

PTP1B deficiency increases glucose uptake in neonatal hepatocytes: involvement of IRA/GLUT2 complexes

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González-Rodríguez Á, Nevado C, Escrivá F, Sesti G, Rondinone CM, Benito M, Valverde ÁM. PTP1B deficiency increases glucose uptake in neonatal hepatocytes: involvement of IRA/GLUT2 complexes. *Am J Physiol Gastrointest Liver Physiol* 295: 338–347, 2008. First published June 5, 2008; doi:10.1152/ajpgi.00514.2007.—The contribution of the liver to glucose utilization is essential to maintain glucose homeostasis. Previous data from protein tyrosine phosphatase (PTP) 1B-deficient mice demonstrated that the liver is a major site for PTP1B action in the periphery. In this study, we have investigated the consequences of PTP1B deficiency in glucose uptake in hepatocytes from neonatal and adult mice. The lack of PTP1B increased basal glucose uptake in hepatocytes from neonatal (3–5 days old) but not adult (10–12 wk old) mice. This occurs without changes in hexokinase, glucokinase, and glucose 6-phosphatase enzymatic activities. By contrast, the glucose transporter GLUT2 was upregulated at the protein level in neonatal hepatocytes and livers from PTP1B-deficient neonates. These results were accompanied by a significant increase in the net free intrahepatic glucose levels in the livers of PTP1B^{-/-} neonates. The association between GLUT2 and insulin receptor (IR) A isoform was increased in PTP1B^{-/-} neonatal hepatocytes compared with the wild-type. Indeed, PTP1B deficiency in neonatal hepatocytes shifted the ratio of isoforms A and B of the IR by increasing the amount of IRA and decreasing IRB. Moreover, overexpression of IRA in PTP1B^{-/-} neonatal hepatocytes increased the amount of IRA/GLUT2 complexes. Conversely, hepatocytes from adult mice only expressed IRB. Since IRA plays a direct role in the regulation of glucose uptake in neonatal hepatocytes through its specific association with GLUT2, we propose the increase in IRA/GLUT2 complexes due to PTP1B deficiency as the molecular mechanism of the increased glucose uptake in the neonatal stage.

liver; insulin receptor isoforms; glucose transport; insulin sensitivity

TYPE 2 DIABETES MELLITUS is a polygenic disease affecting over 100 million people worldwide. Affected patients manifest insulin resistance, hyperinsulinemia, and hyperglycemia (33). Among the peripheral tissues involved in the control of glucose homeostasis, the liver plays a major role because this organ has the ability to consume and produce glucose. Accordingly, deregulation between glucose uptake and production by the liver is thought to contribute to the onset of type 2 diabetes (9). It is now known that in the postprandial state the liver takes up

approximately one-third of an oral glucose load, which is incorporated into glycogen and fatty acids or oxidized into CO₂ (31, 32). Previous studies have demonstrated that under physiological conditions a “portal signal” induced by the negative arterial-portal glucose gradient seen with feeding creates an important signal to enhance net hepatic glucose uptake (NHGU) or hepatic glycogen deposition in rodents (8), dogs (1), and humans (10). NHGU was increased by a rise in insulin and further stimulated by the presence of a portal signal. Thus NHGU is dependent on the amount of glucose reaching the liver, the insulin level within the hepatic sinusoids, and a signal generated by portal glucose delivery. Therefore, the roles of hyperglycemia and hyperinsulinemia in regulating hepatic glucose uptake have extensively been studied.

Among the animal models mimicking hepatic insulin resistance, liver-specific insulin receptor knockout (LIRKO) mice showed an impaired insulin tolerance and also a postprandial hyperglycemia in the fed state. Euglycemic/hyperinsulinemic clamps also revealed a significant decrease of total glucose utilization in response to insulin in fed LIRKO mice compared with their controls, an indication of insulin resistance (16). At the molecular level, in a recent study in neonatal hepatocytes, bearing or void of insulin receptor (IR), we have demonstrated that IR plays a direct role in the regulation of basal glucose uptake through its specific association with endogenous (GLUT2), but not with exogenous (GLUT4), glucose transporters (28).

Several mice models showing increased hepatic insulin sensitivity phenotype have been reported. Among them, protein tyrosine phosphatase (PTP)1B-deficient mice have revealed a unique role of this phosphatase as a negative modulator of insulin signaling and insulin action in the liver (13, 22, 25). In fact, the suppression of hepatic glucose production (HGP) by insulin is increased in 8–10-wk-old PTP1B-deficient mice (13, 22). However, we have recently shown that hepatic insulin sensitization due to the lack of PTP1B is acquired during postnatal development in mice since adult, but not neonatal, PTP1B-deficient mouse hepatocytes showed increased insulin signaling and a more pronounced inhibition of gluconeogenic gene expression compared with the wild-type (20). Since no data has been reported regarding the effect of PTP1B defi-

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ciency on glucose uptake in hepatocytes, the aim of this study was to investigate whether or not PTP1B deficiency improves glucose uptake in hepatocytes in the neonatal (3–5 days old) or adult (8–12 wk old) postnatal developmental stages that display substantial differences in insulin sensitivity.

MATERIALS AND METHODS

Materials. Fetal calf serum and culture media were obtained from Gibco (Gaithersburg, MD). The reagents for the measurement of glucokinase (GK), hexokinase (HK), and glucose 6-phosphatase (G6Pase) activities, insulin, hygromycin, puromycin, and monoclonal anti- β -actin (clone AC-15, no. A5441) antibody were from Sigma Chemical (St. Louis, MO). Protein A-agarose was from Roche Molecular Biochemicals (Mannheim, Germany). The anti-IR β -subunit (sc-711) and anti-GLUT2 (sc-9117) antibodies were purchased from Santa Cruz Biotechnology (Palo Alto, CA). The anti-GLUT1 (Ref. CBL 242) antibody was from Chemicon International (Temecula, CA). The anti-GK antibody was a gift of J. J. Guinovart (Barcelona, Spain). The antibody against IRB isoform (+*exon 11*) was previously described (38).

Primary culture of neonatal hepatocytes. PTP1B-deficient mice (25) were obtained from Abbott Laboratories (Abbott Park, IL). All animal studies were approved by the Consejo Superior de Investigaciones Científicas (CSIC) Animal Care and Use Committee. Pools of 4–6 livers from wild-type (PTP1B^{+/+}) and PTP1B^{-/-} neonates (3–5 days old) were submitted to collagenase dispersion and isolated as previously described (15). Cells were plated in growing medium (199 MEM supplemented with 10% FBS, 25 ng/ml EGF, 0.1 μ M dexamethasone, and 50 nM insulin) and used for further experiments.

Immortalized neonatal hepatocyte cell lines. Viral BOSC 23 packaging cells were transfected at 70% confluence with 3 μ g/6-cm dish of the puromycin-resistance retroviral vector pBabe encoding SV40 Large T antigen (kindly provided by J. de Caprio, Dana Farber Cancer Institute, Boston, MA) as previously described (41). Then, primary neonatal hepatocytes were infected at 60% confluence with polybrene-supplemented virus (4 μ g/ml) for 48 h and maintained in culture medium for 72 h before selection with puromycin (0, 5–1 μ g/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% FBS and ornithine to avoid growth of nonparenchymal cells. The experiments were performed in three independent cell lines from each genotype obtained by immortalization of three different preparations of neonatal hepatocytes.

