

Molecular Mechanisms of Insulin Resistance in IRS-2-Deficient Hepatocytes

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To assess the role of insulin receptor (IR) substrate (IRS)-2 in insulin action and resistance in the liver, immortalized neonatal hepatocyte cell lines have been generated from IRS-2^{-/-}, IRS-2^{+/-}, and wild-type mice. These cells maintained the expression of the differentiated liver markers albumin and carbamoyl phosphate synthetase, as well as bear a high number of IRs. The lack of IRS-2 did not result in enhanced IRS-1 tyrosine phosphorylation or IRS-1-associated phosphatidylinositol (PI) 3-kinase activity on insulin stimulation. Total insulin-induced PI 3-kinase activity was decreased by 50% in IRS-2^{-/-} hepatocytes, but the translocation of PI-3,4,5-trisphosphate to the plasma membrane in these cells was almost completely abolished. Downstream PI 3-kinase, activation of Akt, glycogen synthase kinase (GSK)-3 (α and β isoforms), Foxo1, and atypical protein kinase C were blunted in insulin-stimulated IRS-2^{-/-} cells. Reconstitution of IRS-2^{-/-} hepatocytes with adenoviral IRS-2 restored activation of these pathways, demonstrating that IRS-2 is essential for functional insulin signaling in hepatocytes. Insulin induced a marked glycogen synthase activity in wild-type and heterozygous primary hepatocytes; interestingly, this response was absent in IRS-2^{-/-} cells but was rescued by infection with adenoviral IRS-2. Regarding gluconeogenesis, the induction of phosphoenolpyruvate carboxykinase and glucose 6-phosphatase by dibutyryl cAMP and dexamethasone was observed in primary hepatocytes of all genotypes. However, insulin was not able to suppress gluconeogenic gene expression in primary hepatocytes lacking IRS-2, but when IRS-2 signaling was reconstituted, these cells recovered this response to insulin. Suppression of gluconeogenic gene expression in IRS-2-deficient primary hepatocytes was also restored by infection with dominant negative $\Delta 256$ Foxo1. *Diabetes* 52:2239–2248, 2003

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dbcAMP, dibutyryl cAMP; CPS, carbamoyl phosphate synthetase; FITC, fluorescein isothiocyanate; G6Pase, glucose 6-phosphatase; GFP, green fluorescent protein; GRP1, general receptor for phosphoinositides-1 domain; GS, glycogen synthase; GSK, GS kinase; HGP, hepatic glucose production; IR, insulin receptor; IRS, IR substrate; LTAG, large tumor antigen; MOI, multiplicity of infection; PGC-1, peroxisome proliferator-activated receptor- γ coactivator-1; PH, pleckstrin homology; PI, phosphatidylinositol; PIP, PI phosphate; PKC, protein kinase C.

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Type 2 diabetes is a complex metabolic disease with an environmental and genetic component affecting $\geq 5\%$ of the population in Western societies. The pathogenesis of type 2 diabetes involves abnormalities in both insulin action and secretion (1). At the molecular level, insulin resistance, the first detectable defect in type 2 diabetes, correlates with impaired insulin signaling in peripheral tissues. The transgenic and knockout technology used to create animal models of type 2 diabetes have had a major impact on the assessment of the function of insulin signaling molecules implicated in the regulation of glucose homeostasis in vivo (2). The insulin receptor (IR) substrate (IRS) proteins 1 and 2 are key mediators of many responses in insulin sensitive tissues, especially those that are associated with somatic growth and carbohydrate metabolism (3). During the past few years, knockout mice have been generated that define the specific roles of these molecules (4–7). IRS-1-deficient (IRS-1^{-/-}) mice were growth retarded and mildly insulin resistant but did not develop diabetes. Rather, these mice developed β -cell hyperplasia, resulting in compensatory insulin secretion (4,5). The residual insulin action in IRS-1^{-/-} mice led to the discovery of IRS-2 as an alternative signaling protein (8,9). IRS-2^{-/-} mice developed diabetes as a result of severe insulin resistance paired with β -cell failure (6,7,10). Previous experiments performed in peripheral tissues of IRS-1^{-/-} mice by Yamachi et al. (11) suggested that IRS-2 could be a major player in hepatic insulin action. These results have been assessed by other groups and, taken together, suggest that IRS-2 can compensate IRS-1 deficiency more effectively in liver and β -cells than in muscle or adipose tissues (12,13). However, to what extent reduced IRS-2 contributes to insulin resistance in the liver remains uncertain.

More recently, a kinase cascade that mediates the posttranscriptional actions of insulin in liver and other tissues has been defined (3,14). The cascade begins when activation of the IR results in tyrosine phosphorylation of IRS proteins (3). Then phosphorylated IRSs bind proteins containing Src homology 2 domains, such as the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase (15), which has a central role in the metabolic actions elicited by insulin. Analysis of human subjects and animal models has indicated the existence of a correlation between impaired activation of PI 3-kinase in insulin target tissues and insulin resistance in vivo (16–18). Downstream from PI 3-kinase, the serine/threonine kinase Akt triggers

insulin effects on the liver, such as glycogen synthesis and the suppression of hepatic glucose production. One mechanism by which Akt might contribute to the insulin-mediated suppression of glycogenolysis is by driving glycogen synthesis through the activation of glycogen synthase (GS). It has been demonstrated extensively that an Akt-mediated inactivation of GS kinase (GSK)-3 contributes to a reduction in the net phosphorylation and, subsequently, activation of GS (19). Nonetheless, the absolute contribution of Akt to the regulation of GS remains uncertain because several signaling molecules not influenced by Akt also modulate this process. Regarding gluconeogenesis, the ability of insulin to suppress PEPCK transcription is sensitive to PI 3-kinase inhibition (20,21). Attempts to elucidate the signaling pathways downstream of PI 3-kinase that mediate inhibition of gluconeogenic gene expression have produced conflicting results, as these studies (22,23) have relied on the overexpression of genes encoding constitutively active and inactive mutants.

In the present study, we have developed new tools to study the molecular mechanisms of IRS-2-mediated hepatic insulin resistance. To analyze IRS-2 signals, we have generated immortalized neonatal hepatocytes from wild-type, heterozygous, and IRS-2-deficient mice by means of SV40 large tumor antigen (LTag) retroviral gene transfer. Using these cell lines, as well as primary hepatocytes derived from those animals, we have demonstrated *in vitro* that IRS-2 signaling, through Akt/GSK-3 (α and β isoforms) and atypical protein kinase C (PKC) ζ/λ , is essential in triggering two major metabolic actions of insulin in the liver, i.e., activation of glycogen synthesis and inhibition of gluconeogenic gene expression.

