Developmental Switch from Prolonged Insulin Action to Increased Insulin Sensitivity in Protein Tyrosine Phosphatase 1B-Deficient Hepatocytes

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Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of insulin signaling and a therapeutic target for type 2 diabetes. The purpose of this study was to evaluate the differences in insulin sensitivity between neonate and adult hepatocytes lacking PTP1B. Immortalized neonatal hepatocytes and primary neonatal and adult hepatocytes have been generated from PTP1B$^{-/-}$ and wild-type mice. PTP1B deficiency in immortalized neonatal hepatocytes prolonged insulin-induced tyrosine phosphorylation of the insulin receptor (IR) and IR substrates (IRS) -1, -2 compared with wild-type control cells. Endogenous IR and IRS-2 were down-regulated, whereas IRS-1 was up-regulated in PTP1B$^{-/-}$ neonatal hepatocytes and livers of PTP1B$^{-/-}$ neonates. Insulin-induced activation of phosphatidylinositol 3-kinase/Akt pathway was prolonged in PTP1B$^{-/-}$ immortalized neonatal hepatocytes. However, insulin sensitivity was comparable to wild-type hepatocytes. Rescue of PTP1B in deficient cells suppressed the prolonged insulin signaling, whereas RNA interference in wild-type cells promoted prolonged signaling. In primary neonatal PTP1B$^{-/-}$ hepatocytes, insulin prolonged the inhibition of gluconeogenic mRNAs, but the sensitivity to this inhibition was similar to wild-type cells. By contrast, in adult PTP1B-deficient livers, p85α was down-regulated compared with the wild type. Moreover, primary hepatocytes from adult PTP1B$^{-/-}$ mice displayed enhanced Akt phosphorylation and a more pronounced inhibition of gluconeogenic mRNAs than wild-type cells. Hepatic insulin sensitivity due to PTP1B deficiency is acquired through postnatal development. Thus, changes in IR and IRS-2 expression and in the balance between regulatory and catalytic subunits of phosphatidylinositol 3-kinase are necessary to achieve insulin sensitization in adult PTP1B$^{-/-}$ hepatocytes. (*Endocrinology* 148: 594–608, 2007)

I NSULIN RESISTANCE IN insulin target tissues such as liver, adipose tissues, and skeletal muscle is a key pathogenic feature of type 2 diabetes. Among them, hepatic insulin resistance is considered to be a dominant component in the pathogenesis of fasting hyperglycemia in type 2 diabetes (1, 2). The molecular mechanism underlying hepatic insulin resistance is not completely understood; however, it is believed to involve the impairment of the insulin receptor (IR) signaling network. The cascade begins when activation of the IR by autophosphorylation on several tyrosine residues leads to the recruitment and tyrosine phosphorylation of IR substrate (IRS) proteins (3, 4). Then, phosphorylated IRSs bind proteins containing Src homology 2 domains such as phosphatidyl-

inositol (PI) 3-kinase (5), which has a central role in the metabolic actions elicited by insulin. Over the last years, the role of insulin signaling pathways in the liver has also been studied in mice lacking hepatic IR (6), IRS-2 (7–12), phosphoinositide-dependent protein kinase-1 (13), protein kinase B β (PKB/Akt β) (14), and also in mice overexpressing a dominant negative mutant of PI 3-kinase (15). Various lines of genetic evidence support the concept that insulin negatively regulates hepatic glucose production through a main pathway involving IR, IRS-2, PI 3-kinase, and Akt β.

Given the importance of insulin signaling in multiple biologic responses, activation of this pathway must be tightly regulated. Protein tyrosine phosphatases (PTPs) catalyze the dephosphorylation of tyrosyl-phosphorylated proteins (16) and are known to be important negative regulators of IR signaling (17). Protein tyrosine phosphatase 1B (PTP1B) directly interacts with the activated IR (18, 19) and exhibits high specific activity for IRS-1 (20). It has been reported that overexpression of PTP1B in hepatic cellular models such as Fao hepatoma cells impairs insulin-stimulated glucose metabolism (21). Conversely, reduction of PTP1B in these cells increased insulin signaling (22). Furthermore, *in vivo* experiments have demonstrated that reduction of PTP1B expression by 60% in the liver of ob/ob mice increased IRS-1/2 tyrosine phosphorylation and Akt activation in response

to insulin (23). Mice lacking the *ptpn1* gene (PTP1B$^{-/-}$) exhibited increased insulin sensitivity at 10–14 wk of age owing to enhanced phosphorylation of IR in liver and skeletal muscle, resistance to weight gain on a high-fat diet, and an increased basal metabolic rate (24, 25). However, the molecular mechanism by which the increased insulin sensitivity is acquired during postnatal development of PTP1B-deficient mice has not been investigated. In fact, there are few published reports related to the developmental aspects of glucose tolerance and insulin sensitivity. To address this important issue, we have generated immortalized neonatal hepatocyte cell lines from wild-type and PTP1B$^{-/-}$ mice. These cell lines are novel and unique tools designed to elucidate the molecular mechanism of PTP1B-mediated insulin action in the neonatal liver given that their expression of hepatocyte markers and insulin signaling molecules is similar to liver extracts from wild-type and PTP1B-deficient neonates. In this article, we demonstrate that, in contrast to adult PTP1B knockout mice that show increased hepatic insulin sensitivity after *in vivo* insulin administration (24, 26), the lack of PTP1B in neonatal hepatocytes causes a significant prolongation of insulin signaling through PI 3-kinase/Akt without altering insulin sensitivity. As a result, prolonged inhibition of gluconeogenic gene expression occurred in these cells. Interestingly, our studies reveal that hepatic insulin sensitivity due to PTP1B deficiency is acquired during postnatal development, paralleling changes to the expression of IR and IRS-2 and the establishment of a molecular balance between the regulatory and the catalytic subunits of PI 3-kinase. These molecular alterations are absolutely required to achieve an insulin-sensitizing effect in adult hepatocytes lacking PTP1B.

Materials and Methods

Materials

Fetal serum (FS) and culture media were obtained from Invitrogen (Gaithersburg, MD). Insulin and antimouse IgG-agarose were from Sigma Chemical Co. (St. Louis, MO). IGF-I was from Calbiochem (Calbiochem-Novabiochem International, La Jolla, CA). Protein A-agarose was from Roche Molecular Biochemicals (Mannheim, Germany). (γ^{32}P)-ATP (3000 Ci/mmol), (α^{32}P)-dCTP (3000 Ci/mmol), and the cDNA labeling kit were from Amersham (Aylesbury, UK).

Culture of neonatal hepatocytes and retroviral infections

PTP1B-deficient mice (25) were obtained from Abbott Laboratories (Abbott Park, IL). All animal experimentation described in this article was conducted in accord with accepted standards of human animal care. Four pools of four to six livers from wild-type (PTP1B$^{+/+}$) and PTP1B$^{-/-}$ neonates (3.5–5 d old) were submitted to collagenase dispersion for the preparation of primary cultures as previously described (27). Viral Bosc-23 packaging cells were transfected at 70% confluence with a 3 μg/6-cm dish of the puromycin-resistance retroviral vector pBabe encoding Simian virus 40 large T antigen (LTAg) (kindly provided by J. de Caprio, Dana Farber Cancer Institute, Boston, MA) as previously described (11). Then, neonatal hepatocytes were infected at 60% confluence with polybrene (4 μg/ml)-supplemented virus for 48 h, maintained in culture medium for 72 h, and selected with puromycin (0.5–1 μg/ml) for 1 wk. Pools of infected cells rather than individual clones were selected to avoid potential clone-to-clone variations. Immortalized cell lines were further cultured for at least 2 wk with arginine-free medium supplemented with 10% FS and ornithine to avoid growth of nonparenchymal cells. Experiments were performed using three independent cell lines from each genotype, which were obtained by immortalization of distinct preparations of neonatal hepatocytes.

PTP1B$^{-/-}$ neonatal hepatocytes were reconstituted with retroviral Myc-tagged PTP1B (kindly provided by M. L. Tremblay, McGill Cancer Center, Quebec, Canada) and four pools of infected cells were selected with hygromycin B (200 μg/ml) for 2 wk. As a control, PTP1B-deficient hepatocytes were infected with an empty vector (pBabe hygro). The expression of PTP1B in the different cell lines was assessed by Western blot.

Primary culture of adult hepatocytes

Hepatocytes were isolated from nonfasting male wild-type and PTP1B-deficient mice (10–12 wk old) by perfusion with collagenase as described (28). Cells were plated on 60-mm primaria dishes (Falcon; BD Biosciences, San Jose, CA) and cultured in William's E medium supplemented with 20 ng/ml epidermal growth factor, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FS for 48 h. Then, cells were deprived of serum and used for experiments.

Immunofluorescence and confocal imaging

Immortalized neonatal hepatocytes were grown in glass coverslips until 80% confluence was reached. Then, cells were washed twice with PBS, fixed in methanol (−20 C) for 2 min, and processed to immunofluorescence. Monoclonal anti-vimentin (VIM) (cloneV9) antibody was from Roche Molecular Biochemicals, polyclonal anti-albumin (ALB) antibody was from Nordic Immunology Laboratories (Tilbury, The Netherlands), and monoclonal anti-carbamoyl phosphate synthetase (CPS) antibody was a gift of Dr. P. Martín-Sanz (Consejo Superior de Investigaciones Científicas, Madrid, Spain). Primary antibodies were applied for 1 h at 37 C in PBS 1% BSA followed by four 5-min washes in PBS, a 45-min incubation with fluorescence-conjugated secondary antibodies [fluorescein isothiocyanate (FITC)-conjugated sheep antimouse and Cy3-conjugated goat antirabbit], and four final washes of 5 min each in PBS. Immunofluorescence was examined in an MRC-1024 (Bio-Rad, Hemel Hempstead, UK) confocal microscope adapted to an inverted Nikon Eclipse TE 300 microscope. Immunofluorescence mounting medium was from Vector Laboratories (Burlingame, CA). Images were taken with 488-nm laser excitation for FITC-conjugated antibodies and 514-nm laser excitation for Cy3-conjugated antibodies. Fluorescence emissions were detected through a 513/24-nm bandpass filter for FITC and a 605/15-nm bandpass filter for Cy3.

Transient transfection with small interfering RNA (siRNA)

siRNA oligos were synthesized by Ambion Inc. (Austin, TX). PTP1B siRNA oligo1 corresponded to nucleotides 209–229 of the mouse sequence, and PTP1B siRNA oligo2 was previously described (29). Wild-type immortalized hepatocytes were seeded in 10-cm dishes for 2–3 d before transfection (60–70% confluence). Cells were trypsinized and electrophoresed with the indicated specific siRNA oligonucleotides (1.5 μg) by using Amaxa Nucleofector technology (Amaxa GmbH, Cologne, Germany) following the manufacturer's instructions. Cells were replated in six-well plates and maintained in growing medium (10% FS-DMEM) for a further 48 h. Then, hepatocytes were serum starved for 15 h and stimulated with 10 nm insulin for several time periods.