Coding sequence for the human IR lacking *exon 11* (isoform A) cloned into pBabe-hygro retroviral vector was a gift of C. R. Kahn (Joslin Diabetes Center, Boston, MA). Viral particles were obtained, and PTP1B^{-/-} hepatocytes were infected with polybrene-supplemented (4 μ g/ml) virus for 48 h and then placed in selection medium containing hygromycin (200 μ g/ml) for at least 2 wk. As a control, PTP1B^{-/-} cells were infected with viral particles expressing empty vector (pBabe hygro).

Immortalized neonatal hepatocytes expressing IRA or IRB isoforms have been previously described (28).

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 (4). Cells were plated on 60-mm Primaria dishes (Falcon; BD Biosciences, San Jose, CA) and cultured in Williams' E medium supplemented with 20 ng/ml EGF, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FBS for 48 h. Then, cells were serum starved and used for experiments.

Homogenization and preparation of tissue extracts. Frozen livers were homogenized in 16 volumes (wt/vol) 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 μ g/ml phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml

pepstatin A, and 1 μ g/ml leupeptin, using the Brinkman 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 -80°C.

Isolation of plasma membranes. Partially purified fractions of liver plasma membranes were isolated following Hiol et al. (23), with modifications. Livers were homogenized with buffer H (0.25 M sucrose, 1 mM MgCl₂, and 5 mM Tris·HCl pH 7.4, containing protease inhibitors). The homogenate underwent centrifugation at 300 g for 5 min, the supernatant was saved, and the pellet was homogenized and centrifuged as above. The first and second supernatants were combined and centrifuged at 1,500 g for 10 min. The pellet was resuspended in buffer H and then buffer F (2 M sucrose, 1 mM MgCl₂, and 5 mM Tris·HCl, pH 7.4) was added to obtain a final sucrose concentration of 1.42 M. The suspension was transferred to a nitrocellulose tube and overlaid with buffer H. The samples were then centrifuged for 60 min at 120,000 g (Beckman SW-60 Ti rotor); the pellicle at the interface was then collected, resuspended in buffer H, and centrifuged for 10 min at 1,500 g. Finally, the pellet (plasma membrane fraction) was resuspended in 20 mM EDTA, 1% Nonidet P-40, and 50 mM Tris·HCl pH 7.4.

Immunoprecipitations and Western blot. Quiescent cells (15 h serum-starved) were lysed as previously described (20). After protein content determination, equal amounts of protein (600 μ g-1 mg) were immunoprecipitated at 4°C with the corresponding antibodies. The immune complexes were collected on protein A-agarose beads and submitted to SDS-PAGE. Proteins were transferred to Immobilon membranes and incubated overnight with several antibodies as indicated. Immunoreactive bands were visualized using the enhanced chemiluminescence Western blotting protocol (Amersham, Piscataway, NJ).

Protein determination. Protein determination was performed by the Bradford dye method (7) using the Bio-Rad (Hercules, CA) reagent and BSA as the standard.

Measurement of glucose uptake. Hepatocytes were plated at high density (60–70% confluence) and maintained in growing medium for 48 h. Cells were then cultured in serum-free medium for a further 15 h, and glucose uptake was measured as previously described (42). Cells were washed three times with Krebs-Ringer-phosphate buffer (KRP) containing 135 mM NaCl, 5.4 mM KCl, 1.4 mM CaCl₂, 1.4 mM MgSO₄, and 10 mM sodium pyrophosphate, pH 7.4, and then incubated with 1 ml KRP buffer with or without insulin (10 nM) for 10 min at 37°C. 2-Deoxy-D[1-³H]glucose was added to this solution to a final concentration of 0.1 mM and 250 nCi/ml, and the incubation was continued for 10 min. The cells were then washed three times with ice-cold KRP buffer and solubilized in 1 ml 1% SDS. The radioactivity of a 200- μ l aliquot was determined in a scintillation counter. After protein content determination, individual values were expressed as picomoles of glucose per 10 min per milligrams protein, and results were expressed as fold increase over basal wild-type cells.

Measurement of enzymatic activities. For enzymatic assays, liver homogenates (10% wt/vol) were prepared in sucrose-Tris buffer (10 mM Tris·HCl pH 7.4, 0.25 M sucrose, and 0.5 mM EDTA) at 4°C. The homogenates were centrifuged at 12,000 g for 20 min, and the resulting supernatants were centrifuged at 105,000 g for 45 min. The supernatants were used for GK and HK assays, and the pellets (microsomes) were used for the G6Pase determination. GK and HK activities were measured by spectrophotometric assays in presence of 45 mM Tris·HCl, pH 7.4, 110 mM KCl, 8 mM MgCl₂, 0.5 mM NADP, 5 mM ATP and 0.9 U/ml of glucose-6-phosphate (G6P) dehydrogenase. Glucose (100 mM or 0.5 mM) was added for GK and HK determination, respectively (35). G6Pase activity was measured spectrophotometrically in the microsome fraction previously treated with sodium deoxycholate. The assay is based in the Pi liberation from G6P according to Zakko et al. (44).

Measurement of net free intrahepatic glucose. The freeze-clamped livers were extracted with 4 volumes of ice-cold 6% (wt/vol) HClO₄.

The protein precipitates were removed by centrifugation at 10,000 *g* for 10 min. The supernatant was neutralized with KOH. Glucose was assayed by using a glucose oxidase method (40).

RNA isolation and quantitative real-time PCR analysis. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using the reverse transcription system kit (Promega, Madison, WI). PCR reactions were run in triplicate using SYBR Green Master Mix Plus (Eurogentec, Seraing, Belgium) and quantitated using the ABI Prism 7700 sequence detection system (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's instructions. PCR primers for GLUT2 were described in Ref. 26. Cycle threshold values were normalized to 18S rRNA, and results were expressed as fold change of mRNA compared with the wild-type condition, which was arbitrarily assigned a value of 1.

RNA extraction and Northern blot analysis. RNA was isolated from livers of 3–5-day-old mice, and 20 μ g were submitted to Northern blot analysis. Blots were hybridized with the cDNA probe for GLUT2. Membranes were subjected to autoradiography, and the relative densities of the hybridization signals were determined by densitometric scanning of the autoradiograms.

Semi-quantitative RT-PCR. To analyze IR isoforms (IRA and IRB) in neonatal and adult primary hepatocytes by PCR, 1 μ g of total RNA was primed with oligodT (deoxythymidine) in the presence of murine mammary tumor virus reverse transcriptase (Invitrogen) to synthesize cDNA. The samples were diluted fivefold, and 5% of the total volume was used for subsequent PCR. Primers used were the following: mouse IR *exon 11* primer 1, 5'-ATCAGAGTGAGTATGACGACTCGG-3' and primer 2, 5'-TCCTGACTTGTGGGCACAATGGTA-3'; human IR *exon 11* primer 1, 5'-ACCAGAGTGAGTATGAGGATTCGG-3' and primer 2, 5'-TCCGGACTCGTGGGCACGCTGGTC-3'. For amplification of mouse β -actin, the following primers were used: primer 1, 5'-GGTATGGAATCCTGTGGCATCCATGAAA-3' and primer 2, 5'-GTGTAACGCAGCTCAGTAA-

CAGTCC-3'. PCR reactions were performed as described (14). Reaction products were resolved on 2% agarose gels.