RESEARCH DESIGN AND METHODS

Materials. FCS and culture media were obtained from Gibco (Gaithersburg, MD). Insulin and anti-mouse IgG-Agarose were obtained from Sigma Chemical (St. Louis, MO). Protein A-Agarose was from Roche Molecular Biochemicals (Mannheim, Germany). The anti-IRS-1 (06-248), anti-tyrosine phosphorylation (clone 4G10) (05-321), and anti-IRS-2 (06-506) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). The anti-tyrosine phosphate (Py20) (sc-508) and anti-PKC ζ/λ (c-20) (sc-216) antibodies were purchased from Santa Cruz Biotechnology (Palo Alto, CA). The anti-phospho Akt (Ser473 no. 9271), anti-phospho p70s6k (Ser424/Thr421 no. 9204), anti-Akt (no. 9272), anti-p70s6k (no. 9202), antiphospho GSK-3 α/β (32 P) (239331), anti-phospho Foxo1 (serine256 32 P) (239461), and anti-Foxo1 (32 P) (239462) antibodies were purchased from Cell Signaling (Beverly, MA). For immunofluorescence, fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse immunoglobulins and monoclonal anti-vimentin (cloneV9) antibody were purchased from Roche Molecular Biochemicals, polyclonal antialbumin antibody was from Nordic Immunology Laboratories (Tilbury, the Netherlands), and monoclonal anti-CPS antibody was a gift from Dr. P. Martín-Sanz (Consejo Superior de Investigaciones Científicas, Madrid, Spain). Cy3-conjugated goat anti-rabbit immunoglobulins, [γ - 32 P]-ATP (3,000 Ci/mmol), [α - 32 P]-dCTP (3,000 Ci/mmol), and 125 I-labeled insulin (80 μ Ci/ μ g) were from Amersham (Aylesbury, U.K.). UDP-[U- 14 C] glucose was from NEN Chemicals. Immunofluorescence mounting medium was from Vector (Burlingame, CA).

Cell culture and retroviral infections. Primary hepatocytes were obtained from individual livers of 3.5- to 4-day-old neonatal mice from two to three pregnant IRS-2 $^{+/-}$ mice mated with male IRS-2 $^{+/-}$ mice and further submitted to collagenase dispersion and primary culture as previously described (24). Viral Bosc-23 packaging cells were transfected at 70% confluence by calcium phosphate coprecipitation with 3 μ g/6-cm dish of the puromycin-resistance retroviral vector pBabe encoding SV40 LTag (kindly provided by J. de Caprio, Dana Farber Cancer Institute, Boston, MA). Then, neonatal hepatocytes were infected at 60% confluence with polybrene (4 μ g/ml)-supplemented virus for 48 h and maintained in culture medium for 72 h, before selection with puromycin (1 μ g/ml) for 1 week. One immortalized cell line from each animal was generated and further cultured for 2–3 weeks with arginine-free medium

supplemented with 10% fetal bovine serum to avoid growth of nonparenchymal cells.

Immunofluorescence and confocal imaging. Cells were grown in glass coverslips until 80% confluence was reached. Cells were then washed twice with PBS, fixed in methanol (-20°C) for 2 min, and processed with immunofluorescence. Primary antibodies (antialbumin, anti-CPS, and antivimentin) 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 antibodies (FITC-conjugated sheep anti-mouse and Cy3-conjugated goat anti-rabbit), and four final washes of 5 min each in PBS. Immunofluorescence was examined with an MRC-1024 confocal microscope (Bio-Rad, Hempstead, U.K.) adapted to an inverted Nikon Eclipse TE 300 microscope. 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.

Transfection with the pleckstrin homology domain of general receptor for phosphoinositides-1 domain-green fluorescent protein construct.

Cells were grown to 80% confluence and then transiently transfected with 8 μ g/6-cm dish of pleckstrin homology (PH) domain of general receptor for phosphoinositides-1 domain (GRP1)-green fluorescent protein (GFP) cDNA construct accordingly with the calcium phosphate-mediated protocol (Stratagene). Cells were serum starved for 4 h (24 h after transfection) and subsequently stimulated with insulin (100 nmol/l) for 5 and 10 min. Cells were then washed twice with PBS, fixed in methanol (-20°C) for 2 min, and processed with confocal immunofluorescence.

Transduction of IRS-2-deficient hepatocytes by adenoviral infection. Immortalized or primary hepatocytes were infected with 10 MOI (multiplicity of infection or viral particles per cell) IRS-2 or 10 MOI mock (β -galactosidase). In other experiments, primary hepatocytes from IRS-2 knockout mice were infected with 10 MOI Δ 256Foxo1 mutant. Cells (80–90% confluence) were routinely infected for 24–48 h, after which IRS-2-deficient cells recovered IRS-2 expression. Then, cells were serum starved for 16–20 h and subsequently used for further experiments.

Immunoprecipitations and Western blot. Quiescent cells (20 h serum starved) were treated without or with several doses of insulin and lysed as previously described (25). After protein content determination, equal amounts of protein (500–600 μ g) were immunoprecipitated at 4°C with the corresponding antibodies. The immune complexes were collected on protein A-Agarose or anti-mouse IgG-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).

PI 3-kinase activity. PI 3-kinase activity was measured in the anti-IRS-1, anti-IRS-2, or anti-tyrosine phosphorylation immunoprecipitates by *in vitro* phosphorylation of PI as previously described (25).

PKC ζ/λ activity. Quiescent cells were treated for 10 min without or with several doses of insulin and subsequently lysed. PKC ζ/λ activity was measured in anti-PKC ζ/λ immunoprecipitates by an *in vitro* kinase assay as previously described (25).

