procedures.” B, phosphorylation of IRS1 (Ser307), p85-PI3K (p85), total IRS1, and total JNK1/2 were analyzed by Western blot at early time periods (2–8 h). C, PTP1B, SHP1, and TCPTP mRNA levels (analyzed by RT-PCR), PTP1B protein expression (analyzed by Western blot), and PTP1B enzymatic activity (see “Experimental Procedures”) were determined in primary human hepatocytes treated with 10 mM APAP for 24 h. p85-P13K (p85) antibody was used as loading control. Representative autoradiograms are shown. The results of PTP1B enzymatic activity are expressed as pmol of released phosphate/min/µg protein. **, p < 0.01 APAP-treated versus untreated hepatocytes (n = 3 independent experiments performed in duplicate).
that occupied by total pancreatic cells (28). Images of stained sections were analyzed by automated image analysis software (Histolab; Microvision Instruments, Evry, France). The blood was collected from overnight fasted mice, and serum insulin was determined by radioimmunoassay (27).

**Determination of APAP-Protein Adducts**—Analysis of NAPQI covalently bound to cysteine groups on proteins was measured in homogenized liver samples. Following gel filtration, samples were treated with protease digestion and the resulting peptides were analyzed by HPLC with electrochemical detection for APAP-Cys as described previously (29).

**Data Analysis**—The data are expressed as means ± S.E. Comparisons between groups were made using Student’s t test (two-tailed) or by one-way analysis of variance with Turkey’s post test. Differences were considered to be statistically significant at \( p < 0.05 \).

**RESULTS**

**Effect of APAP in Insulin-mediated Signaling in Human Primary Hepatocytes**—We previously demonstrated that APAP increased PTP1B protein levels in human primary hepatocytes in a dose- and time-dependent manner in parallel to the activation of JNK, and this effect preceded cell death (9). Because PTP1B is a negative modulator of the insulin signaling cascade at the level of the IR (12, 13) and JNK elicits a negative cross-talk on IRS1 (10), we hypothesized that APAP could interfere with the early steps of the insulin signaling in human hepatocytes. Based on our previous work (9), we treated human primary hepatocytes with 10 mM APAP that neither decreased GSH levels nor induced cell death as assessed by released LDH activity (Fig. 1A). Under these experimental conditions, we analyzed JNK phosphorylation and PTP1B levels. As shown in Fig. 1B, 10 mM APAP increased IRS1 Ser307 phosphorylation in human primary hepatocytes. Regarding PTP1B, APAP (10 mM) treatment of human primary hepatocytes for 24 h increased PTP1B mRNA levels (Fig. 1C). To rule out compensatory regulation of PTPs with implication in hepatic insulin signaling, we analyzed SHP1 and TCPTP

**FIGURE 2. Effect of APAP in insulin-mediated signaling in human primary hepatocytes.** A, human primary hepatocytes were treated with APAP (10 mM) for 24 h. Then hepatocytes were further stimulated with 10 nM insulin for 10 min, and insulin signaling cascade was analyzed by Western blot with the antibodies against phospho-IR (Tyr1150/1151), phospho-IR (Tyr1162/1163), IR, phospho-IRS1 (Tyr1179), IRS1, IRS2, phospho-Akt (Ser473), phospho-Akt (Thr308), and Akt. Immunoprecipitation was performed with anti-IR antibody followed by Western blot with the anti-phosphotyrosine antibody. Representative autoradiograms are shown. B, autoradiograms were quantitated by scanning densitometry. The results are means ± S.E. *, \( p < 0.05 \); **, \( p < 0.01 \) APAP-treated versus untreated hepatocytes (n = 3 independent experiments performed in duplicate). I.P., immunoprecipitation; W.B., Western blot.
mRNA levels, and no differences were found between APAP-treated and control cells. Likewise, mRNA levels of other tyrosine phosphatases with a minor importance in insulin signaling such as LAR, PTPα, PTPε, and SHP2 remained unchanged (results not shown). In agreement with increased PTP1B mRNA levels, protein expression and enzymatic activity were increased in APAP-treated human hepatocytes as compared with untreated cells (Fig. 1C).