Statistical analysis. Experiments performed at least three times are presented as mean values \pm SE. Comparisons among groups were made using two-tailed Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of PTP1B deficiency on glucose uptake in neonatal and adult hepatocytes. Hepatic glucose uptake and utilization is essential to maintaining whole body glucose homeostasis. We investigated whether the lack of PTP1B leads to changes in the rate of glucose entry in hepatocytes in a postnatal developmental manner. For this goal, we obtained primary hepatocytes from 3–5-day-old and 10–12-wk-old wild-type (PTP1B^{+/+}) and PTP1B-deficient (PTP1B^{-/-}) mice that represent distant stages of postnatal development. Primary hepatocytes were plated at high density (60–70% confluence) and maintained in growing medium for 48 h. Cells were then cultured in serum-free medium for a further 15 h, and glucose uptake was measured after 10-min incubation in the presence of ³H-2-deoxyglucose. As shown in Fig. 1A, the lack of PTP1B increased by 50% basal glucose uptake in neonatal hepatocytes compared with the corresponding wild-type controls. As expected, glucose uptake in neonatal hepatocytes was insulin independent.

We have recently generated immortalized hepatocyte cell lines from wild-type and PTP1B-deficient neonates (3–5 days old) (20) that have been proven to be unique tools to study insulin signaling and insulin actions due to the experimental

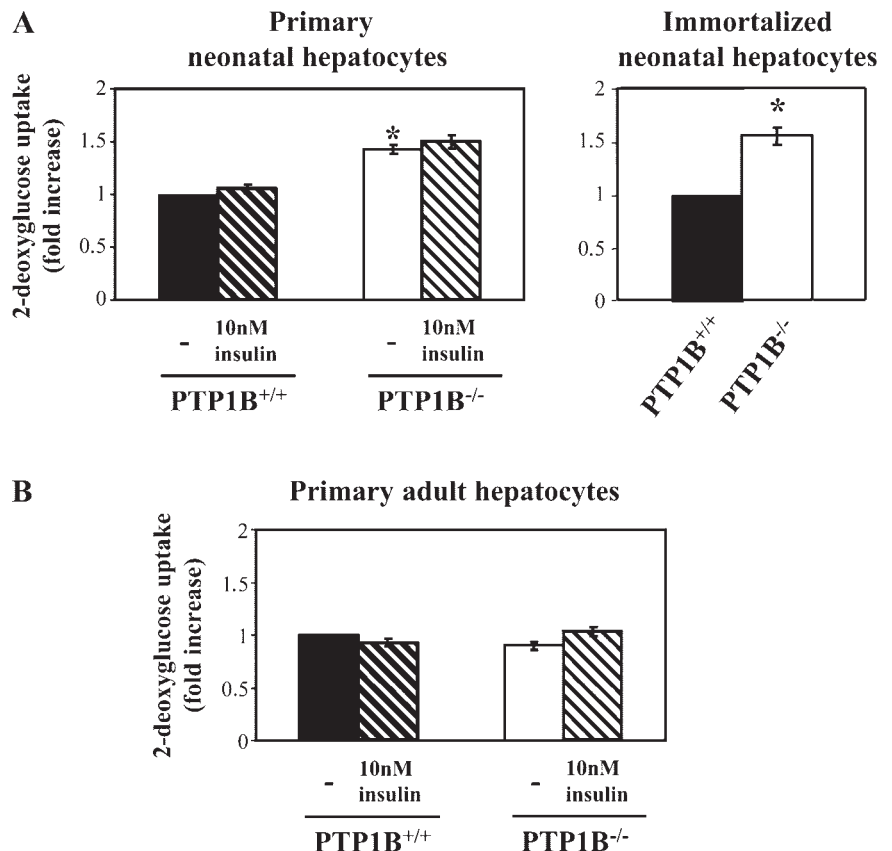


Fig. 1. Effect of protein tyrosine phosphatase (PTP)-1B deficiency on glucose uptake in neonatal and adult primary hepatocytes. Primary and immortalized neonatal (A) and primary adult (B) hepatocytes (obtained from 3–5-day-old and 10–12-wk-old mice, respectively) were cultured to confluence and then serum-starved for 15 h. Then, cells were incubated with the mix of 2-deoxy-D (1-³H)-glucose and cold glucose for 10 min. Glucose uptake was measured as described in MATERIALS AND METHODS. Results are expressed as fold increase over basal wild-type cells (set to 1) and are means \pm SE from 3 independent experiments with duplicate dishes. Statistical significance was carried out by Student's *t*-test by comparison of PTP1B^{-/-} with PTP1B^{+/+} cells (* $P < 0.05$).

limitations of the primary cultures. Glucose uptake was measured in immortalized neonatal hepatocytes from both genotypes under the same experimental conditions as in primary cells. Figure 1A shows similar increases in immortalized hepatocytes lacking PTP1B compared with the wild-type controls. Interestingly, glucose uptake did not change between wild-type and PTP1B^{-/-} primary hepatocytes from adult mice either in the absence or presence of 10 nM insulin (Fig. 1B).

Effect of PTP1B deficiency in the endogenous expression of GLUT2, GLUT1, and GK in neonatal and adult livers and hepatocytes. Next, we analyzed the differential expression of the ubiquitous glucose transporter GLUT1, the liver-specific glucose transporter GLUT2, and the hepatic glucose sensor GK in livers and primary hepatocytes from neonatal and adult mice lacking PTP1B. Of note, primary hepatocytes were cultured under the same experimental conditions as those used for the functional experiments. Figure 2A shows that the expression of GK was very low in neonatal livers and neonatal hepatocytes. These results agree with those previously reported (6, 34). Conversely, adult hepatocytes expressed high levels of GK, with no differences found between both genotypes. The ex-

pression of GLUT1 was detectable but remained unchanged in livers and primary hepatocytes from both neonatal and adult PTP1B-deficient mice compared with their respective wild-type controls. By contrast, GLUT2 was upregulated in liver extracts, plasma membranes, and hepatocytes from neonatal, but not adult, PTP1B-deficient mice (Fig. 2, A and B). To determine whether transcriptional changes may explain the differences observed in GLUT2, we examined transcript levels in livers from 3–5-day-old mice by real-time PCR and Northern blot analysis. As shown in Fig. 2C, no differences were found in GLUT2 expression at the mRNA level between neonatal livers of both genotypes.

Effect of PTP1B deficiency on HK, GK, and G6Pase activities and net free intrahepatic glucose in livers of neonatal and adult mice. Net hepatic glucose flux is the balance between the rate of glucose phosphorylation catalyzed by GK and the rate of dephosphorylation of G6P catalyzed by G6Pase. Importantly, in hepatocytes from suckling mice, constitutively expressed HK is the main enzyme responsible for glucose phosphorylation (6, 34). Next, we investigated whether changes in the activities of HK and G6Pase could account for the net