Measurement of GS activity. Cells were grown to confluence and then serum starved for 20 h. Insulin (100 nmol/l) was added for 15 min, and control cells were incubated in the absence of the hormone. After washing with PBS, cells were scraped in 100 μ l GS assay buffer containing 100 mmol/l NaF, 35 mmol/l EDTA, and 0.5% glycogen (wt/vol) at pH 7.4. Cell lysates were then sonicated three times for 3 s and centrifuged at 12,000g for 10 min at 4°C . The clarified supernatants were aliquoted with GS assay buffer to equal protein content. To measure GS activity, 20 μ l supernatant (6–8 μ g protein per μ l) was added to 100 μ l GS activity buffer containing 72 mmol/l glycylglycine, 12% glycogen (wt/vol), 12 mmol/l Na_2SO_4 , and 0.3 mmol/l UDP-[U- 14 C] glucose (0.1 μ Ci/tube) and then incubated for 15 min at 20°C . The reaction was stopped at 4°C by the addition of 200 μ l of 0.5N KOH, and after adding 35 μ l of 10% glycogen (wt/vol) as carrier, the total glycogen was extracted by 2 ml ethanol (66% final) over 30 min. The samples were centrifuged at 600g for 5 min, and the precipitate was washed once with 5 ml of 66% ethanol and further centrifuged at 3,000g for 30 min. The pellet was dissolved in water and examined for its radioactive content in a scintillation counter. For total GS activity, the reaction mixture included 7.2 mmol/l glucose 6-phosphate, and Na_2SO_4 was omitted, as described (26).

Protein determination. Protein determination was performed by the Bradford dye method (27), using the Bio-Rad reagent and BSA as the standard.

RNA extraction and Northern blot analysis. Primary hepatocytes were isolated from individual livers and cultured to confluence. Cells were serum starved for 20 h and further incubated for 6 h in the presence of 0.5 mmol/l dibutyryl cAMP (dbcAMP) plus 1 μ mol/l dexamethasone in either the

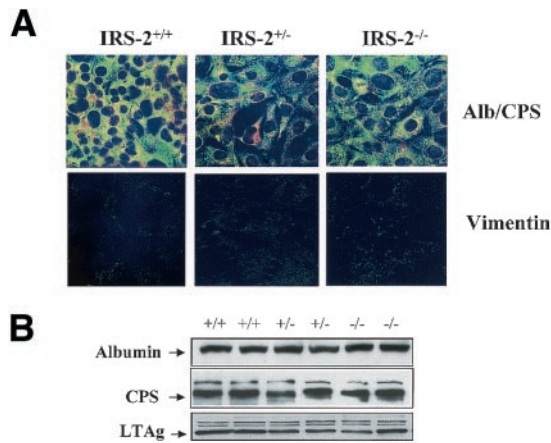


FIG. 1. Characterization of immortalized hepatocyte cell lines. **A:** Immunofluorescence detection of albumin (Alb), CPS, and vimentin of growing immortalized neonatal hepatocytes (wild-type, IRS-2^{+/-}, and IRS-2^{-/-}). **B:** Cells were grown to confluence in the presence of 10% fetal serum. Whole-cell lysates (50 μ g protein) were submitted to SDS-PAGE and analyzed by Western blot using antibodies against albumin, CPS, and LTAg. A representative experiment is shown.

absence or presence of insulin (100 nmol/l). In other experiments, cells were infected with adenovirus as described above. Then, cells were serum starved and further cultured for 6 h in the presence of 0.5 mmol/l dbcAMP plus 1 μ mol/l dexamethasone in either the absence or presence of insulin (100 nmol/l). At the end of the culture time, total RNA was isolated as described (28) and submitted to Northern blot analysis. Blots were hybridized with probes for PEPCK, glucose 6-phosphatase (G6Pase), and 18S ribosomal probe to normalize RNA loading. Membranes were subjected to autoradiography, and the relative densities of the hybridization signals were determined by densitometric scanning of the autoradiograms.

Insulin binding. Cells cultured for 20 h in serum-free medium were incubated for 3 h at 20°C with 0.03 nmol/l ¹²⁵I-labeled insulin in 1 ml of binding buffer containing 25 mmol/l HEPES/PBS, pH 7.4, and 1 mg/ml BSA in the absence or presence of graded concentrations of unlabeled insulin. At the end of incubation, monolayers were rinsed three times with ice-cold PBS/BSA and then dissolved in 0.1 mol/l NaOH, 1% SDS, and 2% Na₂CO₃. Radioactivity was counted by a Packard γ -counter. Nonspecific binding was defined as the radioactivity that remained bound in the presence of a 1,000-fold excess of unlabeled ligand. Bound/free plots and Scatchard plots were calculated for triplicate dishes from three independent experiments.

RESULTS

Generation and characterization of immortalized neonatal hepatocyte cell lines. Immortalized neonatal hepatocyte cell lines were generated from individual livers of wild-type (IRS-2^{+/+}), heterozygous (IRS-2^{+/-}), and IRS-2-deficient (IRS-2^{-/-}) mice. Hepatocytes were isolated from single neonatal livers, and primary cultures were prepared as previously described (24). All cell lines were obtained after infection of neonatal hepatocytes in primary culture with retrovirus encoding LTA_g, followed by antibiotic (puromycin) selection for at least 3 weeks. Then, immortalized cells were further cultured for 10–15 days in arginine-free medium supplemented with epidermal growth factor (20 ng/ml) to select hepatocytes with a functional urea cycle. Then, we proceeded to characterize the phenotype of these cell lines. For this purpose, we performed immunofluorescence on growing cells with antibodies against albumin and carbamoyl phosphate synthetase (CPS). As shown in Fig. 1A, wild-type, IRS-2^{+/-}, and IRS-2^{-/-} neonatal hepatocytes express albumin (a plasma protein secreted exclusively by hepatocytes) and CPS (a urea cycle marker), indicating that after immortalization these cells maintain hepatocyte phenotypic features.