Homogenization and preparation of tissue extracts

Frozen livers were homogenized in 16 vol (weight/volume) of ice-cold lysis buffer containing 50 mm Tris-HCl, 1% Triton X-100, 2 mm EGTA, 10 mm EDTA, 100 mm NaF, 1 mm Na$_4$P$_2$O$_7$, 2 mm Na$_3$VO$_4$, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin. Liver was homogenized in the same lysis buffer using the Brinkmann PT 10/35 Polytron. Extracts were kept ice cold at all times. Liver extracts were cleared by microcentrifugation at 15,000 × *g* for 20 min at 4 C. The supernatant was aliquoted and stored at −70 C.

Immunoprecipitations and Western blot

Quiescent cells (15 h serum starved) were treated without or with several doses of insulin or IGF-I for several times and lysed as previously described (30). After protein content determination, equal amounts of

protein (600 μg–1 mg) were immunoprecipitated at 4 C with the corresponding antibodies. The immune complexes were collected on agarose beads and submitted for Western blot analysis. The anti-IRS-1 (06-248), anti-phosphotyrosine [Tyr(P)] (clone 4G10, 05-321), and anti-IRS-2 (06-506) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). The anti-phospho Akt (Ser473 no. 9271), anti-phospho p70S6 kinase 1 (S6K1) (Ser424/Thr421 no. 9204), anti-Akt (no. 9272), anti-phospho glycogen synthase kinase (GSK) 3 α/β (Ser 21/9 no. 9331), and anti-phospho Foxo1 (Ser256 no. 9461) antibodies were purchased from Cell Signaling Technology (Beverly, MA). The anti-IR β-chain antibody used for immunoprecipitations was from Oncogene (Cambridge, MA) (Ab-3, GR07) and for Western blot (C-19, sc-711) was from Santa Cruz Biotechnology (Palo Alto, CA). The anti-β-actin antibody was from Sigma. Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham).

PI 3-kinase activity

PI 3-kinase activity was measured in the immunoprecipitates by *in vitro* phosphorylation of PI as previously described (30).

Protein determination

Protein determination was performed by the Bradford dye method (Bio-Rad).

RNA extraction from primary hepatocytes and Northern blot analysis

Primary hepatocytes were isolated from livers of 3.5- to 5-d-old neonates or 10- to 12-wk-old mice and cultured as described (27, 28). Then, cells were serum starved for 12–15 h and further incubated for 6 h in the presence of 0.5 mM dibutyryl cAMP plus 1 μM dexamethasone (dex/cAMP) either in the absence or in the presence of insulin (0.1–100 nM) for several time periods. At the end of the culture time, total RNA was isolated with Trizol (Invitrogen) and 20 μg were submitted to Northern blot analysis. Blots were hybridized with cDNA probes for phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase). Membranes were subjected to autoradiography and the relative densities of the hybridization signals were determined by densitometric scanning of the autoradiograms.

Statistical analysis

All values are presented as mean $\pm$ SE. Comparisons between groups were made using unpaired two-tailed Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

Results

Generation and characterization of immortalized hepatocyte cell lines from PTP1B$^{+/+}$ and PTP1B$^{-/-}$ neonates

Immortalized neonatal hepatocyte cell lines were generated from four pools of four to six livers of either wild-type (PTP1B$^{+/+}$) or PTP1B$^{-/-}$ mice at postnatal d 3.5–5. It is important to note that immortalized neonatal hepatocytes were further cultured for 10–15 d in arginine-free medium (supplemented with ornithine) to select hepatocytes having functional urea cycle. As shown in Fig. 1A, all PTP1B$^{+/+}$ immortalized neonatal hepatocytes expressed similar levels of PTP1B; as expected, no expression of this phosphatase was detected in immortalized PTP1B$^{-/-}$ neonatal hepatocytes. In addition, levels of LTAg were similar in all cell lines on immortalization as assessed by Western blot.

Wild-type and PTP1B$^{-/-}$ neonatal hepatocytes expressed ALB (a plasma protein secreted exclusively by hepatocytes) together with CPS (a urea cycle marker), indicating that on immortalization, these cells maintained hepatocyte phenotypic features (Fig. 1B). As a negative control, we performed immunofluorescence experiments with immortalized β-cells previously generated in our laboratory (31). Immunofluorescence was not detected in these immortalized β-cells, thereby confirming the specificity of ALB and CPS antibodies. Moreover, the absence of VIM (a cytoskeletal marker characteristic of fibroblasts) staining demonstrated that the maintenance of cell lines in arginine-free medium was sufficient to eliminate contaminating fibroblasts in the primary culture.

Next, we determined whether the lack of PTP1B in immortalized neonatal hepatocytes modifies the endogenous expression of various proteins of the insulin signaling cascade. As depicted in Fig. 1C, a significant reduction in the expression of IR β-chain was observed in immortalized PTP1B$^{-/-}$ hepatocytes compared with wild-type controls. Interestingly, IRS-2 expression was also down-regulated but, in contrast, IRS-1 protein content was up-regulated in immortalized PTP1B$^{-/-}$ neonatal hepatocytes compared with the wild type. Importantly, the net increase in IRS-1 and the net decrease in IR and IRS-2 expression were similar in PTP1B-deficient immortalized neonatal hepatocytes, primary neonatal hepatocytes, and liver extracts, strongly suggesting that the absence of PTP1B rather than the immortalization process mediates the observed alterations in protein expression. No differences in the levels of p85α, Akt, and S6K1 were noted between immortalized PTP1B$^{+/+}$ and PTP1B$^{-/-}$ neonatal hepatocytes and liver extracts (Fig. 1C). Thus, this novel cellular model is physiologically relevant and has allowed us to analyze the molecular mechanism of PTP1B deficiency in insulin signaling in neonatal hepatocytes.

Lack of insulin sensitivity in PTP1B-deficient neonatal hepatocytes

To determine whether PTP1B deficiency improves insulin sensitivity in immortalized neonatal hepatocytes, quiescent PTP1B$^{+/+}$ and PTP1B$^{-/-}$ cells were stimulated for 5 min with various doses of insulin and IR β-chain tyrosine phosphorylation was analyzed. Maximal IR β-chain tyrosine phosphorylation was elicited at 10 nM concentration in both cell types (Fig. 2A). Unexpectedly, IR tyrosine phosphorylation was higher in wild-type cells. However, after normalization with the total amount of IR β-chain immunoprecipitated in each condition, no differences were found. In addition, insulin-induced anti-Tyr(P)-associated PI 3-kinase activity was unaffected by the lack of PTP1B (Fig. 2A). Next, we investigated whether the lack of PTP1B in immortalized neonatal hepatocytes increased insulin sensitivity with respect to Akt phosphorylation. This experiment was performed because it has been reported that Akt associates with and is dephosphorylated by protein phosphatase 2A (PP2A) (32) and negatively regulates insulin's metabolic signaling pathways (33). More importantly, overexpression of PTP1B decreases tyrosine phosphorylation and, consequently, increases PP2A activity in rat hepatocytes (34). Therefore, we stimulated wild-type and PTP1B$^{-/-}$ hepatocytes with various doses of insulin for 5 min and analyzed Akt phosphorylation by direct Western blot. As shown in Fig. 2A, phosphorylation of Akt was maximal at 10 nM insulin
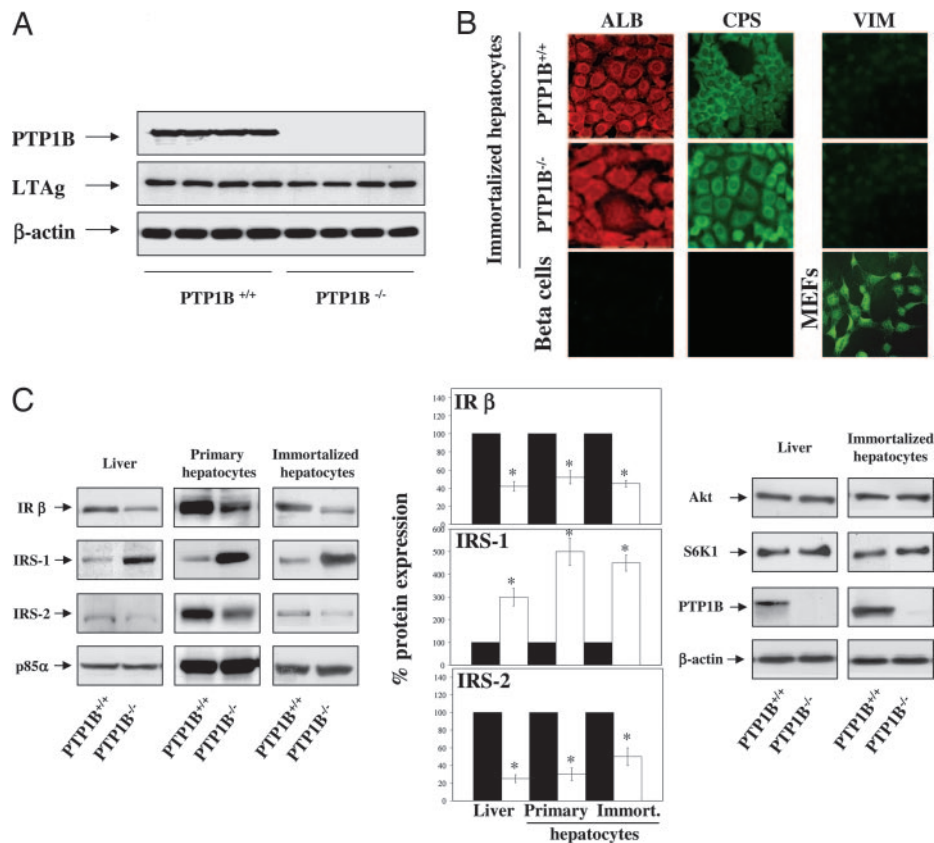
FIG. 1. Characterization of immortalized neonatal hepatocyte cell lines. A, Immortalized neonatal hepatocytes from wild-type and PTP1B-deficient newborn mice (3.5–5 d old) were generated as described in *Materials and Methods*. Growing cells were lysed and PTP1B and LTAg expression of four independent cell lines from each genotype was analyzed in whole-cell lysates by Western blot. B, Immunofluorescence detection of ALB, CPS, and VIM of growing immortalized neonatal hepatocytes (PTP1B$^{+/+}$ and PTP1B$^{-/-}$). As a negative control for ALB and CPS antibodies, immortalized β-cells were used for immunofluorescence. As a positive control for VIM, mouse embryo fibroblasts were used. Representative images are shown. C, Cell lysates from immortalized neonatal hepatocytes, primary neonatal hepatocytes, or livers from six to eight PTP1B$^{+/+}$ and PTP1B$^{-/-}$ neonates were analyzed by Western blot with antibodies against IR, IRS-1, IRS-2, p85α, S6K1, and Akt. A representative experiment is shown. The autoradiograms showing IR, IRS-1, and IRS-2 expression in three different cell lines of immortalized neonatal hepatocytes, primary neonatal hepatocytes, and neonatal livers from each genotype were quantitated by scanning densitometry. Results are expressed as percentage of increase of IRS-1 expression or percentage of decrease of IR and IRS-2 expression of primary and immortalized PTP1B$^{-/-}$ hepatocytes and PTP1B$^{-/-}$ liver extracts compared with the wild type (set to 100%) and are means ± SE. Statistical significance was carried out by Student's *t* test. *, $P < 0.05$ was considered significant.