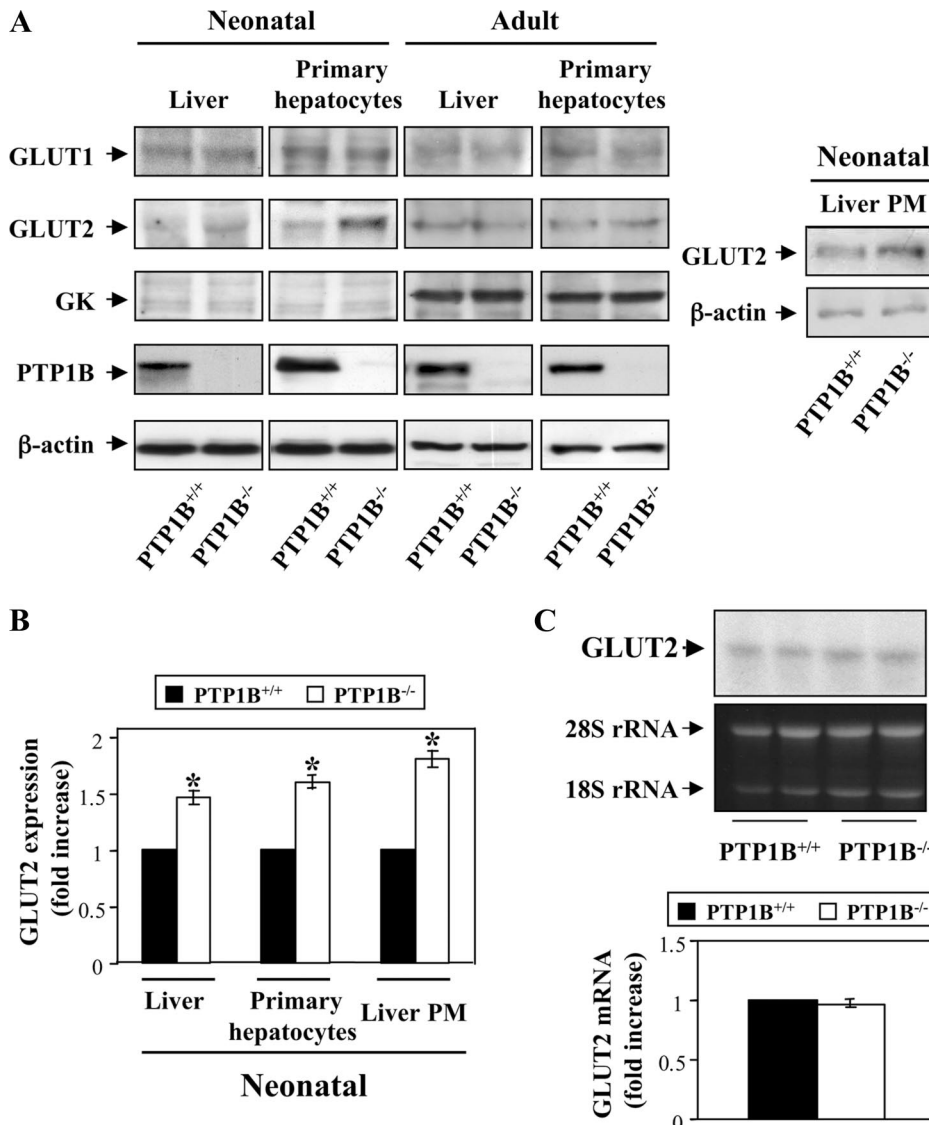


Fig. 2. Effect of PTP1B levels in the endogenous expression of glucose transporter 1 (GLUT1), GLUT2, and glucokinase (GK) in livers and primary hepatocytes from neonatal and adult wild-type and PTP1B-deficient mice. *A, left:* whole cell lysates from neonatal and adult primary hepatocytes and liver extracts from 6–8 neonatal and adult mice of each genotype were analyzed by SDS-PAGE followed by Western blot with antibodies against GLUT1, GLUT2, GK, and β-actin as a loading control. A representative experiment is shown. *Right:* plasma membrane (PM) fraction was isolated from neonatal livers, and the expression of GLUT2 was detected by Western blot. *B:* autoradiograms corresponding to GLUT2 protein content in liver extracts, hepatocytes, and PMs from wild-type and PTP1B^{-/-} neonates were quantitated by scanning densitometry. Results are expressed as fold change of GLUT2 protein content compared with the wild-type condition, which was arbitrarily assigned a value of 1, and are means ± SE. Statistical significance was carried out by Student's *t*-test comparing PTP1B^{-/-} primary hepatocytes, and PMs and total extracts from livers of neonatal mice with the respective values of wild-type controls. **P* < 0.05 was considered significant. *C, top:* RNA was isolated from livers of PTP1B^{+/+} and PTP1B^{-/-} neonatal mice, and GLUT2 mRNA levels were determined by Northern blot. *Bottom:* GLUT2 mRNA levels were determined by real-time PCR as described in MATERIALS AND METHODS. Results are expressed as fold change of mRNA compared with the wild-type condition, which was arbitrarily assigned a value of 1, and are means ± SE from 3 independent experiments.

increase in basal glucose uptake in neonatal PTP1B^{-/-} hepatocytes. For this goal, we prepared liver extracts from wild-type and PTP1B-deficient neonates, and enzymatic activities were measured in the cytosolic and microsomal fractions, respectively. Neither HK nor G6Pase activities were significantly different between neonatal wild-type and PTP1B-deficient livers (Table 1). Notably, in agreement with the similar expression of GK in PTP1B^{+/+} and PTP1B^{-/-} adult hepatocytes, GK enzymatic activity did not change in adult PTP1B^{-/-} livers compared with the wild-type. Together these data reinforce the notion that the increase in glucose uptake in PTP1B-deficient neonatal hepatocytes is not affected by changes in the rate of glucose phosphorylation/dephosphorylation. To investigate whether the increase in glucose uptake in PTP1B-deficient neonatal hepatocytes reflects intracellular changes in glucose levels in neonatal liver, we measured the net free intrahepatic glucose. As shown in Table 1, the net free intrahepatic glucose was significantly higher in livers from neonatal, but not adult, PTP1B-deficient mice compared with their respective wild-type controls. Although contamination of blood glucose could be present in the assay used to determine intrahepatic glucose on freeze-clamped livers, the results presented here are the means of 10–12 independent determinations for each genotype, and we can assume that the degree of possible contamination effects would be similar in both genotypes.

The lack of PTP1B in neonatal hepatocytes increased the ratio of IRA/IRB isoforms in neonatal, but not in adult, hepatocytes. We have previously shown that glucose uptake in neonatal hepatocytes expressing IRA isoform is higher than in those cells expressing IRB (28). Accordingly, our next goal was to investigate whether the changes in glucose uptake in PTP1B^{-/-} neonatal vs. adult hepatocytes correlated with alterations in the profile of IR isoforms (IRA - exon 11 and IRB + exon 11). As shown in Fig. 3A, primary hepatocytes from adult wild-type mice expressed only IRB mRNA. These results agree with those published by Giddings and Carnaghi (17) examining rat liver mRNA levels. Interestingly, the lack of PTP1B in adult hepatocytes did not change the profile of IR isoforms. At the protein level, similar expression of IRB was found in wild-type and PTP1B^{-/-} adult hepatocytes (Fig. 3B). Conversely, wild-type neonatal hepatocytes expressed both isoforms, IRB mRNA levels being nearly twofold higher than IRA mRNA (Fig. 4A). Moreover, the lack of PTP1B in neonatal hepatocytes increased the IRA/IRB ratio by doubling the amount of IRA mRNA in parallel with a 50% decrease in IRB mRNA. At the protein level, IRB was analyzed by immuno-

precipitation of total cell lysates from wild-type and PTP1B^{-/-} immortalized neonatal hepatocytes with a specific anti-IRB antibody (38). The resulting immune complexes were then analyzed by Western blot with the anti-IR antibody. As shown in Fig. 4B, IRB decreased by 60% in PTP1B^{-/-} neonatal hepatocytes compared with the wild-type controls. The expression of IRA was analyzed by immunoprecipitation of the cell lysates depleted of IRB with the anti-IR antibody. Figure 4B shows a twofold increase in IRA expression in neonatal hepatocytes lacking PTP1B. Since a specific anti-IRB antibody for immunoblotting is not available, we cannot check that the preclearing of IRB neonatal hepatocytes in wild-type and PTP1B^{-/-} is 100% efficient, despite two rounds of immunoprecipitation with the anti-IRB antibody having been performed. However, our results clearly show a direct correlation between the changes in the profile of IR isoforms at the mRNA and protein levels. In addition, we performed a control experiment by immunoprecipitating total cell lysates from neonatal hepatocytes expressing only IRB isoform (HIR RecB) with the anti-IRB antibody. The anti-IR Western blot revealed that at least 90% of IRB was depleted (Fig. 4B, left).