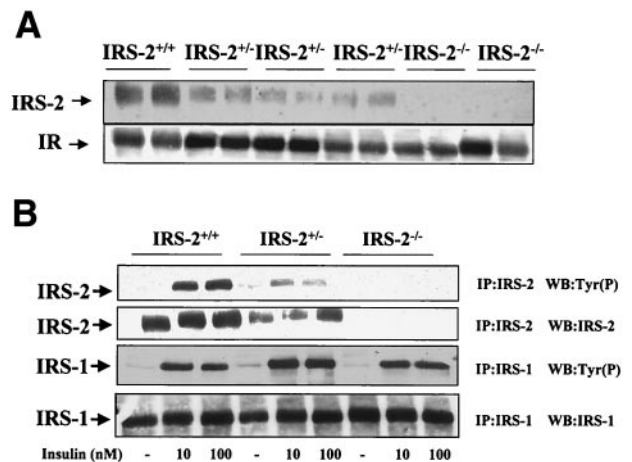


FIG. 2. IR and IRS-1 and -2 expression and tyrosine phosphorylation in immortalized wild-type, IRS-2^{+/-}, and IRS-2^{-/-} neonatal hepatocytes. **A:** Wild-type (IRS-2^{+/+}), heterozygous (IRS-2^{+/-}), and IRS-2-deficient (IRS-2^{-/-}) cells were grown to confluence in the presence of 10% fetal serum. Whole-cell lysates (50 μ g protein) were analyzed by Western blot using antibodies against IRS-2 and IR β -chain. **B:** Quiescent (20-h serum-starved) hepatocytes were stimulated with insulin (10–100 nmol/l) for 5 min. Control cells were cultured in the absence of the hormone. At the end of the culture time, cells were lysed and 600 μ g of total protein was immunoprecipitated with anti-IRS-1 and anti-IRS-2 antibodies. The resulting immune complexes were analyzed by Western blotting with anti-tyrosine phosphate, anti-IRS-1, or anti-IRS-2 antibodies, as indicated in each panel. The results shown are representative of three experiments.

Conversely, all cell lines showed negative immunofluorescence with the antivimentin antibody. Vimentin is a cytoskeletal marker characteristic to fibroblasts and should be absent in parenchymal hepatocytes to confirm that the maintenance of cell lines in arginine-free medium has sufficiently eliminated possible contamination of fibroblasts in the primary culture. The expression of albumin and CPS in all cell lines, regardless of the absence of IRS-2, was also confirmed by Western blot analysis (Fig. 1B). In addition, all cell lines showed similar levels of LTA_g.

Western blot experiments revealed that immortalized neonatal hepatocyte cell lines express IRS-2 protein at a level consistent with the corresponding animal genotype, with no IRS-2 being detected in IRS-2^{-/-} neonatal hepatocytes (Fig. 2A). Both wild-type and IRS-2^{+/-} hepatocytes displayed a high number of high-affinity insulin binding sites ($303 \times 10^3 \pm 33 \times 10^3$ sites/cell, $K_d = 66 \pm 9$ nmol/l). IR β -chain expression (Fig. 2A) and its tyrosine phosphorylation in response to insulin (results not shown) were similar among the different cell lines analyzed. Thus, we conclude that, based on these characteristics, immortalized neonatal hepatocyte cell lines are insulin target cells. To test whether the lack of IRS-2 was compensated by IRS-1 as an alternative docking protein, quiescent cells were stimulated with insulin (10–100 nmol/l) and total protein was immunoprecipitated with anti-IRS-1 or IRS-2 antibodies and submitted to Western blot analysis with anti-tyrosine phosphate antibody. Whereas IRS-2 tyrosine phosphorylation was consistent with IRS-2 expression in the three cell types, neither IRS-1 protein content nor its tyrosine phosphorylation was increased in IRS-2-deficient hepatocytes (Fig. 2B).

Effect of IRS-2 deficiency on the activation of PI 3-kinase and generation of PI(3,4,5) trisphosphate. Next, we investigated the activation of PI 3-kinase, which

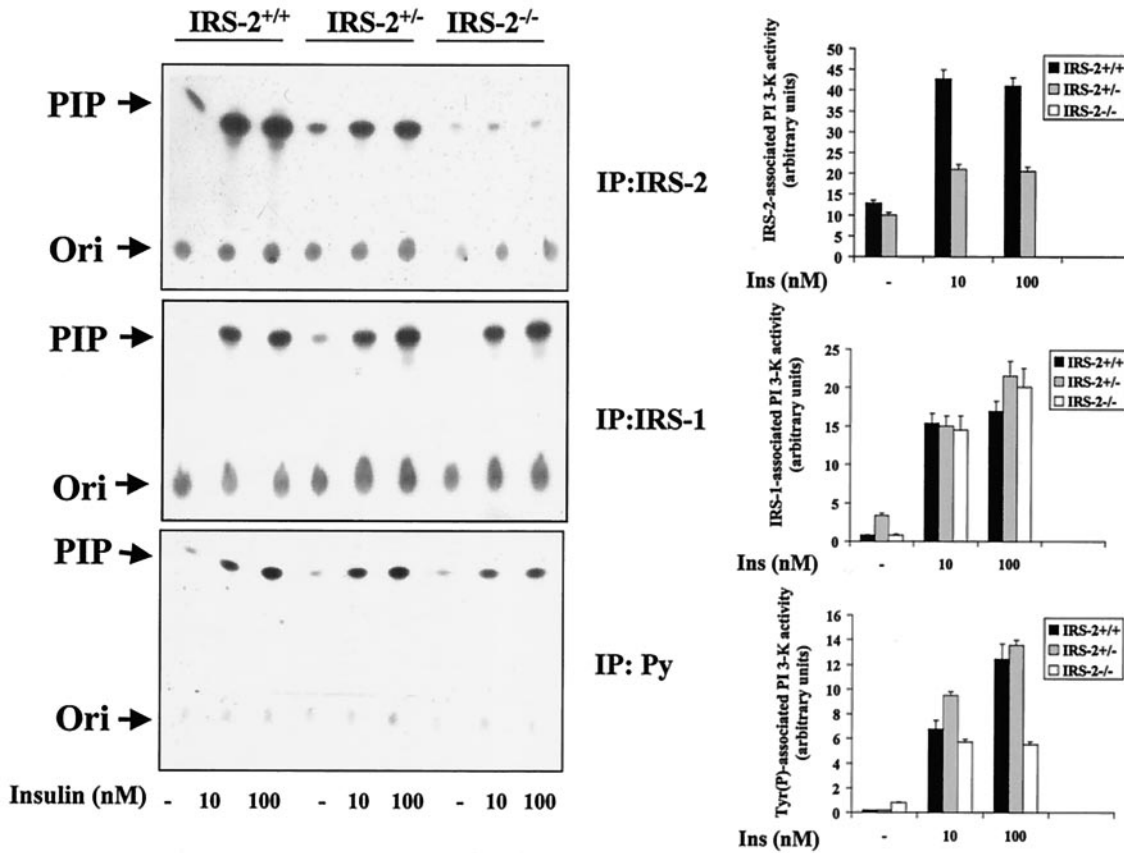


FIG. 3. Insulin-stimulated PI 3-kinase activity in immortalized wild-type, IRS-2^{+/-}, and IRS-2^{-/-} neonatal hepatocytes. Quiescent hepatocytes were cultured and stimulated with insulin as described in Fig. 2B. At the end of the culture time, cells were lysed and 600 μg total protein immunoprecipitated with anti-IRS-1, anti-IRS-2, or anti-tyrosine phosphate antibodies and the resulting immune complexes were washed and immediately used for an in vitro PI kinase assay. The conversion of PI to PIP in the presence of [³²P]ATP was analyzed by thin-layer chromatography. The origin of the thin-layer chromatography (Ori) and the migration of PIP are indicated by arrows. Representative autoradiograms are shown. The autoradiograms corresponding to three independent experiments were quantitated by scanning densitometry. Results are expressed as arbitrary units of PI 3-kinase and are means ± SE.