concentration in both cell types. However, PTP1B$^{-/-}$ hepatocytes displayed significant Akt phosphorylation in the basal state and after stimulation with lower doses of insulin (0.1–1 nM) compared with the wild-type controls. To further explore this effect, we performed anti-PP2A Western blot analysis of anti-Tyr(P) immunoprecipitates. In wild-type cells, phosphorylation of both PP2A tyrosine and Akt serine 473 was low in the absence of insulin. After stimulation, PP2A and Akt phosphorylation increased in a dose-dependent manner. By contrast, in PTP1B-deficient hepatocytes, both proteins were highly phosphorylated in the absence of insulin. After treatment with low doses of insulin (0.1–1 nM), phosphorylation of PP2A and Akt was greater than in wild-type controls. However, at higher insulin concentrations (10–100 nM), no differences were observed between both cell types. Given that no significant differences in either IR β-chain tyrosine phosphorylation or anti-Tyr(P)-associated PI 3-kinase activity were noted between wild-type and PTP1B$^{-/-}$ neonatal hepatocytes, these results suggest that

possible downstream mechanisms such as inactivation of PP2A in a PTP1B-dependent manner might modulate Akt phosphorylation.

There are recent observations *in vivo* in adult mice suggesting that the liver is a major site of the peripheral action of PTP1B in regulating glucose homeostasis (26). Accordingly, our next step was to study *in vitro* whether the lack of PTP1B in neonatal hepatocytes modifies the negative regulation of gluconeogenic gene expression by insulin. Toward this end, we prepared primary hepatocytes from PTP1B$^{+/+}$ and PTP1B$^{-/-}$ neonates (3.5–5 d old). The reason for using primary cells to conduct these experiments is the fact that, in immortalized neonatal hepatocytes, PEPCK and G6Pase were only modestly increased after dexamethasone plus dibutyryl cAMP (dex/cAMP) treatment. As shown in Fig. 2B, both PEPCK and G6Pase mRNAs were highly induced by dex/cAMP in primary neonatal hepatocytes regardless of PTP1B expression. These experimental conditions allowed us to study the effect of insulin on both genes. Insulin treat-
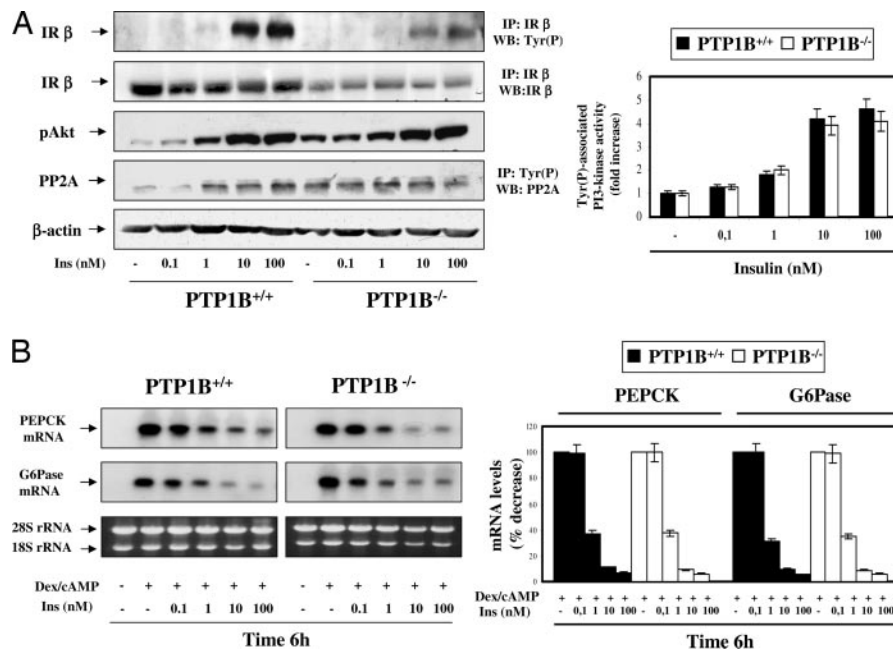
FIG. 2. Dose-response effects on insulin signaling and gluconeogenic gene expression in wild-type and PTP1B-deficient neonatal hepatocytes. A (*left panel*), Quiescent immortalized wild-type (PTP1B$^{+/+}$) and PTP1B-deficient (PTP1B$^{-/-}$) neonatal hepatocytes were stimulated with insulin (0.1–100 nM) for 5 min. Control cells were cultured in the absence of the hormone. At the end of the culture time, cells were lysed and 1 mg of total protein was immunoprecipitated with the anti-IR β-chain antibody. The resulting immune complexes were analyzed by Western blotting with the anti-Tyr(P) antibody or the anti-IR β-chain antibody. Akt phosphorylation was measured by direct Western blot analysis with the anti-phospho Akt antibody. Tyrosine phosphorylation of PP2A was measured by immunoprecipitating 600 μg of total protein with anti-Tyr(P) antibody followed by Western blot with anti-PP2A antibody. The experiment was repeated twice with similar results. *Right panel*, Cells were stimulated as described previously. At the end of the culture time, cells were lysed and 600 μg of total protein, immunoprecipitated with the anti-Tyr(P) antibody, was immediately used for an *in vitro* PI 3-kinase assay. The conversion of PI to PIP in the presence of (γ^{32}-P) ATP was analyzed by thin layer chromatography (TLC). The autoradiograms corresponding to three independent experiments, performed in three different cell lines from each genotype, were quantitated by scanning densitometry. The value of nonstimulated PTP1B$^{+/+}$ and PTP1B$^{-/-}$ cells was set to 1. Results are expressed as fold increase by insulin of Tyr(P)-associated PI 3-kinase activity and are means ± SE. B, Primary neonatal hepatocytes, obtained from pools of 3.5- to 5-d-old mice, were cultured to confluence. Cells were serum starved for 12–15 h and further cultured for 6 h without or with 0.5 mM dibutyril cAMP plus 1 μM dexamethasone (dex/cAMP). Then, various doses of insulin were added for a further 6 h. At the end of the culture time, total RNA was isolated, submitted to Northern blot analysis, and hybridized with labeled PEPCK and G6Pase cDNAs. Representative autoradiograms are shown. The autoradiograms corresponding to four independent experiments were quantitated by scanning densitometry. The value of dex/cAMP-treated cells was set to 100%. Results are expressed as percentage of decrease by insulin of PEPCK and G6Pase mRNAs of primary PTP1B$^{-/-}$ neonatal hepatocytes compared with the wild type and are means ± SE.

ment inhibited dex/cAMP-induced PEPCK and G6Pase mR-NAs in a dose-dependent manner with total inhibition being observed at 10 nM in both cell types.

Prolonged IR tyrosine phosphorylation in immortalized PTP1B-deficient neonatal hepatocytes

Because no differences in insulin sensitivity were found between immortalized wild-type and PTP1B-deficient hepatocytes from neonates, we explored the possibility of a differential time course of insulin signaling. Thus, we performed a detailed time course of IR β-chain tyrosine phosphorylation in both cell types. Quiescent cells were serum starved for 15 h and subsequently stimulated with 10 nM insulin for 1 min–15 h. Then, tyrosine phosphorylation of IR β-chain was analyzed in anti-IR immunoprecipitates. As shown in Fig. 3A, insulin strongly induced tyrosine phosphorylation of IR β-chain in immortalized wild-type neonatal hepatocytes, reaching a maximal effect at 1 min. This response was sustained for 5 min but declined at 15 min, with no tyrosine phosphorylation being observed after 1 h of insulin treatment. Although IR β-chain protein content is

reduced in PTP1B$^{-/-}$ neonatal hepatocytes (Figs. 1C and 2A), insulin-mediated tyrosine phosphorylation of the IR was quite different in these cells; maximal phosphorylation was sustained from 1 min up to 2 h of insulin stimulation.

It is well known that IGF-I receptors (IGF-IR) can mediate actions of insulin at high concentrations. To exclude the possibility that 10 nM insulin triggers the phosphorylation of IGF-IR in neonatal hepatocytes, immortalized wild-type and PTP1B$^{-/-}$ hepatocytes were stimulated with 10 nM insulin for 5 min, and IGF-IR tyrosine phosphorylation was analyzed in anti-IGF-IR immunoprecipitates. As a control, immortalized β-cells were stimulated with 10 nM IGF-I. As depicted in Fig. 3B, insulin at 10 nM concentration did not induce IGF-IR tyrosine phosphorylation in either wild-type or PTP1B-deficient hepatocytes. Of note, IGF-IR expression was comparable in both kinds of cells.