Next, APAP-pretreated human primary hepatocytes were stimulated with insulin (10 nM) for 10 min, and insulin signaling was evaluated compared with cells that did not receive APAP pretreatment. As shown in Fig. 2, insulin-induced IR tyrosine phosphorylation, analyzed with the phospho-specific IR Tyr1162/Tyr1163 and IR Tyr1150/Tyr1151 antibodies, was significantly decreased in primary human hepatocytes pretreated with 10 mM APAP. To assess the impact of APAP pretreatment in the global tyrosine phosphorylation of IR β chain that reflects its intrinsic tyrosine kinase activity, we performed immunoprecipitations with anti-IR antibody followed by Western blot with anti-phosphotyrosine antibody. By using this approach, we confirmed a decreased response to insulin in APAP-pretreated cells. Downstream from the IR, basal levels of IRS1 and its tyrosine phosphorylation in response to insulin were also decreased; this effect was not observed in IRS2. In agreement with these results, insulin-induced Akt phosphorylation at Ser473 and Thr308 residues was impaired by APAP.

**Reduction of PTP1B Enzymatic Activity by Suramin or PTP1B Protein Levels by Rosiglitazone Restored Insulin Signaling in APAP-pretreated Human Primary Hepatocytes—To get insight into the involvement of PTP1B enzymatic activity in the modulation of insulin signaling in hepatocytes by APAP, we treated human primary hepatocytes with APAP (10 mM) in the absence or presence of suramin, a reversible and competitive inhibitor of PTP1B (32), for 24 h. Suramin (20 μM) significantly inhibited PTP1B enzymatic activity in APAP-pretreated human primary hepatocytes without affecting PTP1B protein levels (Fig. 3A). Next, hepatocytes were pretreated with APAP with or without suramin and then stimulated with 10 μM insulin for 10 min. As shown in Fig. 3B, suramin prevented the decrease in IR tyrosine phosphorylation mediated by APAP, resulting in a recovery of Akt phosphorylation.

Because PTP1B inhibitors are not currently available in therapeutics, we investigated whether APAP-mediated attenuation of the insulin signaling cascade in human hepatocytes could be ameliorated by rosiglitazone, a well-known insulin sensitizer that binds to the peroxisome proliferator-activated-γ receptors. Human primary hepatocytes were pretreated with APAP (10 mM) in the presence or absence of rosiglitazone (20 μM) for 24 h. As shown in Fig. 4A, rosiglitazone reduced PTP1B protein levels, and in agreement with previous reports, (33–35), it inhibited JNK phosphorylation in APAP-treated human hepatocytes. Regarding insulin signaling, when cells were stimulated with 10 nM insulin at the end of the treatment, rosiglitazone protected against APAP-mediated decreases in IR and IRS1 tyrosine phosphorylation and degradation of IRS1 and also maintained insulin responsiveness in Akt phosphorylation at both Ser473 and Thr308 residues (Fig. 4B).

**Reduction of PTP1B Levels in Human Hepatocytes Protected against APAP-mediated Inhibition of Insulin Signaling—We have demonstrated that inhibition of PTP1B successfully restored hepatic insulin sensitivity in several in vivo models of insulin resistance such as IRS2-deficient (24) or aged (15) mice. On that basis, we investigated APAP-mediated insulin signaling in human hepatic cells with reduced PTP1B expression.
Because of the limitations of the human primary hepatocytes, similar experiments were conducted in the human Huh7 hepatoma cell line. Initially, GSH content and cellular damage, monitored by microscopy analysis, and released LDH activity were evaluated, and no toxicity was detected at 5 mM concentration of APAP (Fig. 5A). Importantly, in Huh7 cells, APAP at 5 mM concentration increased JNK and IRS1 Ser307 phosphorylations and PTP1B expression (Fig. 5B). In further experiments, we analyzed insulin signaling in APAP-treated human Huh7 hepatic cells infected with scrambled or PTP1B shRNA lentiviral particles. As depicted in Fig. 5C, transient (siRNA) or stable silencing of PTP1B in human Huh7 hepatocytes increased the insulin response in both IR and Akt phosphorylation. As observed in primary hepatocytes, treatment of control (scrambled) Huh7 cells with 5 mM APAP reduced IR and IRS1 tyrosine phosphorylation, IRS1 levels, as well as phosphorylation of Akt (Ser473 and Thr308) (Fig. 5D). Conversely, APAP treatment had a minor negative effect in the insulin signaling cascade in Huh7 cells with reduced PTP1B levels.