The lack of PTP1B increased IRA/GLUT2 complexes in immortalized neonatal hepatocytes and plasma membranes from neonatal liver. The coimmunoprecipitation of IR and GLUT2 has been reported in rat hepatocytes (12) and more recently in immortalized neonatal hepatocytes (28). Furthermore, the lack of IR in neonatal hepatocytes resulted in a significant decrease in basal glucose uptake, supporting the hypothesis that IR and GLUT2 form a receptor-transporter complex in hepatocytes. Importantly, the decrease in glucose uptake in IR-deficient neonatal hepatocytes was reverted by reexpressing the IRA isoform but not IRB (28). Since glucose uptake is increased in PTP1B^{-/-} neonatal hepatocytes in parallel with an increase in IRA isoform, we investigated the possibility of changes in the association between IRA and GLUT2. For that goal, cell lysates, obtained from growing (10% FBS) wild-type and PTP1B^{-/-} immortalized hepatocytes, were depleted of IRB by two rounds of immunoprecipitation with the specific anti-IRB antibody. The resulting cell lysates were then reimmunoprecipitated with the anti-GLUT2 antibody, and the immune complexes were analyzed by Western blot with the anti-IR antibody or with the anti-GLUT2 antibody. As shown in Fig. 5A, IRA associated GLUT2 with a significant increase in PTP1B^{-/-} neonatal hepatocytes compared with the wild-type controls. Importantly, the graph depicted in Fig. 5A shows the quantification of the IRA/GLUT2 complexes in neonatal hepatocytes after normalization with the

Table 1. Effect of PTP1B deficiency on HK, GK, and G6Pase enzymatic activities and net free intrahepatic glucose in livers of wild-type and PTP1B-deficient mice

	Neonatal Liver		Adult Liver	
	PTP1B ^{+/+}	PTP1B ^{-/-}	PTP1B ^{+/+}	PTP1B ^{-/-}
Free hepatic glucose, mg/g	0.30 ± 0.04	0.70 ± 0.07*	2.37 ± 0.09	2.06 ± 0.15
Glucokinase, nmol·min ⁻¹ ·mg protein ⁻¹	—	—	1.06 ± 0.11	0.98 ± 0.0.7
Hexokinase, nmol·min ⁻¹ ·mg protein ⁻¹	1.58 ± 0.27	1.53 ± 0.22	—	—
G6Pase, nmol·min ⁻¹ ·mg protein ⁻¹	40.0 ± 0.53	35.2 ± 3.0	88.3 ± 6.4	86.0 ± 4.8

Values are means ± SE. Hexokinase (HK) and glucokinase (GK) enzymatic activities were measured in cytosolic extracts from 10–12 livers of neonatal and adult wild-type and protein tyrosine phosphatase 1B (PTP1B)^{-/-} mice as described in MATERIALS AND METHODS. Glucose 6-phosphatase (G6Pase) activity was measured in the microsomal fraction. Net free intrahepatic glucose was measured as described in MATERIALS AND METHODS. Statistical significance was carried out by Student's *t*-test by comparison of wild-type and PTP1B^{-/-} livers. **P* < 0.05 was considered significant.

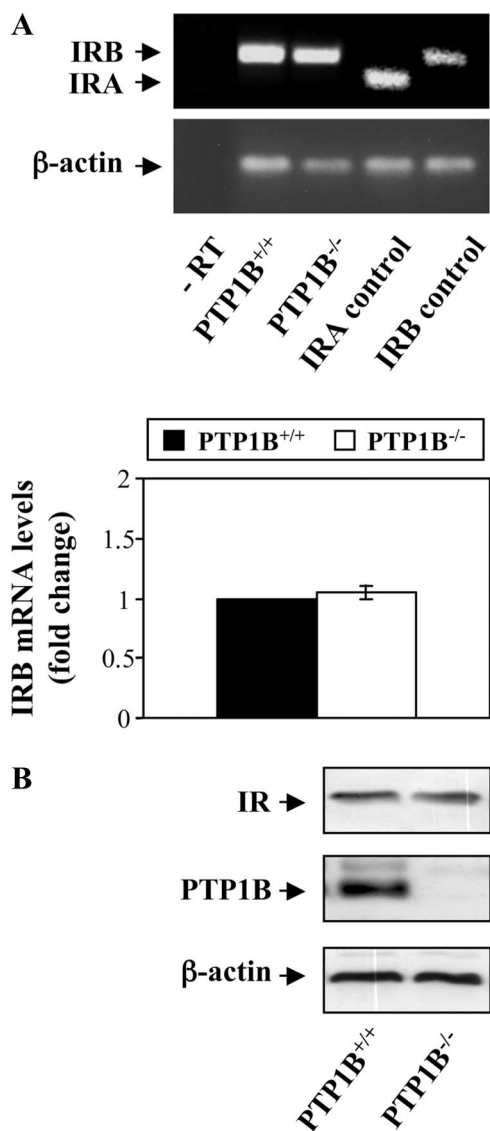


Fig. 3. Expression profile of insulin receptor (IR) isoforms in adult wild-type and PTP1B-deficient hepatocytes. *A*, top: total RNA was isolated from adult primary hepatocytes from each genotype, and RT-PCR was performed as described in MATERIALS AND METHODS. IR-deficient neonatal hepatocytes reconstituted with IRA (IRA control) or IRB (IRB control) were used as controls. As a loading control RT-PCR with mouse β -actin primers has been performed. A representative experiment is shown (bottom). The graph showing IRB mRNA levels from 3 independent experiments were quantitated by scanning densitometry and are expressed as fold change of PTP1B^{-/-} hepatocytes as compared with the wild-type condition, which was arbitrarily assigned a value of 1, and are means \pm SE. *B*: cell lysates from adult primary hepatocytes of each genotype were analyzed by SDS-PAGE followed by Western blot with antibodies against IR, PTP1B, and β -actin as a loading control. A representative experiment is shown.

amount of GLUT2 levels in the same immunoprecipitation. To confirm these results, the same experiment was performed with plasma membrane extracts prepared from pools of livers from 3–5-day-old wild-type and PTP1B-deficient mice. Of note, IRA/GLUT2 complexes, normalized with the amount of GLUT2, were more abundant in the plasma membrane fraction of neonatal livers lacking PTP1B compared with the wild-type controls.