Prolonged IRS-1, -2/PI 3-kinase signaling in immortalized neonatal hepatocytes lacking PTP1B

Our next aim was to study whether the prolonged insulin-induced IR tyrosine phosphorylation provoked by PTP1B
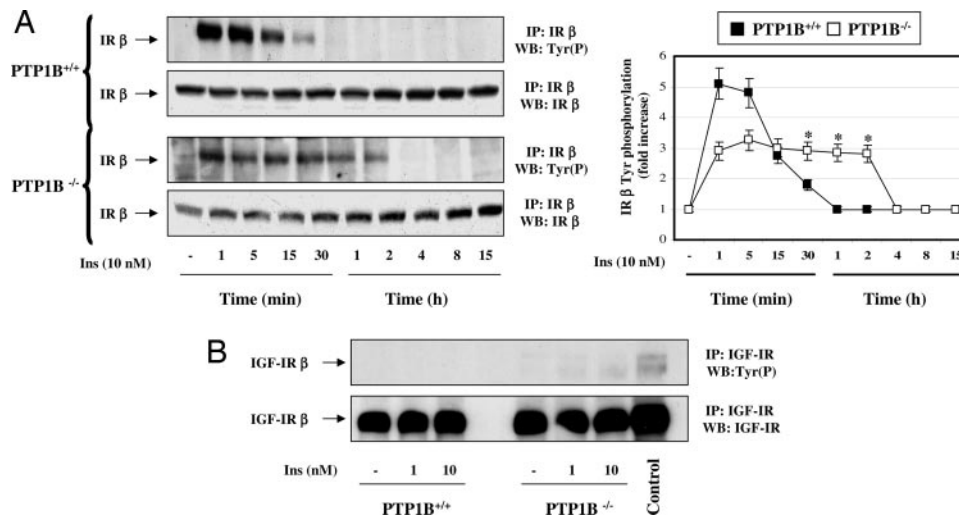
FIG. 3. Time course of IR β-chain tyrosine phosphorylation in insulin-stimulated immortalized wild-type and PTP1B-deficient neonatal hepatocytes. A, Quiescent immortalized neonatal hepatocytes (PTP1B$^{+/+}$ and PTP1B$^{-/-}$) were stimulated with 10 nM insulin for 1 min to 15 h. At the end of the culture time, cells were lysed and 1 mg of total protein was immunoprecipitated with anti-IR β-chain antibody. The resulting immune complexes were analyzed by Western blotting with anti-Tyr(P) or anti-IR antibodies as indicated in each panel. A representative experiment is shown. The autoradiograms corresponding to three independent experiments, performed in three different cell lines from each genotype, were quantitated by scanning densitometry. The value of nonstimulated PTP1B$^{+/+}$ and PTP1B$^{-/-}$ cells was set to 1. Results are expressed as fold increase by insulin of tyrosine phosphorylation of IR and are means ± SE. Statistical significance was carried out by Student's t test comparing immortalized PTP1B$^{-/-}$ neonatal hepatocytes with the respective values of wild-type cells. *, $P < 0.05$ was considered significant. B, Quiescent immortalized neonatal hepatocytes (PTP1B$^{+/+}$ and PTP1B$^{-/-}$) were stimulated with 1–10 nM insulin for 5 min. As a control, immortalized β-cells were treated with 10 nM IGF-I for 5 min. At the end of the culture time, cells were lysed and 600 μg of total protein was immunoprecipitated with anti-IGF-IR antibody. The resulting immune complexes were analyzed by Western blotting with anti-Tyr(P) or anti-IGF-IR antibodies as indicated in each panel. The results shown are representative of three experiments performed in three independent cell lines from each genotype.

deficiency in immortalized neonatal hepatocytes is transduced through IRS-1 and/or IRS-2/PI 3-kinase pathway, which in fact mediates most of the metabolic actions of insulin in the liver. Tyrosine phosphorylation of IRS-1, as well as its association with p85α and activation of IRS-1-associated PI 3-kinase activity, decreased after 15–30 min of insulin stimulation in PTP1B$^{+/+}$ neonatal hepatocytes (Fig. 4, *upper panel*). In sharp contrast, the effect of insulin on IRS-1 tyrosine phosphorylation in PTP1B$^{-/-}$ neonatal hepatocytes was prolonged; tyrosine phosphorylation of IRS-1, IRS-1/p85α association, and IRS-1-associated PI 3-kinase activity was sustained for 4 h in PTP1B$^{-/-}$ neonatal hepatocytes (Fig. 4, *lower panel*). Similar to findings for IRS-1, IRS-2 tyrosine phosphorylation, its binding to p85α, and activation of IRS-2-associated PI 3-kinase activity decreased between 15 and 60 min in insulin-stimulated PTP1B$^{+/+}$ neonatal hepatocytes (Fig. 5, *upper panel*). However, insulin action was prolonged up to 4 h on this signaling pathway in PTP1B$^{-/-}$ neonatal hepatocytes (Fig. 5, *lower panel*). Taken all together, our results suggest that both IRS-1 and IRS-2 signaling contribute to the prolonged PI 3-kinase activation in immortalized neonatal hepatocytes in the absence of PTP1B.

Sustained insulin signaling downstream of PI 3-kinase in immortalized neonatal hepatocytes lacking PTP1B

Downstream of PI 3-kinase, various serine/threonine kinases become activated after insulin stimulation. As shown in Fig. 6, insulin stimulated Akt phosphorylation up to 30 min in immortalized wild-type neonatal hepatocytes. This effect was markedly prolonged (up to 4 h) in PTP1B$^{-/-}$ cells.

It is noteworthy that, in PTP1B$^{-/-}$ neonatal hepatocytes, there is a significant basal phosphorylation of Akt compared with wild-type control cells. Downstream of Akt, both GSK3 (α and β isoforms) and Foxo 1 were phosphorylated after short-term insulin stimulation in wild-type cells. However, in PTP1B$^{-/-}$ neonatal hepatocytes, GSK3 (mainly GSK3β isoform) and Foxo1 displayed prolonged phosphorylation in response to insulin stimulation. In addition, S6K1, a mammalian target of rapamycin target, showed a similar profile of short-term (wild-type) and long-term (PTP1B$^{-/-}$) phosphorylation in response to insulin. Thus, the lack of PTP1B prolonged insulin activation of the PI 3-kinase/Akt signaling pathway, which might have physiological consequences in neonatal hepatocytes.

Effect of PTP1B reconstitution or siRNA-mediated suppression of PTP1B on insulin signaling in immortalized neonatal hepatocytes

To test that the lack of PTP1B is indeed responsible for prolonged insulin signaling in immortalized PTP1B-deficient neonatal hepatocytes, we reconstituted PTP1B expression by retroviral gene transfer. As a control, PTP1B$^{-/-}$ neonatal hepatocytes were infected with an empty retroviral construct. Figure 7A reveals the levels of ectopic expression of PTP1B in four different pools of PTP1B$^{-/-}$ immortalized neonatal hepatocytes (PTP1B$^{-/-}$Rec). Expression of recombinant PTP1B in deficient neonatal hepatocytes restored a decline of IR β-chain tyrosine phosphorylation after 30 min of insulin stimulation, similar to the effects of insulin in wild-type cells (Figs. 3A and 7B). In addition, the time course
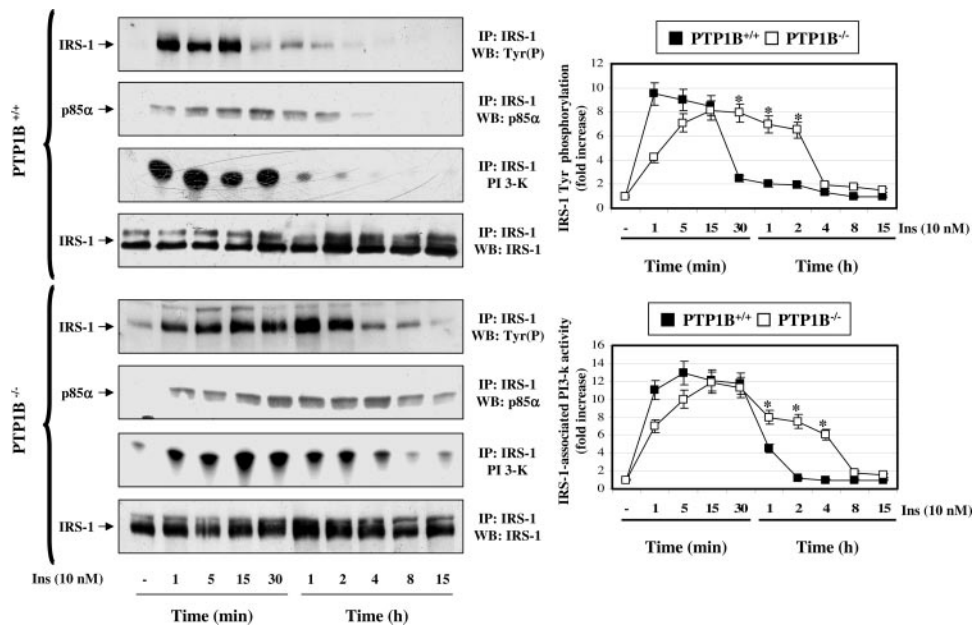
FIG. 4. Insulin effect on IRS-1-tyrosine phosphorylation and IRS-1-associated PI 3-kinase activity in immortalized wild-type and PTP1B-deficient neonatal hepatocytes. Quiescent immortalized neonatal hepatocytes (PTP1B$^{+/+}$ and PTP1B$^{-/-}$) were stimulated with 10 nM insulin for 1 min to 15 h or cultured in the absence of the hormone. At the end of the culture time, cells were lysed and 600 μg of total protein, immunoprecipitated with anti-IRS-1 antibody, was analyzed by Western blot with anti-Tyr(P), anti-p85α, and anti-IRS-1 antibodies or used for an *in vitro* PI 3-kinase assay. The conversion of PI to PIP in the presence of [γ^{32}-P] ATP was analyzed by TLC. A representative experiment is shown. The autoradiograms corresponding to three independent experiments, performed in three different cell lines from each genotype, were quantitated by scanning densitometry. The value of nonstimulated PTP1B$^{+/+}$ and PTP1B$^{-/-}$ cells was set to 1. Results are expressed as fold increase by insulin of tyrosine phosphorylation of IRS-1 or IRS-1-associated PI 3-kinase activity and are means ± SE. Statistical significance was carried out by Student's *t* test comparing immortalized PTP1B$^{-/-}$ neonatal hepatocytes with the respective values of wild-type cells. *, $P < 0.05$ was considered significant.

of insulin-mediated Akt phosphorylation in PTP1B$^{-/-}$Rec neonatal hepatocytes was comparable to that observed in wild-type cells (Figs. 6 and 7B).

To further demonstrate that PTP1B regulates PI 3-kinase/Akt insulin signaling in neonatal hepatocytes, we established RNA interference (siRNA) assays in wild-type cells. As depicted in Fig. 8, reduction of PTP1B expression by 70% in wild-type cells with two different siRNA oligos prolonged the effects of insulin on IR β-chain tyrosine phosphorylation as well as on phosphorylation of Akt, Foxo1, GSK3, and S6K1. Thus, both experimental approaches (rescue of PTP1B and siRNA) reinforce our findings in immortalized PTP1B-deficient neonatal hepatocytes.

PTP1B deficiency prolongs inhibition of gluconeogenic gene expression by insulin in neonatal hepatocytes

The fact that the disruption of the *ptpn1* gene did not increase insulin sensitivity in neonatal hepatocytes prompted us to study *in vitro* whether the prolonged insulin signaling via PI 3-kinase/Akt in PTP1B$^{-/-}$ neonatal hepatocytes could modulate the timing of the suppression of gluconeogenic gene expression by insulin. As depicted in Fig. 9A, insulin inhibited gluconeogenic gene expression in a time-dependent manner in neonatal hepatocytes, with total inhibition of PEPCK and G6Pase mRNAs being observed after 90 min of insulin treatment, independently of PTP1B expression. However, when we analyzed the long-term inhibitory effect of insulin on dex/cAMP-induced PEPCK and G6Pase mRNAs, substantial differences were found.