PTP1B-deficient Hepatocytes Are Protected against APAP-mediated Effects on Insulin Signaling—To further understand the role of PTP1B on insulin signaling in APAP-treated hepatocytes, we used immortalized hepatocytes from wild-type (PTP1B+/+) and PTP1B−/− mice generated in our laboratory. Importantly, the expression levels of the insulin signaling mediators in these cells are similar to that of the primary hepatocytes and liver tissue (20). Because immortalized hepatocytes are highly sensitive to APAP (9), lower concentrations of APAP were used in these studies. Hepatocytes from both genotypes were treated for 24 h with APAP (0.5 mM) and then stimulated with insulin for 10 min. As shown in Fig. 6A, in wild-type cells insulin-mediated IR and IRS1 tyrosine phosphorylation were severely impaired in parallel with the reduction of IRS1 levels. However, this effect was not observed in IRS2. Downstream from IRS1, phosphorylation of Akt at both Ser473 and Thr308 residues in response to insulin was impaired in APAP-pre-treated cells (Fig. 6B). The same assays were performed in PTP1B−/− immortalized hepatocytes that, as recently demonstrated (9), showed a lower response to APAP in the activation of stress kinases. PTP1B−/− hepatocytes pretreated with 0.5 mM APAP were resistant to the decrease in IR and IRS1 tyrosine phosphorylation and phosphorylation of Akt at both residues. In addition, total levels of IRS1 in these cells were unaffected by APAP (Fig. 6, A and B).
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In addition, immortalized hepatocytes from wild-type and PTP1B−/− mice were exposed to 0.5 mM APAP for short time periods (1–4 h), and phosphorylation of JNK and IRS1 at Ser307 was examined. As depicted in Fig. 6C, early phosphorylation of JNK and IRS1 Ser307 was observed in wild-type hepatocytes in response to APAP but was delayed and reduced in PTP1B−/− cells.

Effect of Chronic APAP Treatment on Glucose Homeostasis in Wild-type and PTP1B-deficient Mice—Mice deficient in Ptpn1 gene exhibit increased insulin sensitivity due to enhanced IR phosphorylation in liver and skeletal muscle (12, 13). Because of the substantial differences in insulin signaling observed in wild-type and PTP1B-deficient mouse hepatocytes treated with APAP, our next step was to study the impact of an in vivo chronic APAP treatment in wild-type and PTP1B−/− mice on whole body glucose homeostasis and insulin sensitivity. Mice were treated with APAP dissolved in the drinking water for 6 months as detailed under “Experimental Procedures.” For this 6-month treatment interval, no evidence of liver damage was detected, as reflected by the analysis of serum alanine amino transaminase levels and liver histology in all experimental groups (Fig. 7, A and B). In the light of these data, during chronic APAP treatment, liver concentrations of APAP-protein adducts were low in both genotypes of mice as compared with those determined after an acute APAP intoxication protocol (9) (Fig. 7C). However, protein levels of MnSOD and catalase were decreased in APAP-treated wild-type mice, whereas protein levels of both enzymes were increased in APAP-treated PTP1B−/− mice (Fig. 7D). These results suggest the presence of enhanced antioxidant defense in livers of mice lacking PTP1B in agreement with the protection against oxidative stress and liver damage in the acute model of APAP hepatotoxicity recently reported by our group (9).

Control wild-type mice increased body weight during the experiment, but this effect was not observed in mice receiving APAP (Fig. 8A). Of note, APAP-treated wild-type mice did not show symptoms of cachexia including muscle atrophy, fatigue, and weakness. However, no differences in body weight were observed in PTP1B−/− mice during the experimental protocol regardless of APAP treatment.