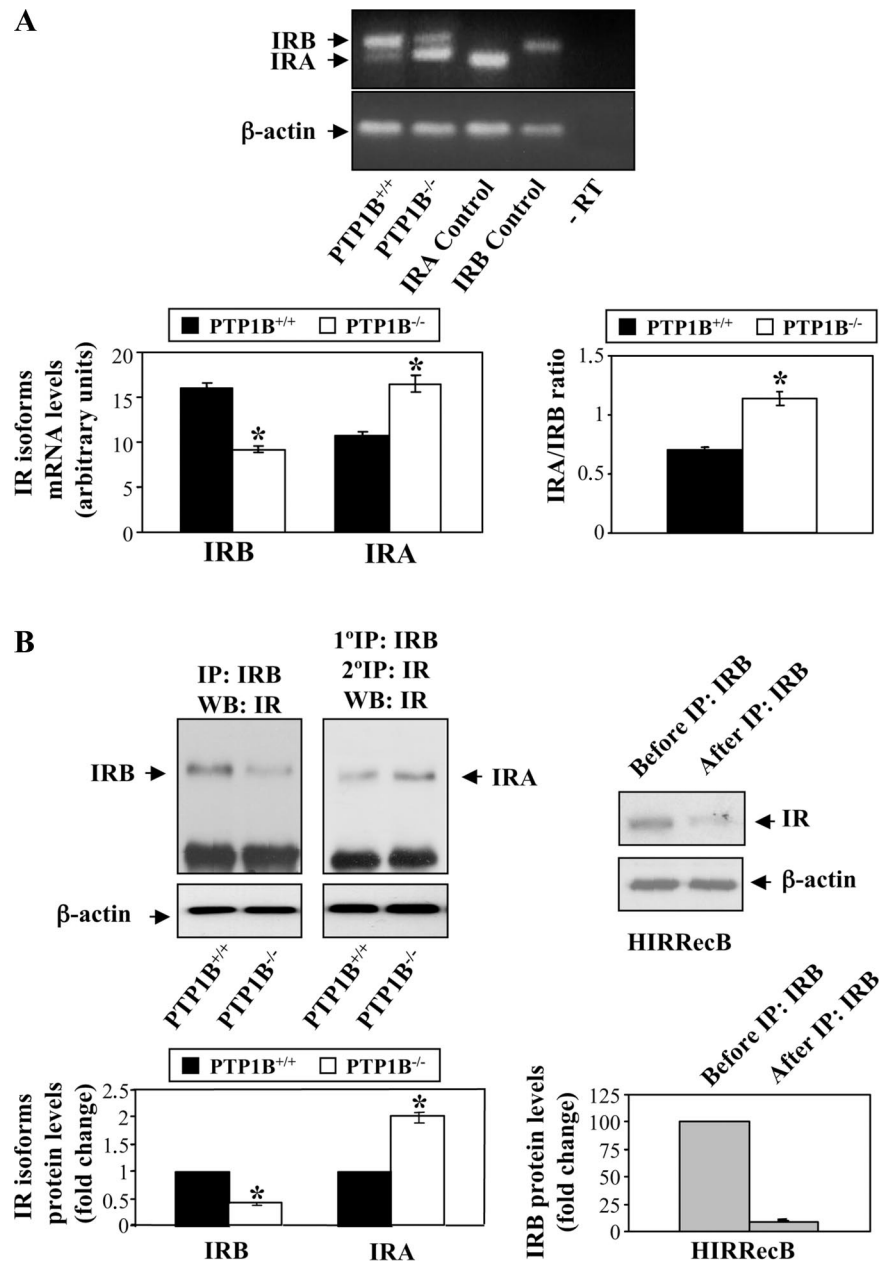
To further demonstrate whether the increase in IRA led to more abundance of IRA/GLUT2 complexes in PTP1B-deficient neonatal hepatocytes, these cells were infected with retroviruses encoding human IRA (PTP1B^{-/-}IRA). As a control, immortalized PTP1B^{-/-} cells were infected with empty vector (PTP1B^{-/-}Hygro). The presence of human IRA in immortalized PTP1B^{-/-}IRA neonatal hepatocytes was assessed by RT-PCR (Fig. 5B). Importantly, the endogenous mouse IR isoforms remained unchanged in PTP1B^{-/-}IRA and PTP1B^{-/-}Hygro cell lines compared with the parental (PTP1B^{-/-}) cell line. At the protein level, in PTP1B^{-/-}IRA cells, IR increased twofold compared with cells infected with empty vector. However, as depicted in Fig. 5B, the endogenous expression of GLUT2 remained unchanged. Next, we performed the two-round immunoprecipitation protocol described in Fig. 5A to measure IRA/GLUT2 complexes normalized by the amount of GLUT2. Figure 5C shows that, despite similar GLUT2 content in the immunoprecipitation, IRA/GLUT2 complexes significantly increased in PTP1B^{-/-} neonatal hepatocytes overexpressing IRA compared with those present in PTP1B-deficient cells infected with empty vector.

DISCUSSION

The liver plays a major role in insulin-regulated glucose homeostasis through the balance and coordination of NHGU and HGP. Classically, the HGP-suppressing effect of insulin is thought to occur via hepatic IR signaling inhibiting glycogenolysis and gluconeogenesis. However, during the last years several studies have challenged this paradigm strongly, suggesting direct and indirect effects of insulin on the control of HGP in the liver (19). By contrast, the contribution of the liver to glucose utilization has been examined less thoroughly.

Insulin signaling is negatively regulated by several mechanisms including downregulation of the IR, serine phosphorylation or degradation of IRS proteins, and IR and IRS dephosphorylation by specific PTPs (43). Of the latter, PTP1B has been extensively studied in recent years as a therapeutic target for type 2 diabetes and obesity. The liver is a major site of PTP1B action in the periphery (10, 11). In fact, liver-specific PTP1B reexpression in PTP1B-deficient mice attenuates the increased insulin signaling and insulin hypersensitivity characteristic of PTP1B^{-/-} animals (22). Euglycemic/hyperinsulinemic clamp studies in PTP1B-deficient mice revealed that glucose utilization is increased compared with wild-type controls. This was accompanied by significant increases in whole body glycolysis and nonoxidative glucose metabolism (25). However, since most of the glucose disposal during a euglycemic/hyperinsulinemic clamp represents uptake into skeletal muscle, the markedly increased glucose utilization in PTP1B^{-/-} mice, compared with control mice, does not illustrate whether PTP1B deficiency affects hepatic glucose utilization. To assess this important issue, we have investigated the effect of PTP1B deficiency in hepatic glucose uptake. We have measured in vitro basal glucose uptake in primary wild-type and PTP1B-deficient hepatocytes from 3–5-day-old neonatal mice and 10–12-wk-old adults representing two distinct stages of postnatal development. The reason for this is the differential effect of PTP1B deficiency in neonatal vs. adult hepatocytes. As we have recently reported, in contrast to the increased insulin

Fig. 4. The lack of PTP1B in neonatal hepatocytes increased the ratio of IRA/IRB isoforms. **A**: total RNA was isolated from neonatal primary hepatocytes from each genotype, and RT-PCR was performed as described in MATERIALS AND METHODS. IR-deficient immortalized neonatal hepatocytes reconstituted with IRA or IRB were used as controls. As a loading control RT-PCR with mouse β -actin primers has been performed. A representative experiment is shown. The photos showing IRA and IRB mRNA expression from 5 independent experiments were quantitated by scanning densitometry, and IRA/IRB ratio was calculated. Results corresponding to IRA and IRB mRNA levels are expressed as arbitrary units. Results corresponding to IRA/IRB ratio are expressed as fold change of PTP1B^{-/-} hepatocytes compared with the wild-type condition and are means \pm SE. Statistical significance was carried out by Student's *t*-test by comparison of wild-type and PTP1B^{-/-} neonatal primary hepatocytes. **P* < 0.05 was considered significant. **B**, *left*: total protein (1 mg) from neonatal immortalized hepatocyte cell lines (whole cell lysates) was immunoprecipitated with the anti-IRB antibody. Then protein A agarose was added for 2 h to collect the IRB immune complexes. The supernatant (depleted of IRB) was reimmunoprecipitated with the anti-IRB antibody and, after that, immunoprecipitated with the anti-IR antibody. The resulting immune complexes and the IRB immune complexes were analyzed by Western blot with the anti-IR antibody. A fraction (50 μ g) of the whole lysates was analyzed by Western blot with the anti- β -actin antibody as a loading control. The photos showing IRA and IRB isoforms protein expression from 5 independent experiments were quantitated by scanning densitometry. Results are expressed as fold change of IR isoforms protein content in PTP1B^{-/-} neonatal hepatocytes compared with the wild-type condition, which was arbitrarily assigned a value of 1, and are means \pm SE. Statistical significance was carried out by Student's *t*-test by comparison of wild-type and PTP1B^{-/-} neonatal primary hepatocytes. **P* < 0.05 was considered significant. *Right*: cell lysates from immortalized neonatal hepatocytes expressing only IRB isoform (HIR RecB) were immunoprecipitated with the anti-IRB antibody. The resulting immune complexes were analyzed by Western blot with the anti-IR antibody. As a loading control, a fraction (50 μ g) of the whole lysates was analyzed by Western blot with the anti- β -actin antibody. A representative experiment is shown.



signaling in adult PTP1B-deficient livers (13, 22) and hepatocytes (20), the lack of PTP1B in neonatal hepatocytes prolonged insulin signaling and insulin action without increasing insulin sensitivity (20). On this basis, we wanted to know whether the effect of PTP1B deficiency in basal glucose uptake in hepatocytes varies in a postnatal developmental manner.