is involved in a number of metabolic actions of insulin in immortalized neonatal hepatocyte cell lines. Quiescent cells were stimulated with insulin (10–100 nmol/l), and total cell lysates immunoprecipitated with antibodies against IRS-1 and IRS-2 were immediately used for an in vitro PI 3-kinase activity assay. Insulin stimulated IRS-2-associated PI 3-kinase activity in wild-type and heterozygous cells but not in IRS-2^{-/-} cells, which is consistent with the IRS-2 protein levels in each cell line (Fig. 3). In contrast, IRS-1-associated PI 3-kinase activity was similar among the three cell types. To assess the relative contributions of IRS-1 and IRS-2 to the total content of insulin-stimulated PI 3-kinase activity, in vitro kinase reactions were performed on anti-tyrosine phosphate immunoprecipitates. As shown in Fig. 3, the overall PI 3-kinase activity decreased by 50% in IRS-2^{-/-} cells as compared with that of wild-type. In addition, the ability of insulin to stimulate the production of PI(3,4,5) trisphosphate (PIP3) was determined by measuring the translocation of the PH GRP1-GFP fusion protein in neonatal hepatocyte cell lines. Several groups have reported that the PH domain of GRP1 specifically binds to PIP3 and acutely localizes in membrane ruffles upon growth factor stimulation (29–31). Whereas insulin stimulated the translocation of the PH GRP1-GFP fusion protein to the membrane in >80% of wild-type cells, only a small fraction of IRS-2-deficient

hepatocytes (<10%) revealed membrane localization of PH GRP1-GFP fusion protein in response to the hormone. A representative experiment is shown in Fig. 4. **Differential activation of PI 3-kinase effectors in wild-type, IRS-2^{+/-}, and IRS-2^{-/-} neonatal hepatocytes.** Downstream from PI 3-kinase, various serine/

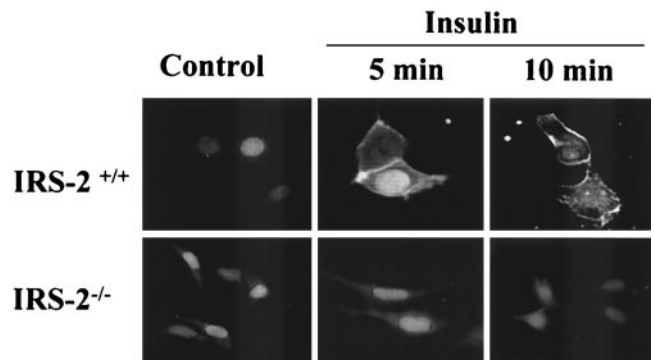


FIG. 4. Differential effects of insulin on PIP3 production in wild-type and IRS-2-deficient neonatal hepatocytes. Cells were grown to 80% confluence and then transiently transfected with a plasmid encoding PH GRP1-GFP fusion protein. After transfection (24 h), cells were serum starved for 4 h and further stimulated with 100 nmol/l insulin for 5 and 10 min. Then, cells were washed twice with PBS, fixed in methanol (–20°C) for 2 min, and processed with confocal immunofluorescence. A representative experiment is shown.