To further understand the effect of APAP on insulin signaling, whole body glucose homeostasis was examined at the end of the treatment by performing a glucose tolerance test. Fig. 8B shows that APAP increased the AUC in wild-type mice reflecting glucose intolerance. By contrast, enhanced glucose tolerance was evident in PTP1B−/− mice that remained insulin-sensitive under APAP treatment. Next, we performed an insulin tolerance test to investigate the effects of chronic APAP treatment in peripheral insulin sensitivity. As shown in Fig. 8C, PTP1B−/− mice displayed increased insulin sensitivity as compared with the wild-type control regardless of APAP treatment. However, chronic APAP treatment did not affect the ability of exogenously injected insulin to decrease glucose levels in both genotypes of mice.

In addition to the reduction in whole body glucose disposal (mainly accounted by skeletal muscle-mediated glucose uptake), increased hepatic glucose production has also been reported to contribute to glucose intolerance. To assess whether increased hepatic glucose production was involved in glucose intolerance of the APAP-treated wild-type animals, we performed a pyruvate tolerance test. In wild-type mice, we found increased glucose levels after pyruvate injection (Fig. 8D), and this effect was enhanced in mice that had received the chronic APAP treatment. By contrast, in PTP1B−/− mice, blood glucose levels were less elevated after pyruvate injection in both APAP-treated and vehicle-treated groups.

Effect of Chronic APAP Treatment in Insulin Signaling in Wild-type and PTP1B-deficient Mice—Next, we analyzed insulin signaling in the livers of both genotypes of mice. At the molecular level, chronic APAP treatment increased hepatic PTP1B enzymatic activity and protein content in wild-type mice (Fig. 9A). As expected, in wild-type mice that received a chronic treatment of APAP, there was a marked decrease in insulin-induced IR and IRS1 tyrosine phosphorylation and Akt phosphorylation as compared with untreated mice (Fig. 9B). However, total levels of IRS1 were unaffected by the chronic treatment. By contrast, in the livers of APAP-treated PTP1B−/− mice, insulin...
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signaling was not decreased compared with the untreated controls. Of note, no effect of APAP was found in insulin signaling in skeletal muscle, indicating that APAP effects were specific to the liver (Fig. 9C).

Effects of Chronic APAP Treatment on EE in Wild-type and PTP1B−/− Mice—The fact that APAP induced substantial changes in body weight during chronic treatment in wild-type mice prompted us to analyze metabolic parameters. As expected for leptin hypersensitivity in the hypothalamus (36), PTP1B−/− mice showed decreased daily food intake compared with their corresponding wild-type controls (Fig. 10A). When the effect of APAP was evaluated in the two genotypes of mice, we found that in wild-type mice, food intake was similar regardless of APAP treatment, whereas food intake was significantly elevated in both APAP-treated wild-type and PTP1B−/− mice after chronic APAP treatment (scale bar represents 100 μm) (n = 6–8 mice of each condition). C, analysis of APAP-protein adducts. The data from acute treatment (1 h) was previously reported (9). *** p < 0.001 APAP-treated PTP1B−/− versus APAP-treated PTP1B+/− mice. D, protein levels on MnSOD and catalase analyzed by Western blot in total liver extracts. p85, PI3K (p85) antibody was used as loading control. Autoradiograms were quantitated by scanning densitometry. The results are means ± S.E. *** p < 0.001 APAP-treated PTP1B−/− versus untreated PTP1B−/− mice. ## p < 0.01; ### p < 0.001 APAP-treated PTP1B−/− versus untreated PTP1B−/− mice (n = 4–6 mice per genotype and condition). ALT, alanine amino transaminase; veh, vehicle.

observed in wild-type and PTP1B−/− mice treated with APAP were associated with an increase in spontaneous locomotor activity as compared with their respective control groups not receiving APAP (Fig. 10D).