Whereas insulin was able to suppress gluconeogenic gene expression for 12 h in wild-type neonatal hepatocytes, this effect was prolonged up to 36 h in PTP1B-deficient cells (Fig. 9B).

Expression profile of key molecules of the insulin signaling cascade in the liver from adult PTP1B-deficient mice

We assessed the possibility that the striking differences in insulin signaling between neonatal PTP1B-deficient hepatocytes (this paper) and the adult liver (26) could be due to a different pattern of endogenous protein expression of the key molecules of insulin signaling network through postnatal development. For this goal, protein extracts were obtained from four to six livers of adult mice (10–12 wk old) from both genotypes. Then, the expression of the key molecules of the insulin signaling was analyzed. Substantial differences were found in the liver between adult (Fig. 10A) and neonatal (Fig. 1C) PTP1B-deficient mice regarding protein expression. Whereas the expression of IR β-chain was down-regulated in neonatal PTP1B-deficient liver as compared with the neonatal wild type, no differences between both genotypes were observed in adult liver. Moreover, IRS-1 and IRS-2 protein content was similar in livers from wild-type and PTP1B$^{-/-}$ adult mice. Conversely, the expression of the p85α regulatory subunit of PI 3-kinase was significantly down-regulated (~55%) in adult (Fig. 10A) but not in neonatal (Fig. 1C) PTP1B-deficient liver as compared with the corresponding wild-type controls. Collectively, these results show that, in PTP1B-deficient mice, the hepatic expression profile of key
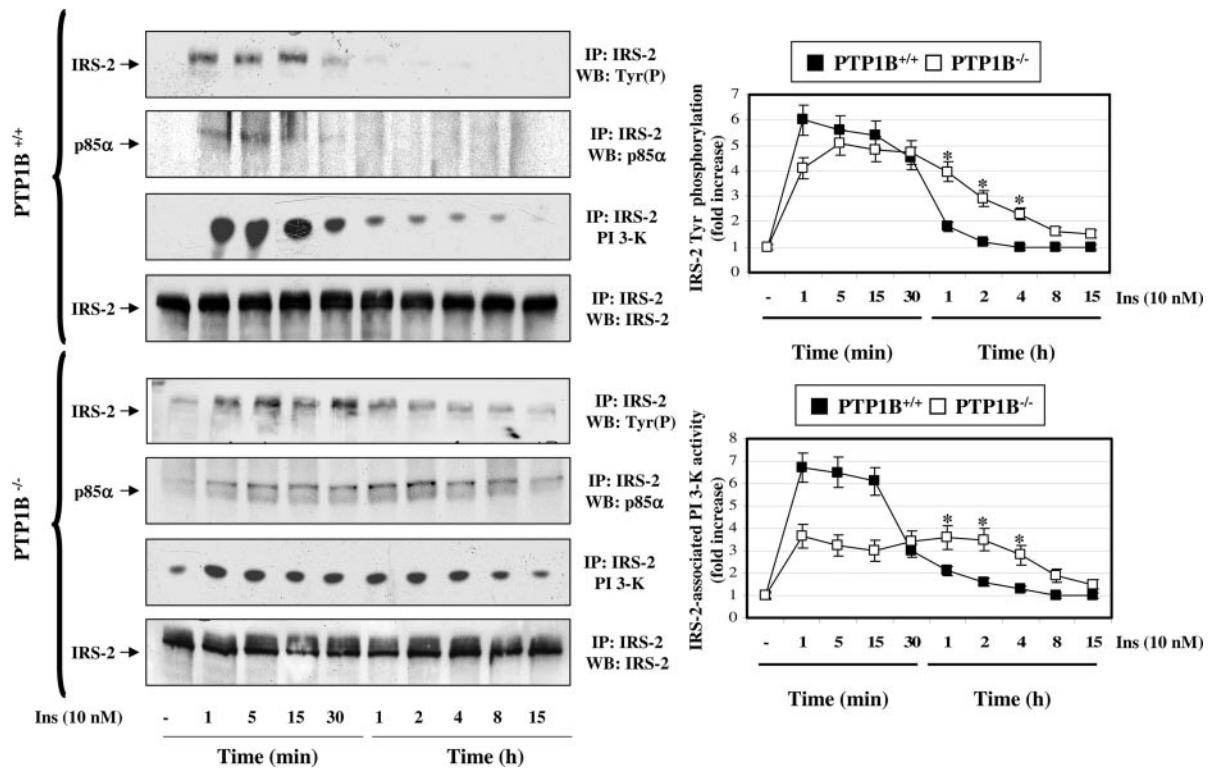
FIG. 5. Insulin effect on IRS-2-tyrosine phosphorylation and IRS-2-associated PI 3-kinase activity in immortalized wild-type and PTP1B-deficient neonatal hepatocytes. Quiescent cells were stimulated with 10 nM insulin for 1 min to 15 h or cultured in the absence of the hormone. At the end of the culture time, cells were lysed and 600 μg of total protein, immunoprecipitated with anti-IRS-2 antibody, was analyzed by Western blot with anti-Tyr(P), anti-p85α, and anti-IRS-2 antibodies or used for an *in vitro* PI 3-kinase assay. The conversion of PI to PIP in the presence of [γ^{32}-P]ATP was analyzed by TLC. A representative experiment is shown. The autoradiograms corresponding to three independent experiments were quantitated by scanning densitometry. The value of nonstimulated PTP1B$^{+/+}$ and PTP1B$^{-/-}$ cells was set to 1. Results are expressed as fold increase by insulin of tyrosine phosphorylation of IRS-2 or IRS-2-associated PI 3-kinase activity and are means ± SE. Statistical significance was carried out by Student's t test comparing immortalized PTP1B$^{-/-}$ neonatal hepatocytes with the respective values of wild-type cells. *, $P < 0.05$ was considered significant.

mediators of insulin signaling switches in the neonatal/adult transition.

Increased insulin sensitivity in primary hepatocytes from adult PTP1B-deficient mice

Next, we investigated whether the expression profile of insulin signaling molecules in adult PTP1B-deficient liver can account for an increase in insulin sensitivity in hepatocytes. Thus, we prepared primary hepatocytes from adult wild-type and PTP1B$^{-/-}$ mice to analyze insulin signaling. Cells were serum deprived for 6–8 h and then several doses of insulin were added for 10 min. As shown in Fig. 10B, phosphorylation of Akt was barely detected after stimulation with 1 nM insulin in adult hepatocytes from wild-type mice. By contrast, at the same dose, maximal phosphorylation of Akt was elicited in PTP1B$^{-/-}$ adult hepatocytes. A similar trend was noted with respect to the phosphorylation of Foxo-1 and GSK-3β.

Finally, we analyzed the ability of insulin to suppress PEPCK and G6Pase mRNAs in adult hepatocytes lacking PTP1B. Primary hepatocytes were incubated with dex/cAMP before treatment with 10 nM insulin for 6 h. Then, the expression of PEPCK and G6Pase was analyzed by Northern blot. As shown in Fig. 10C, addition of insulin completely blocked the induction of PEPCK and G6Pase mRNAs by

dex/cAMP in PTP1B$^{-/-}$ adult hepatocytes. However, a remnant of 25% and 50% of G6Pase and PEPCK mRNAs, respectively, was detected in insulin-treated wild-type cells.

Discussion

The control of insulin signaling is complex, involving the coordinated action of both positive and negative regulatory proteins. Of these, PTP1B is a major negative regulatory protein as demonstrated by the positive effects of deleting the *ptpn1* gene on insulin sensitivity, energy expenditure, and lipoprotein secretion (24, 25, 35). Thus, although it is clear that PTP1B plays a role in insulin sensitivity and glucose homeostasis, the molecular mechanism of how this protein may play a tissue-specific role is not completely understood. Nevertheless, the suppression of PTP1B has been proposed as beneficial treatment for type 2 diabetes, and various pharmacological PTP1B inhibitors are under development (36).

The specific role of PTP1B in insulin action in the liver has been a controversial issue. Wang *et al.* (37) reported that adenovirus-mediated liver-specific overexpression of PTP1B in rats did not alter insulin action. Conversely, it has been recently shown that liver-specific PTP1B rescue in PTP1B-deficient mice led to attenuation of enhanced insulin sensitivity, strongly suggesting that the liver is a major site of PTP1B action in the periphery (26). Moreover, PTP1B defi-
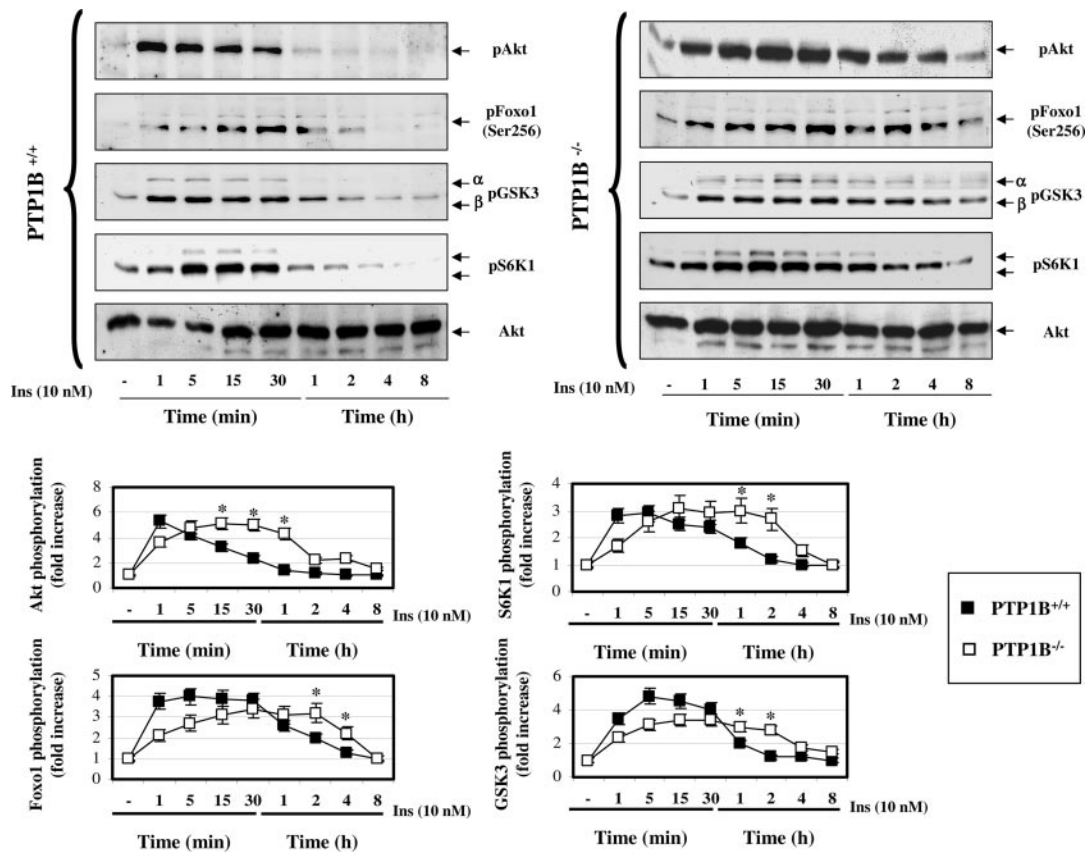
FIG. 6. Differential effect of insulin on the activation of PI 3-kinase effectors in immortalized wild-type and PTP1B$^{-/-}$ neonatal hepatocytes. Quiescent cells (15 h serum starved) were stimulated with 10 nM insulin for 1 min to 8 h or maintained in the absence of the hormone. Cells were then lysed and total protein (50 μg) was submitted to SDS-PAGE and analyzed by immunoblotting with the corresponding antibodies against phospho-Akt, total Akt, phospho-S6K1, phospho-GSK3-(α/β), and phospho-Foxo1. A representative experiment is shown. The autoradiograms corresponding to three experiments, performed in three independent cell lines from each genotype, were quantitated by scanning densitometry. The value of nonstimulated PTP1B$^{+/+}$ and PTP1B$^{-/-}$ cells was set to 1. Results are expressed as fold increase by insulin of Akt, Foxo1, S6K1, and GSK3 phosphorylation and are means ± SE. Statistical significance was carried out by Student's t test comparing immortalized PTP1B$^{-/-}$ neonatal hepatocytes with the respective values of wild-type cells. *, $P < 0.05$ was considered significant.