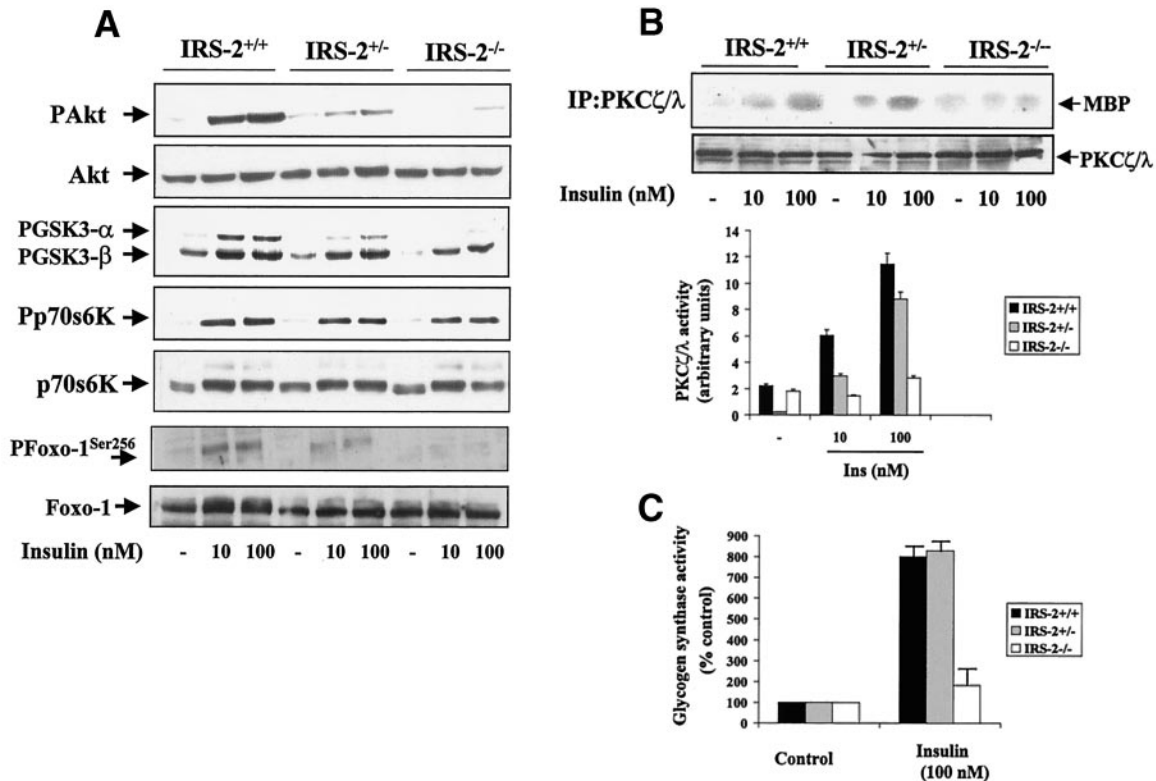


FIG. 5. Differential effect of insulin on the activation of PI 3-kinase effectors in wild-type, IRS-2^{+/-}, and IRS-2^{-/-} neonatal hepatocytes. **A:** Quiescent (20-h serum-starved) wild-type (IRS-2^{+/+}), IRS-2^{+/-}, and IRS-2^{-/-} neonatal hepatocytes were stimulated with insulin (10–100 nmol/l) for 5 min or maintained in the absence of the hormone. Cells were 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-p70s6k total p70s6k, phospho GSK3-(α/β), phospho-Foxo1, and Foxo1. The autoradiograms corresponding to four independent experiments were quantitated by scanning densitometry. A representative experiment is shown. **B:** Serum-starved hepatocytes were stimulated with insulin (10–100 nmol/l) for 10 min. Then cells were lysed, and PKC ζ/λ activity was determined as described in RESEARCH DESIGN AND METHODS. The autoradiograms corresponding to three independent experiments were quantitated by scanning densitometry. Results are expressed as arbitrary units of myelin basic protein (MBP) phosphorylation and are means ± SE. **C:** Immortalized hepatocyte cell lines were grown to confluence and then serum starved for 20 h. Insulin (100 nmol/l) was added for 15 min, and control cells were incubated in the absence of the hormone. GS activity was assayed in the supernatants of cell lysates as described in RESEARCH DESIGN AND METHODS in the absence or presence of 10 mmol/l glucose 6-phosphate. GS activity is expressed as a percentage of the basal nonstimulated values. Data are means ± SE for six to eight different experiments.

threonine kinases become activated following insulin stimulation. The Western blot demonstrated that insulin stimulated Akt phosphorylation in wild-type immortalized neonatal hepatocytes in a dose-dependent manner (Fig. 5A). This effect was slightly reduced in IRS-2^{+/-} cells and severely impaired in IRS-2^{-/-} cells. Downstream from Akt, both GSK-3 (α and β isoforms) and members of the Forkhead family of transcription factors were phosphorylated and inactivated upon insulin treatment. Again, IRS-2^{+/-} hepatocytes showed a reduced response to insulin in GSK-3 phosphorylation (mainly GSK-3α isoform) and Foxo1 (previously known as FKHR), as compared with wild-type cells, but this effect was impaired in IRS-2^{-/-} cells. By contrast, serine/threonine phosphorylation of p70s6k, another Akt target, was similar in the three cell lines upon insulin stimulation, regardless of genotype.

Several studies performed in adipose tissue and skeletal muscle suggest that activation of the atypical PKC isoforms (ζ and λ) is required for insulin stimulation of glucose uptake and GLUT4 translocation (32–34). To test whether insulin activation of PKC ζ/λ in the liver relies on IRS-2/PI 3-kinase signaling, quiescent cells were stimulated with insulin (10–100 nmol/l) for 10 min and PKC ζ/λ activity was determined by an *in vitro* protein kinase assay. As shown in Fig. 5B, insulin increased PKC ζ/λ

activity in wild-type hepatocytes, in which the response is maximal at the 100-nmol/l concentration. Insulin activation of PKC ζ/λ was not affected in cells lacking 50% IRS-2 expression (IRS-2^{+/-}). However, insulin was unable to activate PKC ζ/λ in cells lacking IRS-2. The three cell types showed similar PKC ζ/λ protein content.

It has been proposed that phosphorylation of GSK-3 serves as a physiologically relevant mechanism for regulating GS activity. Immortalized wild-type hepatocytes displayed a substantial increase in GS activity upon insulin stimulation (Fig. 5C). IRS-2^{+/-} cells responded similarly to wild-type cells in the stimulation of GS activity. By contrast, the ability of insulin to activate GS in IRS-2^{-/-} hepatocytes was completely abolished.

Reconstitution of IRS-2 in immortalized hepatocytes from IRS-2 knockout mice by adenoviral gene transfer restores activation of Akt and PKC ζ/λ and inactivation of GSK-3 and Foxo1 by insulin. To test whether the lack of IRS-2 is indeed responsible for impaired insulin signaling in IRS-2-deficient hepatocytes, we reconstituted IRS-2 expression by adenoviral gene transfer. Figure 6A reveals the re-expression of IRS-2 in two different IRS-2^{-/-} hepatocyte cell lines. Following retroviral-mediated gene transfer, the level of IRS-2 expression was similar to that detected in wild-type cells. Under these