Effects of Chronic APAP Treatment in the Pancreas of Wild-type and PTP1B-deficient Mice—As depicted in Fig. 11A and in agreement with previous work (37), fasted insulin levels were significantly lower in PTP1B−/− mice as compared with the wild-type mice. Interestingly, chronic APAP treatment decreased serum insulin in wild-type mice, but not in mice lacking PTP1B, suggesting possible effects of APAP in pancreatic islets. Therefore, we performed histological analysis of pancreas and insulin and glucagon immunostaining in the four experimental groups. Islet density did not show significant differences among groups (Fig. 11B). As described (37), islets of PTP1B−/− mice were significantly smaller than those of the wild-type mice with a significant lower percentage of larger islets (Fig. 11, C and D). Accordingly to smaller islet size, PTP1B−/− mice treated with APAP showed a significant decrease in the beta cell relative volume compared with wild-type mice regardless of APAP treatment (Fig. 11E). Interestingly, although APAP treatment did not alter islet size in wild-type mice (Fig. 11, C and D), the beta cell relative volume was significantly decreased compared with their corresponding
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controls treated with vehicle (Fig. 11E). On the other hand, whereas alpha cell relative volume remained unaffected among groups (Fig. 11F), APAP increased the alpha/beta cell ratio exclusively in wild-type mice (Fig. 11G). In addition, wild-type mice treated with APAP showed a relative disorganization of alpha cells, and this effect was ameliorated in islets of APAP-treated PTP1B−/− mice (Fig. 11H). No differences in IL-6 immunostaining were observed among experimental groups (data not shown).

DISCUSSION

We previously reported elevated PTP1B expression in livers of patients with acute APAP intoxication, as well as in hepatocytes treated with concentrations of APAP that induced death in parallel to the activation of JNK (9). Because both molecules interfere with the early steps of the insulin signaling cascade (10, 12, 13), in this study we evaluated the specific cross-talk between signaling pathways triggered by APAP and insulin signaling in hepatic cells. Importantly, we used APAP concentrations that neither induced GSH depletion nor cell death, but increased JNK phosphorylation, together with PTP1B mRNA, protein levels, and enzymatic activity. Under these conditions, we did not find elevations in other phosphatases that modulate the insulin signaling cascade, suggesting APAP-specific effects on PTP1B.

Our results clearly demonstrated a decreased response to insulin in inducing IR tyrosine phosphorylation in human and mouse hepatocytes pretreated with APAP in parallel with increased PTP1B expression. The data indicate a negative cross-talk between APAP and insulin signaling at the level of IR that, together with IRS proteins, constitute a critical node of the insulin signaling (38). Downstream of IR/IRSs, insulin signaling was also attenuated in APAP-treated hepatocytes as reflected by decreased Akt phosphorylation at both catalytic and regulatory sites. These results were supported by the reduction of APAP-mediated negative effects on insulin signaling in human
primary hepatocytes in the presence of the PTP1B inhibitor suramin. Because PTP1B inhibitors are not yet approved to treat insulin resistance in humans, we have investigated the effect of rosiglitazone, an insulin sensitizer. The rationale for this approach is based on the use of thiazolidinediones to improve insulin sensitivity and decrease hyperglycemia in type 2 diabetic patients (39). In addition, several studies have demonstrated that compounds containing scaffolds similar to thiazolidinediones exhibit inhibitory effects against PTP1B (40). In this regard, Combs et al. (41) identified isothiazolidinione-containing compounds as potent PTP1B inhibitors. Our data in human primary hepatocytes clearly demonstrate that the combination of APAP plus rosiglitazone reduced both JNK phosphorylation, as has been reported in different systems (33–35), as well as PTP1B levels and restored insulin sensitization. Our previous work has demonstrated the beneficial effects of PTP1B inhibition as a mechanism to overcome hepatic insulin resistance caused by IRS2 deficiency (24) or aging (15) in mice. Similarly, the results presented herein reinforce the benefits of inhibiting PTP1B in the liver to ameliorate insulin resistance in this tissue including that induced by APAP.