ciency delays onset of type 2 diabetes in IRS-2-deficient mice (38). The fact that immortalized neonatal hepatocytes developed in our laboratory have provided a unique tool for the *in vitro* study of insulin signaling leading to the expression of genes implicated in liver glucose metabolism (11) and survival (12) prompted us to generate immortalized hepatocytes from wild-type and PTP1B-deficient neonates. By using this model system, we have addressed whether the increase in insulin sensitivity provoked by PTP1B deficiency in adult liver is acquired through postnatal development.

PTP1B$^{+/+}$ and PTP1B$^{-/-}$ immortalized neonatal hepatocyte cell lines were grown in arginine-free medium to select cells with functional urea cycle. Consequently, we have circumvented the potential contamination of the primary cultures with nonparenchymal cells as confirmed by the absence of anti-VIM staining. In addition, all cell lines maintained the expression of hepatocyte markers such as ALB and CPS.

Unexpectedly, our studies reveal that, in contrast to very recent data published in livers from adult mice (26), PTP1B-deficient neonatal hepatocytes did not display increased sensitivity to insulin in the early steps of the insulin signaling network. In particular, tyrosine phosphorylation of IR β-chain and IRS-1, -2-associated PI 3-kinase activity was un-

affected by the lack of PTP1B at a wide range of insulin concentrations (0.1–100 nM). However, we have observed significant differences in the expression of these endogenous proteins between immortalized PTP1B$^{+/+}$ and PTP1B$^{-/-}$ neonatal hepatocytes. Surprisingly, the expression of IR β-chain was down-regulated in immortalized PTP1B$^{-/-}$ neonatal hepatocytes without an accompanying change in the expression of the IGF-IR. This phenomenon might be regulated by tissue-specific developmental mechanisms because this pattern of IR expression was not observed in PTP1B-deficient fibroblasts (39). Alternatively, these results can be explained on the basis of recent data (40) demonstrating basal (insulin-independent) interaction of PTP1B with IR during biosynthesis of the IR precursor in the endoplasmic reticulum. This interaction, which triggers dephosphorylation of tyrosyl residues of the IR precursor in the absence of insulin binding, regulates the IR precursor during its biosynthesis and may be important to prevent insulin-independent autonomous activity of the immature IR precursor. In neonatal hepatocytes, if the lack of PTP1B prevents IR precursor dephosphorylation, this effect might impair IR processing. The fact that IR expression is down-regulated in liver extracts and in primary hepatocytes of PTP1B-deficient
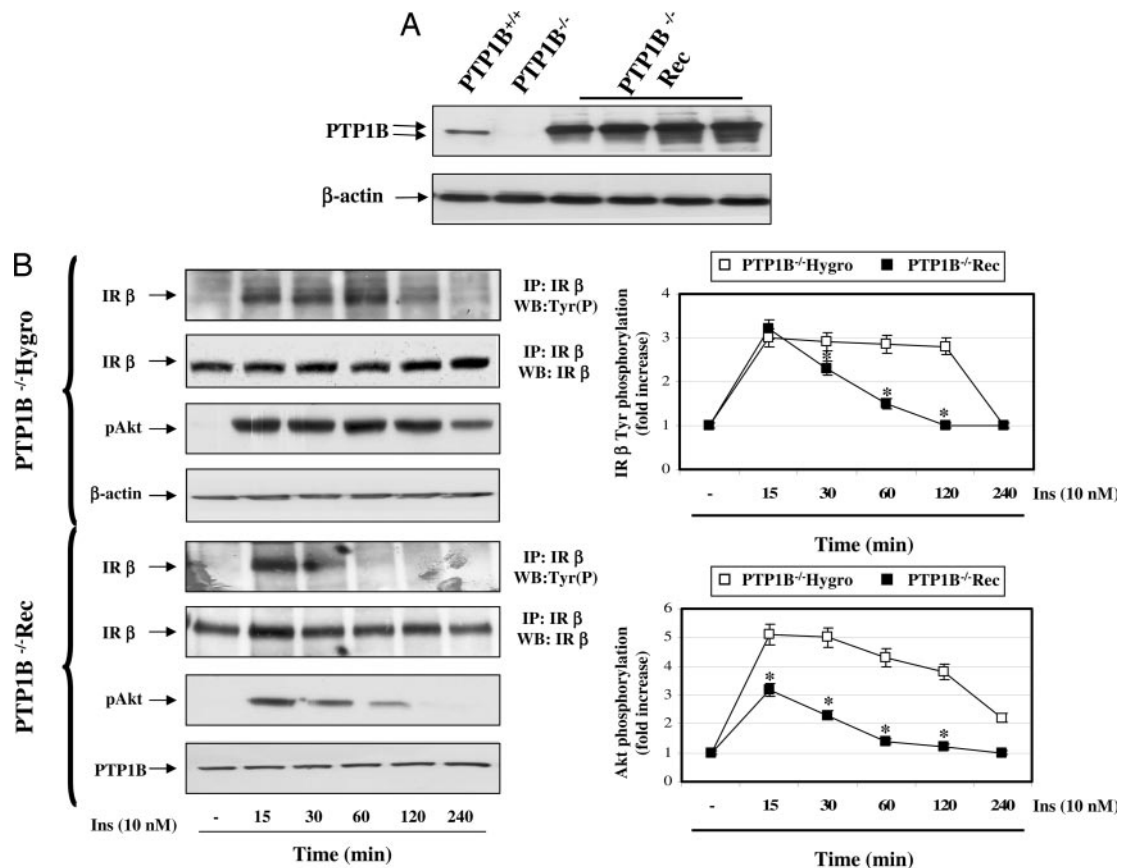
FIG. 7. Effects of PTP1B rescue in insulin signaling of immortalized PTP1B-deficient neonatal hepatocytes. A, Immortalized PTP1B-deficient neonatal hepatocytes were reconstituted with retroviral PTP1B as described in *Materials and Methods*. PTP1B expression in four different cell lines was assessed by Western blot. B, Reconstituted hepatocytes (PTP1B$^{-/-}$Rec) or PTP1B$^{-/-}$ hepatocytes infected with empty retroviral vector (PTP1B$^{-/-}$Hygro) were serum starved for 15 h and further stimulated with 10 nM insulin for various time periods. At the end of the culture time, cells were lysed and 1 mg of total protein was immunoprecipitated with anti-IR β-chain antibody. The resulting immune complexes were analyzed by Western blotting with anti-Tyr(P) or anti-IR antibodies as indicated in each panel. Representative autoradiograms are shown. Phosphorylation of Akt was assessed by direct Western blot. The autoradiograms corresponding to three experiments, performed in three independent cell lines from each genotype, were quantitated by scanning densitometry. The value of nonstimulated PTP1B$^{-/-}$Rec and PTP1B$^{-/-}$Hygro cells was set to 1. Results are expressed as fold increase by insulin of phosphorylated IR and Akt and are means ± SE. Statistical significance was carried out by Student's *t* test comparing immortalized PTP1B$^{-/-}$Rec hepatocytes with the respective values of PTP1B$^{-/-}$Hygro cells. *, $P < 0.05$ was considered significant.

neonates reinforces our data in immortalized neonatal hepatocytes. However, further studies will be needed to address this important issue. Additionally, as discussed subsequently, we found that endogenous levels of IRS-1 were increased in neonatal immortalized and primary hepatocytes lacking PTP1B, whereas IRS-2 expression was down-regulated. Similar changes were also observed in livers of PTP1B-deficient neonates.

Regarding insulin signaling, our experiments demonstrate that in contrast to the adult liver, the lack of PTP1B substantially prolongs phosphorylation of IR β-chain, IRS-1, and IRS-2 in immortalized neonatal hepatocytes compared with wild-type cells (Figs. 3–5) but does not increase insulin sensitivity (Fig. 2A). It is noteworthy that in PTP1B$^{-/-}$ neonatal hepatocytes, there is an increase in basal tyrosine phosphorylation of IRS-1 and IRS-2, which is associated with a detectable basal PI 3-kinase activity. However, the increase in basal signaling is not due to increased IGF-IR expression or increased basal tyrosine phosphorylation of IGF-IR in immortalized neonatal PTP1B-deficient hepatocytes. Thus, the

lack of PTP1B might modulate the basal state by increasing basal tyrosine phosphorylation of IRS proteins. Consistent with this, the net increase in PI 3-kinase activity after insulin stimulation is higher in wild-type than in PTP1B$^{-/-}$ cells. Nevertheless, tyrosine phosphorylation of IRS-1 and IRS-2 was prolonged in PTP1B$^{-/-}$ neonatal hepatocytes in parallel to the association with p85α/PI 3-kinase and, subsequently, activation of its enzymatic activity. These results suggest that, at least in neonatal hepatocytes, a sustained IRS-1,-2/PI 3-kinase signaling could be more important for insulin-regulated gene expression than the net increase during the first 5–10 min.