Our results clearly show that the positive effect of PTP1B deficiency in basal glucose uptake in hepatocytes occurs only in the neonatal state. This period is characterized by a marked hypoinsulinemia and, consequently, very limited insulin-induced glucose consumption by extrahepatic tissues (18). Nevertheless, in hepatocytes glucose uptake is not regulated by translocation of the glucose transporter GLUT2 from intracellular stores to plasma membrane in response to insulin (27, 30). By contrast, expression of GK in hepatocytes and pancreatic β -cells is of major physiological importance to mammalian

glucose homeostasis. Hepatic GK catalyzes the first committed rate-limiting step in the disposal of glucose. Importantly, in hepatocytes from suckling mice, the expression of GK, which is induced by insulin after weaning, is absent. Of note, at this postnatal stage, constitutively expressed HK is the main enzyme responsible for glucose phosphorylation (5, 34). In neonatal hepatocytes lacking PTP1B, insulin signaling and insulin action are prolonged but not enhanced (20). Accordingly, possible effects of PTP1B deficiency on insulin-induced gene expression in these cells can be excluded as a mechanism responsible for the increase in glucose uptake. Paradoxically, our results show that PTP1B deficiency in adult hepatocytes, which resulted in increased insulin signaling (20), did not increase GK expression and/or activity. These results indicate the existence of compensatory mechanisms, possibly mediated through other PTPs, maintaining unaltered GK expression and,

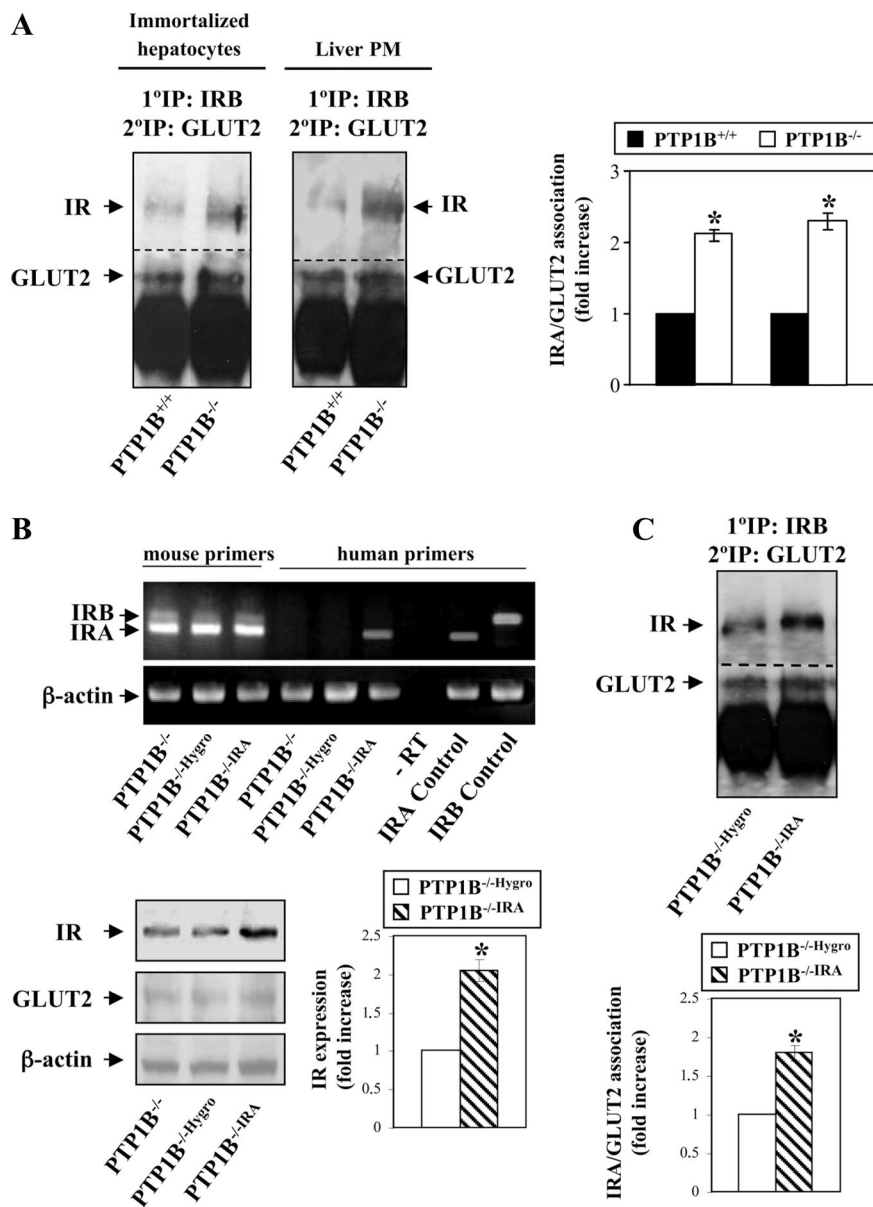


Fig. 5. The lack of PTP1B increased the amount of IRA/GLUT2 complexes in neonatal hepatocytes and plasma membranes from neonatal liver. **A**: purified fractions of liver plasma membranes from PTP1B^{+/+} and PTP1B^{-/-} neonatal mice were isolated as described in MATERIALS AND METHODS. Total protein (1 mg) from these purified fractions and from neonatal immortalized hepatocyte cell lines (whole cell lysates) were immunoprecipitated 2 times with the anti-IRB antibody. Then protein A agarose was added for 2 h to collect the IRB immune complexes. The supernatant (depleted of IRB) was reimmunoprecipitated with the anti-GLUT2 antibody, and the resulting immune complexes were analyzed by Western blot with anti-IR and anti-GLUT2 antibodies. A representative experiment is shown. The autoradiograms showing IRA-associated GLUT2 from 3 independent experiments were quantitated by scanning densitometry. Results are expressed as fold increase of IRA/GLUT2 association (normalized by the amount of GLUT2) in PTP1B-deficient neonatal hepatocytes and plasma membrane fractions compared with the wild-type condition (set to 1) and are means \pm SE. Statistical significance was carried out by Student's *t*-test by comparison between wild-type and PTP1B^{-/-} conditions. **P* < 0.05 was considered significant. **B**, top: immortalized PTP1B^{-/-} neonatal hepatocytes were infected with retroviruses encoding human IRA (PTP1B^{-/-}IRA). As a control, immortalized PTP1B^{-/-} cells were infected with empty vector (PTP1B^{-/-}Hygro). The presence of human IRA and the endogenous mouse isoforms was assessed by RT-PCR. Bottom: the expression of IR and GLUT2 was analyzed by Western blot with β -actin as a loading control. **P* < 0.05 was considered significant. **C**: total protein (1 mg) was submitted to the 2-round immunoprecipitation protocol described in **A** to measure IRA/GLUT2 complexes normalized by the amount of GLUT2. The autoradiograms showing IRA-associated GLUT2 from 3 independent experiments were quantitated by scanning densitometry. Results are expressed as fold increase of IRA/GLUT2 association in PTP1B^{-/-}IRA cells compared with the control condition (PTP1B^{-/-}Hygro set to 1) and are means \pm SE. Statistical significance was carried out by Student's *t*-test by comparison between PTP1B^{-/-}IRA and PTP1B^{-/-}Hygro conditions. **P* < 0.05 was considered significant.

consequently, glucose uptake in adult hepatocytes in the absence of PTP1B.