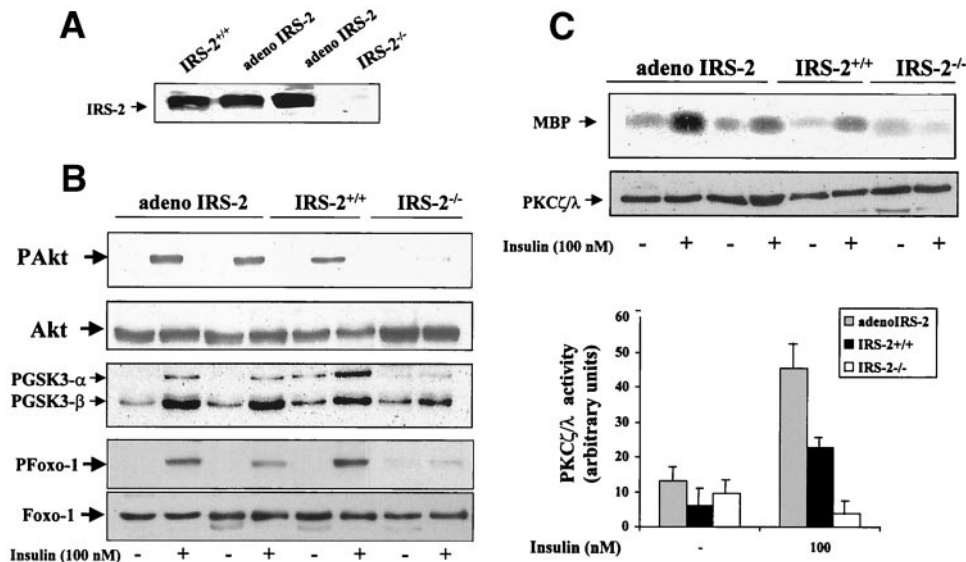


FIG. 6. Reconstitution of IRS-2 expression and its signaling in IRS-2-deficient hepatocytes by adenoviral gene transfer. **A:** Immortalized IRS-2-deficient neonatal hepatocytes were grown to confluence. Cells were then infected with adenovirus encoding wild-type IRS-2 and mock (β -galactosidase). After 24–48 h, cells were lysed, and IRS-2 expression in the different cell lines was assessed by Western blot. **B:** Cells were infected with adenovirus encoding wild-type IRS-2 and mock (β -galactosidase), as described in **A**. Then cells were serum starved for 16–20 h and further stimulated with insulin (10–100 nmol/l) for 5 min. Phosphorylation of Akt, GSK-3, and Foxo1 was assessed by Western blot. A representative experiment out of three is shown. **C:** Cells were infected and further stimulated with insulin as described above. PKC ζ/λ activity was determined in anti-PKC ζ/λ immune complexes as described under RESEARCH DESIGN AND METHODS. The results from three independent experiments were quantitated by scanning densitometry and are means \pm SE.

experimental conditions, we tested whether insulin signaling downstream from PI 3-kinase could be recovered. As shown in Fig. 6B, phosphorylation of Akt and its downstream targets, GSK-3 (α and β isoforms) and Foxo1, were rescued in IRS-2 reconstituted hepatocytes (adenoviral IRS-2), reaching levels comparable with those observed in wild-type cells. Furthermore, activation of PKC ζ/λ by insulin was even higher in adenoviral-infected IRS-2-deficient hepatocytes than in wild-type cells (Fig. 6C).

Insulin fails to activate GS and to inhibit PEPCK and G6Pase mRNA expression in IRS-2-deficient primary hepatocytes. To investigate whether the lack of IRS-2 in hepatocytes could interfere with the metabolic actions of insulin, we performed experiments in a more physiological cellular model. Isolated hepatocytes from wild-type, heterozygous, and IRS-2 knockout neonates were cultured as described in RESEARCH DESIGN AND METHODS. Then, GS activity was measured in 20-h serum-deprived cells following 15 min of insulin stimulation. As shown in Fig. 7A, both insulin-treated wild-type and heterozygous hepatocytes in primary culture displayed an increase in GS activity. By sharp contrast, insulin failed to activate GS in IRS-2^{-/-} primary hepatocytes.

Gluconeogenesis is a highly regulated process catalyzed by several enzymes under the regulation of insulin. In vivo, insulin resistance in the livers of IRS-2 knockout mice appears to result from the failure of the hormone to inhibit hepatic glucose production (HGP). In liver, PEPCK and G6Pase are rate-limiting enzymes for gluconeogenesis. Accordingly, our next step was to study in vitro whether gluconeogenic enzymes were induced by an appropriated stimulus (dexamethasone/cAMP) in primary cultures of wild-type, IRS-2^{+/-}, and IRS-1^{-/-} mouse neonatal hepatocytes. Hepatocytes isolated from single livers were grown to confluence, and after 20 h of serum deprivation a

mixture of dexamethasone/cAMP was added to the medium for a further 6 h. RNA was then isolated and analyzed by Northern blot. As shown in Fig. 7B, both PEPCK and G6Pase mRNA were induced by dexamethasone/cAMP in the three cell types studied, regardless of IRS-2 expression. We studied the effect of insulin on gluconeogenic gene expression. Insulin triggered a substantial inhibition of dexamethasone/cAMP-induced PEPCK and G6Pase mRNAs in wild-type and IRS-2^{+/-} hepatocytes but was incapable of suppressing dexamethasone/cAMP-induced gluconeogenic gene expression in primary hepatocytes derived from IRS-2-deficient mice.

Reconstitution of IRS-2 signaling in primary cultures of neonatal hepatocytes rescues activation of GS and inhibition of gluconeogenic gene expression by insulin. Given the failure of insulin to induce GS activity and to suppress PEPCK and G6Pase mRNA expression in primary IRS-2-deficient hepatocytes, we addressed whether the reconstitution of IRS-2 signaling in these cells could restore insulin sensitivity. IRS-2^{-/-} primary hepatocytes were infected with adenoviral vectors encoding wild-type IRS-2 and mock. After 24 h, cells were serum starved for a further 16–20 h. Then, cells were stimulated with insulin for 15 min, and GS activity was determined. As shown in Fig. 8A, insulin stimulated GS activity in reconstituted hepatocytes (adenoviral IRS-2) to a level similar to that observed in wild-type cells. Insulin failed to activate GS in mock-infected IRS-2-deficient cells. Next, we investigated the ability of IRS-2 to mediate insulin-induced suppression of gluconeogenic gene expression. Primary hepatocytes were infected as indicated above. After 24 h of infection, cells were serum starved for 4 h and then stimulated for 6 h with dexamethasone/cAMP in the absence or presence of insulin (100 nmol/l). Figure 8B demonstrates that PEPCK and G6Pase mRNA expression (induced by dexa-