Downstream of PI 3-kinase, phosphorylation of Akt is a critical step in controlling metabolic actions elicited by insulin. Despite the lack of insulin sensitivity in the early steps of the insulin signaling cascade, the current study reveals that basal Akt phosphorylation and the insulin response at low doses are augmented in neonatal hepatocytes lacking PTP1B. Our results are in agreement with those reported by Shimizu *et al.* (34) indicating that a serial activation of the
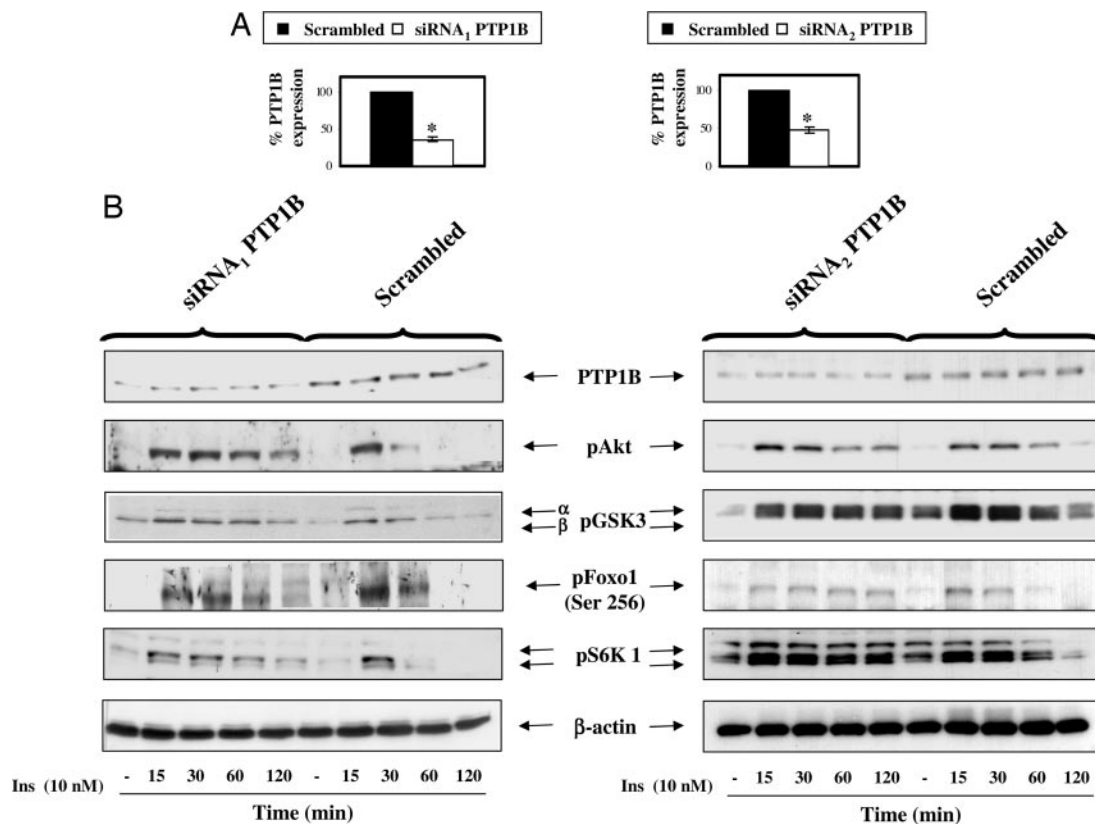
FIG. 8. Effects on insulin signaling of siRNA-mediated suppression of PTP1B in wild-type cells. A, Wild-type cells were electroporated with scrambled or PTP1B specific siRNAs. After 48 h, total lysates were prepared in Laemmli buffer, resolved by SDS-PAGE, and immunoblotted with antibodies against PTP1B or β-actin as a loading control. The autoradiograms corresponding to four independent experiments were quantitated by scanning densitometry. The value of PTP1B content in cells electroporated with scrambled oligo was set to 100%. Results are expressed as percentage of decrease of PTP1B in cells electroporated with siRNA oligos and are means ± SE. Statistical analysis was carried out by Student's *t* test comparing cells treated with PTP1B siRNA oligos with the respective values of cells treated with scrambled oligo (*, $P <$ 0.05). B, Wild-type cells were electroporated with scrambled or PTP1B specific siRNAs. After 48 h, cells were serum starved and further stimulated with 10 nM insulin for various time periods. Total lysates were prepared in Laemmli buffer, resolved by SDS-PAGE, and immunoblotted with specific antibodies. These experiments were repeated twice with each siRNA oligo.

PTP1B/PP2A axis might modulate Akt phosphorylation in an insulin-dependent (low doses) and -independent (basal state) manner. However, we cannot exclude the possibility that other targets of this phosphatase could also regulate activation of Akt in hepatocytes. Nevertheless, it is noteworthy that PTP1B deficiency in neonatal hepatocytes prolonged the insulin effect on Akt phosphorylation and its downstream targets GSK-3, Foxo1, and S6K1. It has been proposed that phosphorylation and inactivation of GSK-3 serves as a physiologically relevant mechanism for activating glycogen synthase (41). In addition, Foxo1 is phosphorylated in an insulin-responsive manner by Akt and then excluded from the nucleus (42–44). Previous studies in hepatoma cells suggest that Foxo1 and Foxo3 regulate the transcription of reporter genes containing insulin response elements from the PEPCK and G6Pase promoters (45, 46). Of note, it has been demonstrated very recently that Foxo proteins promote hepatic glucose production through multiple mechanisms, thereby exerting a pivotal role in the metabolic regulation of the adaptation to fasting and feeding in the liver (47). Indeed, insulin signaling through Akt/Foxo1 has been shown to inhibit the expression of the coactivator PGC-1α, which is induced in the liver in the fasting state and in various mouse

models deficient for insulin action and is required for the expression of PEPCK and G6Pase (48). Thus, the prolonged phosphorylation of Akt/Foxo1 may have metabolic consequences in the regulation of gluconeogenic gene expression in PTP1B$^{-/-}$ neonatal hepatocytes.

The molecular consequences on insulin signaling provoked by PTP1B deficiency in neonatal hepatocytes have been validated by two different approaches. First, rescue of PTP1B in deficient cells suppressed the prolonged insulin signaling. Second, reduction of PTP1B by two different siRNA oligos in wild-type cells prolonged signaling similar to our observations in PTP1B-deficient cell lines. Collectively, these results indicate that the lack of PTP1B in neonatal hepatocytes prolongs the insulin effect on IR/IRS-1,-2/PI 3-kinase/Akt/Foxo1 signaling, which results in a sustained activation of downstream proteins that control insulin's metabolic actions in the liver.

Suppression of glucose output is a critical mechanism of hepatic insulin sensitivity. Another unexpected finding in this study was the fact that PTP1B deficiency in neonatal hepatocytes did not decrease the dose at which insulin down-regulated PEPCK and G6Pase mRNAs despite the slight effect of PTP1B deletion on the enhanced response of Akt
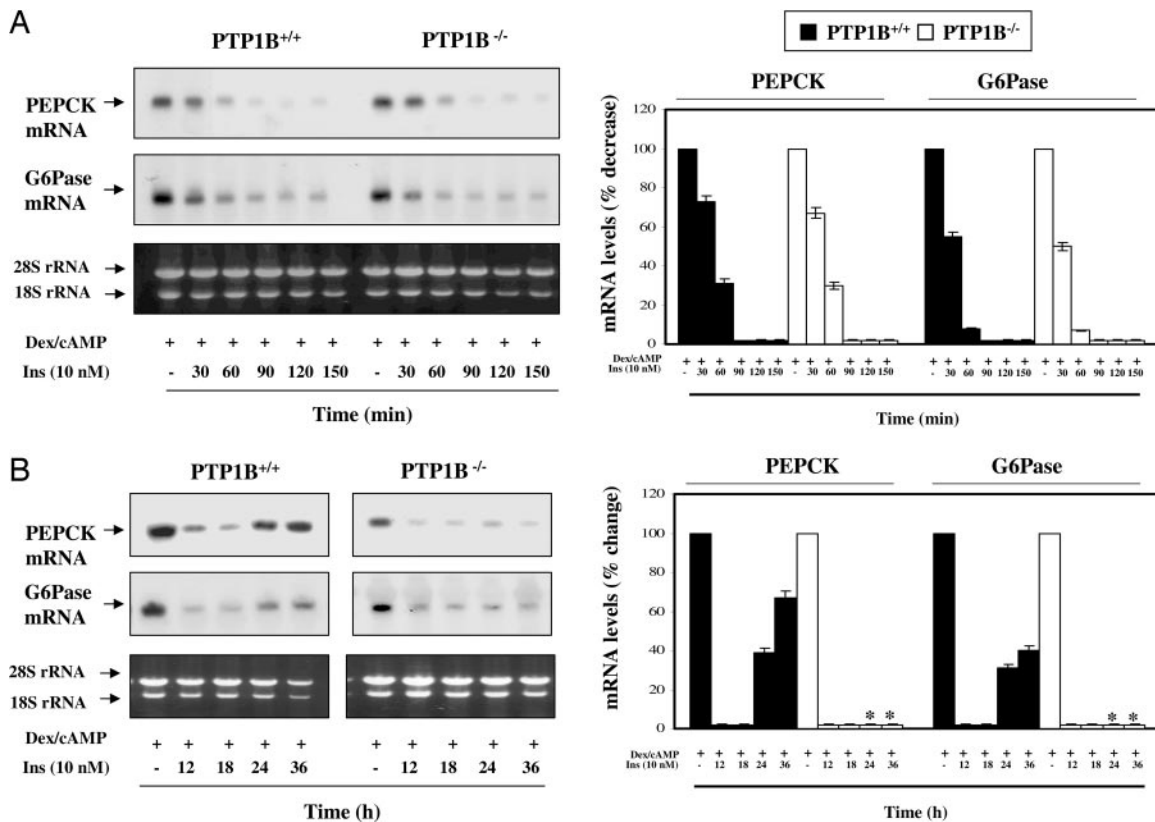
FIG. 9. Prolonged inhibition by insulin of dex/cAMP-induced PEPCK and G6Pase gene expression in primary PTP1B-deficient neonatal hepatocytes. A, Primary hepatocytes, obtained from a pool of 3.5- to 5-d-old mice, were cultured to confluence as described in *Materials and Methods*. Then, cells were cultured in serum-free medium for 6 to 8 h and further stimulated with dex/cAMP as described in Fig. 2. Then, insulin (10 nM) was added for 30 min to 3.5 h. At the end of the culture time, RNA was isolated and submitted to Northern blot analysis. Representative autoradiograms are shown. The autoradiograms corresponding to four independent experiments were quantitated by scanning densitometry. The value of dex/cAMP-treated cells was set to 100%. Results are expressed as percentage of decrease by insulin of PEPCK and G6Pase mRNAs and are means ± SE. B, Primary neonatal hepatocytes were cultured as described previously. Insulin (10 nM) was added for 12 to 36 h. At the end of the culture time, RNA was isolated, submitted to Northern blot analysis, and hybridized with labeled PEPCK and G6Pase cDNAs. Representative autoradiograms are shown. The autoradiograms corresponding to four independent experiments were quantitated by scanning densitometry. The value of dex/cAMP-treated cells was set to 100%. Results are expressed as percentage of decrease by insulin of PEPCK and G6Pase mRNAs and are means ± SE. Statistical significance was carried out by Student's t test comparing primary PTP1B$^{-/-}$ neonatal hepatocytes with the respective values of wild-type cells. *, $P < 0.05$ was considered significant.

phosphorylation at low insulin concentrations. Moreover, we have not found differences in the time course of inhibition of gluconeogenic enzymes on insulin treatment. However, the prolonged IR/IRS-1,-2/PI3-kinase/Akt/Foxo1 signaling of PTP1B$^{-/-}$ neonatal hepatocytes was accompanied by a prolonged inhibitory effect of insulin on PEPCK and G6Pase mRNAs. In addition, phosphorylation of GSK-3, which was prolonged in the absence of PTP1B, could also regulate gluconeogenic gene expression through the inactivation of transcription factors such as CCAAT/enhancer-binding protein β or cAMP response element binding protein, which have potent stimulatory effects on PEPCK gene transcription (49). Of note, the restoration of PEPCK and G6Pase mRNAs after long insulin treatment in wild-type cells did not correlate with an increase in PTP1B content (data not shown). Thus, the agreement of our signaling and gluconeogenic gene expression data in neonatal hepatocytes indicate that in these cells, the effect of PTP1B deletion is manifested by prolonged insulin action rather than in higher short-term responses.