Importantly, the net increase in basal glucose uptake in PTP1B-deficient neonatal hepatocytes paralleled with an up-regulation of GLUT2 at the protein level but without changes in the expression of GLUT1. Since glucose metabolism is required for the regulation of GLUT2 expression in the liver (34), it is not possible to determine whether the increased expression of GLUT2 in PTP1B^{-/-} neonatal hepatocytes is a consequence of increased glucose uptake and, subsequently, increased glucose metabolism or whether it is a direct/indirect effect of the lack of PTP1B. The latter possibility agrees with a role of PTP1B in the regulation of the expression of genes involved in glucose or lipid metabolism in the liver. Regarding this, Shimizu et al. (39) have reported that PTP1B activates hepatic lipogenesis by increasing the expression of sterol regulatory element-binding protein-1c, a mechanism mediated by protein phosphatase 2A dephosphorylation and activation.

Moreover, since GLUT2 protein content is also increased in the plasma membrane fraction, the possibility that PTP1B deficiency can increase GLUT2 protein stability and/or cellular distribution should not be excluded. Nevertheless, since the transport capacity via GLUT2 is present in vast excess of the GK trapping reaction in hepatocytes and, subsequently, GLUT2 acts as a low-affinity, high-capacity transport system to allow the uninhibited (non-rate-limiting) flux of glucose into or out of hepatocytes (27, 30), the upregulation of GLUT2 in neonatal PTP1B^{-/-} hepatocytes does not seem by itself to be the molecular mechanism by which basal glucose uptake is increased in these cells. Of note, under the same experimental conditions (basal state), no differences in G6Pase enzymatic activity were found between wild-type and PTP1B^{-/-} livers from neonates. Moreover, basal glucose uptake was also increased in PTP1B^{-/-} immortalized neonatal hepatocytes compared with immortalized wild-type control. Importantly, both kinds of immortalized neonatal hepatocytes do not express

phosphoenolpyruvate carboxykinase and G6Pase (results not shown). This is an important issue since a higher accumulation of 2-deoxyglucose in hepatocytes from PTP1B^{-/-} mice may result from increased glucose uptake, decreased glucose released by the cell, or both. Accordingly, our results clearly indicate that glucose uptake is increased in neonatal hepatocytes in the absence of PTP1B. More importantly, the increase in basal glucose uptake of PTP1B^{-/-} neonatal hepatocytes correlated with increased net free intrahepatic glucose content in the livers from PTP1B-deficient neonates, indicating a positive correlation of our results in vitro and in vivo.

Our previous results have demonstrated the involvement of IR on basal glucose uptake in neonatal hepatocytes since deletion of IR decreased basal glucose uptake by 50%. More importantly, in IR-deficient neonatal hepatocytes, glucose uptake was restored to similar levels as in control cells when IRA, but not IRB, was reexpressed (28). These results, together with those discussed below, suggest the importance of the expression ratios of IR isoforms during normal development or in the pathogenesis of human diseases such as type 2 diabetes and cancer. However, many of the studies on the relative expression of IRA and IRB isoforms in type 2 diabetes have reported conflicting results. Several studies indicate that the expression of IRB is increased in skeletal muscle or adipocytes of type 2 diabetic patients relative to control subjects (24, 36). By contrast, Norgren et al. (29) reported an increase in the relative abundance of IRA in the skeletal muscle of a patient with type 2 diabetes with markedly impaired insulin-mediated glucose utilization. Furthermore, other groups have reported no differences in the relative amounts of IR isoforms in skeletal muscle among lean normal, obese nondiabetic, and type 2 diabetic subjects (2, 3). However, numerous studies have shown that there is a preferential expression of IRA in fetal tissues such as the kidney, muscle, and liver, as well as in many types of cancer (reviewed in 37, 11). Concurring with this, in our study we have demonstrated increased IRA, decreased IRB, and, consequently, increased IRA/IRB ratio in neonatal hepatocytes lacking PTP1B. In PTP1B^{-/-} neonatal hepatocytes, the increased glucose uptake correlates with the upregulation of IRA. By contrast, hepatocytes from adult mice lack IRA, IRB being the isoform expressed at this stage of development. Moreover, in these cells the lack of PTP1B did not modify the profile of IR isoforms and, subsequently, glucose uptake remained unchanged compared with wild-type cells. Thus our data demonstrate a novel role of PTP1B in regulating the expression of IR isoforms in neonatal hepatocytes and further reinforce the suggested differential role of IR isoforms in glucose transport. Moreover, our results show an increase in IRA/GLUT2 complexes in PTP1B^{-/-} neonatal hepatocytes, resulting in a more efficient role of IR to form a receptor-transporter complex with GLUT2. Indeed, the amount of these complexes augmented by IRA overexpression in PTP1B-deficient cells. More importantly, the increased abundance of IRA/GLUT2 complexes in PTP1B^{-/-} neonatal hepatocytes compared with wild-type cells was significant after the normalization for GLUT2 levels in the immunoprecipitation. This result is particularly interestingly since it is the first evidence that shows a direct correlation between increases in IRA/GLUT2 complexes and glucose uptake. Furthermore, as discussed above, in IR-deficient neonatal hepatocytes, glucose uptake, which was decreased compared with cells expressing

IR, was restored after reconstitution with IRA but not IRB (28). Notably, because hepatocytes from adult mice only expressed IRB, the association of IRA/GLUT2 cannot play a role in glucose uptake at this developmental stage. It is intriguing why this regulatory mechanism for glucose uptake operates in neonatal, but not adult, hepatocytes. A possible explanation that might account for this differential regulation is the fact that neonatal liver is a growing tissue with higher mitogenic capacity than the adult liver. Since growth factor receptors such as EGF-R or IGF-I-R are PTP1B substrates (21), one would expect that PTP1B deficiency increases the proliferative capacity of liver cells and, subsequently, the requirements of glucose uptake. The fact that PTP1B^{-/-} hepatocytes have increased IRA, together with our previous results showing increased IRS-1 (20), is consistent with this idea. Moreover, since the regulation of hepatic metabolism differs between neonatal and adult liver, current investigations are focused on the physiological role of increased free glucose and glucose utilization in PTP1B-deficient neonatal vs. adult livers.

In summary, results presented here demonstrate that deletion of PTP1B markedly increased glucose uptake in neonatal, but not adult, hepatocytes. GLUT2 is upregulated in PTP1B^{-/-} neonatal hepatocytes at the protein level. Moreover, PTP1B deficiency alters the ratio of IRA/IRB isoforms by increasing IRA isoform and decreasing IRB, resulting in a more abundance of IRA/GLUT2 complexes. Thus the amount of IRA/GLUT2 complexes plays a direct role in the regulation of glucose uptake in neonatal hepatocytes. Consistent with this, neonatal PTP1B^{-/-} hepatocytes might represent a suitable model in which to study the role of IR isoforms in glucose metabolism.

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