IRS1 is susceptible to proteasomally mediated degradation by different triggers including oxidative stress. Acute oxidative stress leads to a short term activation of insulin signaling (42), but if prolonged it induces additional effects such as IRS1 degradation. In this regard, in 3T3-L1 adipocytes, it has been shown that urea, at concentrations that increase ROS levels, induces modification of insulin signaling molecules by O-GlcNAc and reduces insulin-stimulated IRS1 and Akt phosphorylation and glucose transport (43). Our data in three hepatic cellular models clearly show that, in parallel to JNK phosphorylation, APAP induces a rapid IRS1 phosphorylation at Ser307 that triggers its degradation. Therefore, in hepatocytes, the negative cross-talk elicited by JNK on IRS1 and by PTP1B on the IR might synergize to decrease Akt phosphorylation, which is known to be a critical mediator for the metabolic actions of insulin in the liver (38). Regarding IRS2, there are few data identifying the triggers and molecular mechanisms responsible for its degradation (31). However, in rat testis, decreased levels of IRS2 were observed following exposure to oxidative stress induced by bisphenol A (44). Another study demonstrated IRS2 degradation in muscle cells treated with hydrogen peroxide (45). Our data show that IRS2 levels were not affected by APAP in human and mouse hepatocytes, indicating that IRS2 may be more resistant to degradation compared with IRS1 as reported in primary mouse hepatocytes (30). These results suggest that the susceptibility to IRS2 degradation by oxidative stressors might vary among different cellular systems.

To assess the impact of PTP1B in APAP-mediated effects in glucose homeostasis, we extended our investigations to an in vivo model of chronic APAP treatment in mice. This treatment mimicked doses administered in humans with chronic pain and, importantly, did not induce liver damage in both genotypes of mice excluding possible indirect effects of APAP in glucose metabolism secondary to hepatotoxicity. Glucose intolerance was found exclusively in wild-type mice that received APAP. Regarding peripheral insulin sensitivity, no sig-
Significant differences were detected in these mice by performing an insulin tolerance test, probably because of the higher contribution of skeletal muscle for glucose disposal (46). However, the pyruvate tolerance test revealed decreased insulin suppression of hepatic glucose production in wild-type mice receiving APAP, but not in PTP1B-deficient mice. At the molecular level, PTP1B protein content and its enzymatic activity were up-regulated in the liver of wild-type mice treated chronically with APAP in parallel with decreased antioxidant defense as revealed by MnSOD and catalase protein levels. As a consequence, insulin signaling was impaired in liver tissue. Remarkably, insulin signaling was preserved in skeletal muscle of wild-type mice reinforcing the tissue specificity of the modulation of insulin signaling by APAP. By contrast, PTP1B−/− mice were totally protected against all these deleterious effects of APAP in the liver. In this scenario, our data highlight PTP1B as a main modulator of APAP-mediated hepatic insulin resistance in vivo.

Wild-type mice that did not receive APAP gained weight thorough the 6 months of the study, an effect that was not observed in PTP1B−/− mice. These data agree with our recent study demonstrating protection of PTP1B deficiency against weight gain and adiposity during aging (36). Unexpectedly, weight loss was observed in wild-type mice receiving APAP for 6 months, whereas weight loss did not occur in APAP-treated PTP1B−/− mice. One possible explanation for such an effect in wild-type mice could be decreased food intake and/or increased energy expenditure. In this regard, daily food intake did not change in wild-type mice treated with APAP compared with wild-type mice left untreated. However, in wild-type mice receiving APAP, there...
was a significant increase in EE, probably as a result of increased locomotor activity. Notably, APAP decreased body temperature in wild-type mice that might increase locomotor activity to warm-up, therefore protecting against weight gain. Recently, enhanced EE caused by increased locomotor activity has been found in mice with chronic activation of the glucagon receptor (47). Thus, another explanation for the effect of APAP in EE and locomotor activity in wild-type mice might be the increased alpha:beta cell ratio observed in the islets analysis. Of note, in these mice APAP also decreased insulin levels. In PTP1B−/− mice that did not gain weight during APAP treatment in spite of these mice APAP also decreased insulin levels. In PTP1B−/− mice, these additional effects on EE can be associated with insulin resistance in the liver.

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