As mentioned previously, the overall results presented in this study challenge recently published findings regarding insulin action in livers of adult PTP1B-deficient mice (26). Our data indicate that, in PTP1B-deficient mice, there is a switch from prolonged insulin action to enhanced insulin sensitivity in a postnatal developmental manner. One possible molecular explanation for our hypothesis is the developmental changes in the expression profile of key proteins that control insulin signaling (*i.e.* IR, IRS-1,-2, p85α), which might regulate the transition from prolonged insulin action to increased insulin sensitivity in liver of PTP1B-deficient mice. In fact, reduced expression of IR in the liver and hepatocytes of PTP1B-deficient neonates compared with the wild type was not observed in adult mice. It is possible that, at this stage of development, other tyrosine phosphatases might compensate the lack of PTP1B, allowing IR precursor dephosphorylation and processing like in wild-type hepatocytes. More importantly, the significant down-regulation of IRS-2 in neonatal PTP1B-deficient hepatocytes compared with the wild type was not observed in adult PTP1B-deficient hepatocytes. As IRS-2 mediates PI phosphate (PIP)3/Akt signaling in hepatocytes (11), its recovery in adult PTP1B-deficient hepatocytes may contribute to the gain of insulin
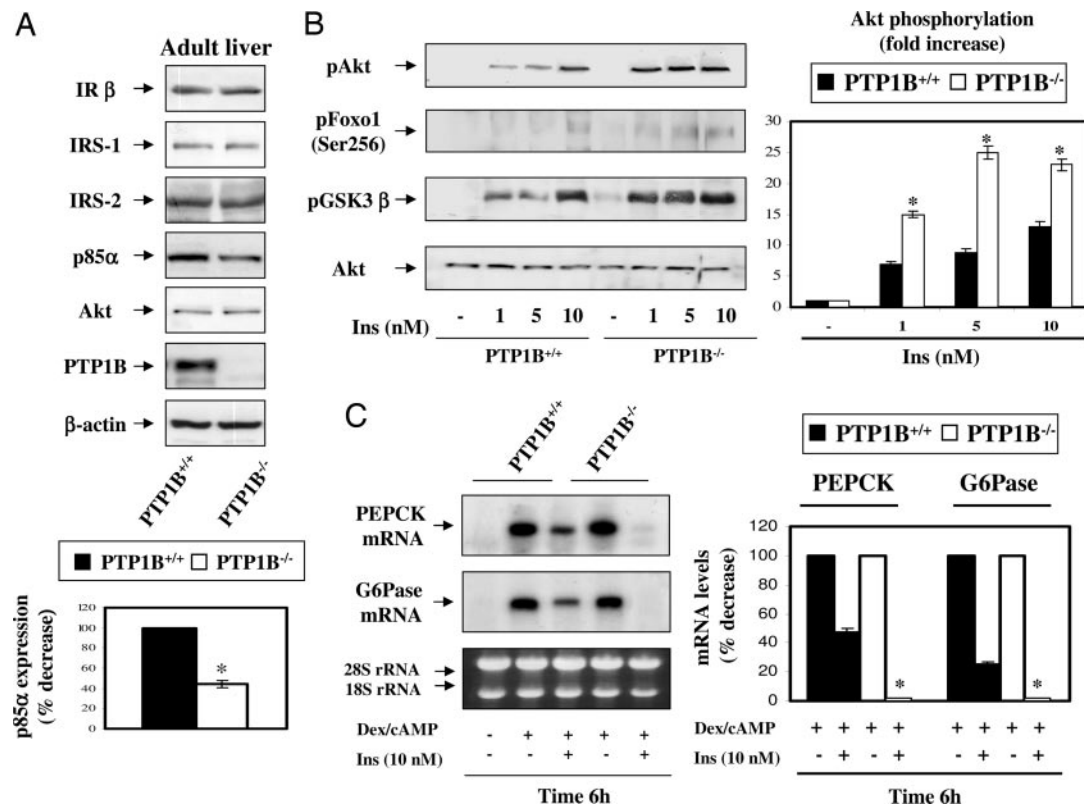
FIG. 10. The expression profile of key molecules of the insulin signaling cascade in PTP1B$^{-/-}$ adult liver confers insulin sensitivity in hepatocytes. A, Lysates from six to eight adult livers from PTP1B$^{+/+}$ and PTP1B$^{-/-}$ genotypes were analyzed by Western blot with antibodies against insulin signaling molecules. Representative autoradiograms corresponding to liver extracts from one mouse are shown. The autoradiograms showing p85α expression from six to eight animals were quantitated by scanning densitometry. Results are expressed as percentage of decrease of p85α content in PTP1B$^{-/-}$ liver extracts compared with the wild type (set to 100%) and are means $\pm$ SE. Statistical significance was carried out by Student's *t* test comparing PTP1B$^{-/-}$ livers with the respective values of the wild type. *, $P < 0.05$ were considered significant. B, Quiescent primary hepatocytes, isolated from three adult wild-type (PTP1B$^{+/+}$) and PTP1B$^{-/-}$ mice, were stimulated with various doses of insulin for 10 min or maintained untreated. Cells were lysed and total protein (50 μg) was submitted to SDS-PAGE and analyzed by immunoblotting with the corresponding antibodies against phospho-Akt (ser473), total Akt, phospho-GSK3, and phospho-Foxo1 (ser256). Representative autoradiograms corresponding to primary hepatocytes isolated from one animal are shown. The autoradiograms corresponding to the experiments performed in hepatocytes isolated from three separate mice from each genotype were quantitated by scanning densitometry. The value of nonstimulated cells was set to 1. Results are expressed as fold increase by insulin of Akt, Foxo1, and GSK-3 phosphorylation and are means $\pm$ SE. Statistical significance was carried out by Student's *t* test comparing primary PTP1B$^{-/-}$ adult hepatocytes with the respective values of wild-type cells. *, $P < 0.05$ was considered significant. C, Primary hepatocytes obtained from adult mice were cultured as described in *Materials and Methods*. Then, cells were cultured in serum-free medium for 12 to 15 h and further stimulated with dex/cAMP as described in Fig. 2. Then, insulin (10 nM) was added for a further 6 h. At the end of the culture time, RNA was isolated and submitted to Northern blot analysis. Representative autoradiograms corresponding to primary hepatocytes isolated from one mouse are shown. The autoradiograms corresponding to the experiments performed in hepatocytes isolated from three separate mice from each genotype were quantitated by scanning densitometry. The value of dex/cAMP-treated cells was set to 100%. Results are expressed as percentage of decrease by insulin of PEPCK and G6Pase mRNAs and are means $\pm$ SE. Statistical significance was carried out by Student's *t* test comparing PTP1B$^{-/-}$ adult hepatocytes with the respective values of wild-type cells. *, $P < 0.05$ was considered significant.

sensitization as occurs in suppressor of cytokine signaling1$^{-/-}$ mice (50). In contrast to IR and IRS-2 expression, IRS-1 is highly expressed in the livers and hepatocytes of PTP1B-deficient neonates, but its expression decreased in the adult (data not shown). Preliminary studies in our laboratory indicate that PTP1B-deficient neonates have a slight but significant increase in liver weight compared with wild-type neonates. Given that IRS-1 signaling mediates somatic growth and development (51), the up-regulation of IRS-1 in the livers of PTP1B-deficient neonates might be responsible for this phenotype. Finally, another possible explanation for the differences in insulin sensitivity between neonatal and adult PTP1B-deficient hepatocytes is the p85α regulatory subunit of PI 3-kinase. During recent years, significant evi-

dence has accumulated indicating that changes in the molecular balance between the regulatory subunit and the catalytic subunit may affect the PI 3-kinase-dependent signaling and insulin sensitivity. Thus, p85α can play a dual role in insulin action mediating PI 3-kinase activation by bridging IRS proteins and the catalytic p110 subunit but can also act as a competitive inhibitor in PI 3-kinase signaling in its monomeric state. Interestingly, p85$^{+/-}$ mice exhibited increased insulin sensitivity (52) and display an increase in Akt in livers. This appears to be due to an improved stoichiometry of the p85/p110/IRS complex and enhanced PI 3-kinase-dependent signaling. Our data clearly demonstrate that although p85α expression was significantly down-regulated in the liver of adult PTP1B-deficient mice, this effect does not

occur just after birth. Indeed, insulin sensitivity was observed in primary hepatocytes from adult PTP1B-deficient mice in Akt signaling and, of note, in the inhibition of gluconeogenic gene expression.

In conclusion, the results presented in this paper demonstrate a direct effect of PTP1B deficiency in prolongation of insulin signaling and inhibition of gluconeogenic gene expression in neonatal hepatocytes without significant changes in insulin sensitivity. During postnatal development of the liver of PTP1B-deficient mice, changes in the expression of IR and IRS-2 together with the establishment of a molecular balance between the regulatory (p85α) and the catalytic subunits of PI 3-kinase in the liver of PTP1B-deficient mice may mediate a switch from prolonged insulin action (neonatal hepatocytes) to the acquisition of insulin sensitivity (adult hepatocytes). Consequently, inhibition of PTP1B has different effects on insulin signaling and gluconeogenic gene expression depending on the stage of liver development. However, cross talk between insulin target tissues may contribute to hepatic insulin action after birth or in adult animals and should not be excluded.

Acknowledgments

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