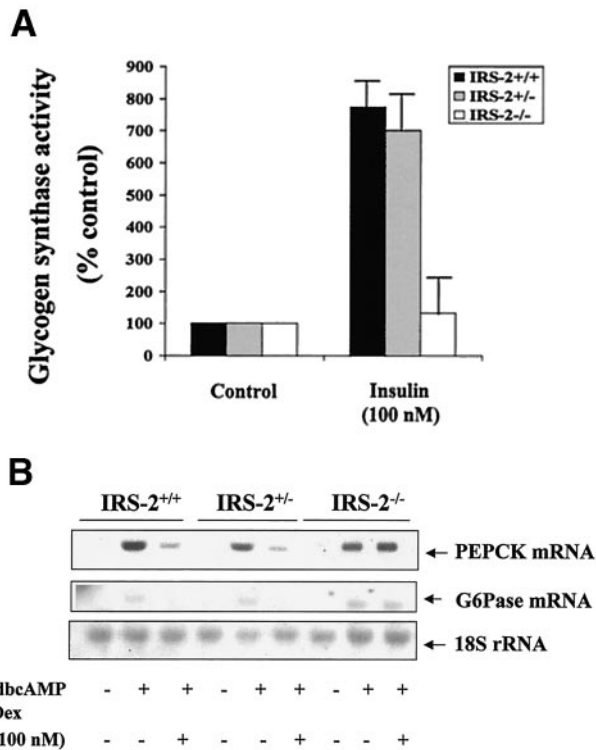


FIG. 7. Insulin failed to activate GS and inhibit dexamethasone/cAMP-induced PEPCK and G6Pase gene expression in IRS-2-deficient primary neonatal hepatocytes. **A:** Primary hepatocytes obtained from 3-day-old mouse livers were cultured to confluence as described in RESEARCH DESIGN AND METHODS and then serum starved for 20 h. Insulin (100 nmol/l) was added for 15 min, and control cells were incubated in the absence or presence of 10 mmol/l glucose 6-phosphate. GS activity was assayed in the supernatants of cell lysates as described in RESEARCH DESIGN AND METHODS in the absence or presence of 10 mmol/l glucose 6-phosphate. GS activity is expressed as a percentage of the basal nonstimulated values. Data are means \pm SE for six to eight different experiments. **B:** Primary hepatocytes were cultured to confluence. Then cells were serum starved for 20 h and further cultured for 6 h in the presence of 0.5 mmol/l dbcAMP plus 1 μ mol/l dexamethasone in either the absence or presence of 100 nmol/l insulin. At the end of the culture, RNA was isolated, submitted to Northern blot analysis, and hybridized with labeled PEPCK, G6Pase, and 18S rRNA cDNA. Representative autoradiograms are shown.

methasone/cAMP) was significantly suppressed by insulin in reconstituted IRS-2^{-/-} hepatocytes (adenoviral IRS-2) compared with IRS-2^{-/-} hepatocytes, which lacked this effect.

Finally, we investigated an alternative mechanism to overcome insulin resistance in the gluconeogenic pathways of IRS-2-deficient hepatocytes. Nakae et al. (35) have recently proposed that the truncated Foxo1 mutant that lacks the transactivation domain (Δ 256) could be a useful reagent for inhibiting gluconeogenesis in experimental systems. Accordingly, we tested this approach in our in vitro model of IRS-2-deficient primary hepatocytes by assessing the effect of Δ 256Foxo1 on gluconeogenic gene expression. Transduction of IRS-2^{-/-} hepatocytes with adenoviral vector encoding Δ 256Foxo1 mutant, which competes with endogenous Foxo1 and exerts a dominant negative effect, totally eliminated dexamethasone/cAMP-induced PEPCK and G6Pase mRNA expression in the absence of insulin (Fig. 8B).

DISCUSSION

Hepatic insulin resistance is a common feature of animal models of insulin resistance and type 2 diabetes. IRS-2

knockout is a genetic mouse model with increased HGP (6,7). In addition, ablation of IRS-2 results in an impaired development of pancreatic β -cells as a result of a lack of IGF-I signaling (36). However, the combined defect of β -cell failure and peripheral insulin resistance has complicated the analysis of hepatic insulin resistance as a primary cause of the type 2 diabetic phenotype of IRS-2-deficient mice. In this study, we generated immortalized hepatocyte cell lines derived from individual livers of wild-type, heterozygous, and IRS-2 knockout mice. By using this approach we attempted to define the role of IRS-2 in the insulin-signaling network of hepatocytes and relate signaling defects to the abnormalities in the hepatic carbohydrate metabolism described in the IRS-2-deficient mice. These cells provided an excellent tool for in vitro study of insulin signaling leading to the expression of genes implicated in liver glucose metabolism.

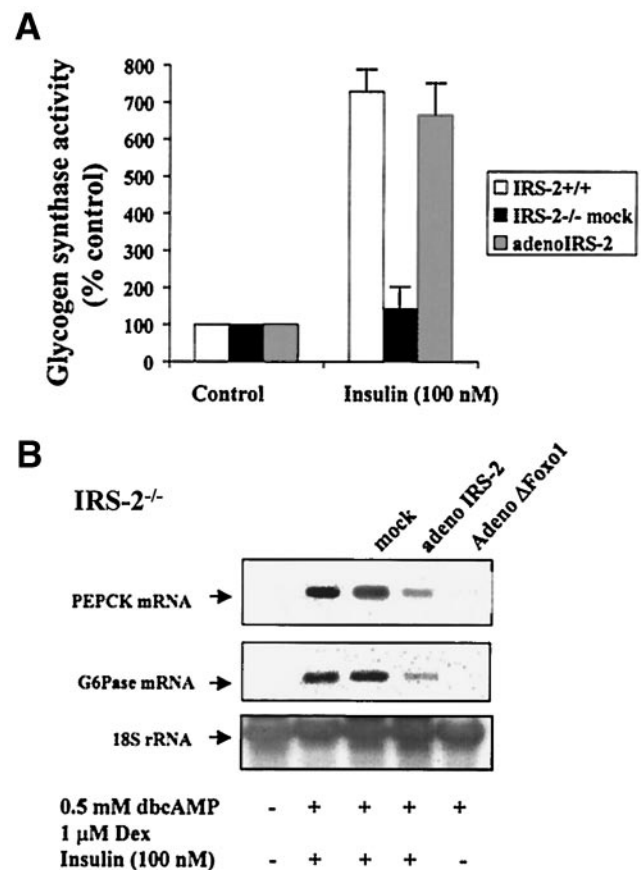


FIG. 8. Reconstitution of IRS-2 in IRS-2-deficient neonatal primary hepatocytes restores activation of GS and inhibition of gluconeogenic gene expression by insulin. **A:** IRS-2-deficient primary hepatocytes were infected with adenovirus encoding wild-type IRS-2 and mock (β -galactosidase) as described above. After infection (24 h), cells were serum starved for 16–20 h and further stimulated with 100 nmol/l insulin for 15 min. GS activity was assayed in the supernatants of cell lysates as described in RESEARCH DESIGN AND METHODS in the absence or presence of 10 mmol/l glucose 6-phosphate. GS activity is expressed as a percentage of the basal nonstimulated values. Data are means \pm SE for six to eight different experiments. **B:** Primary hepatocytes were cultured to confluence as described in RESEARCH DESIGN AND METHODS. Then cells were infected with adenoviral constructs encoding IRS-2, Δ 256Foxo1, and mock (β -galactosidase). After infection (24 h), cells were serum starved for 4 h and further stimulated for 6 h in the presence of 0.5 mmol/l dbcAMP plus 1 μ mol/l dexamethasone in either the absence or presence of 100 nmol/l insulin. At the end of the culture time, RNA was isolated and submitted to Northern blot analysis and hybridized with labeled PEPCK, G6Pase, and 18S rRNA cDNA. Representative autoradiograms are shown.

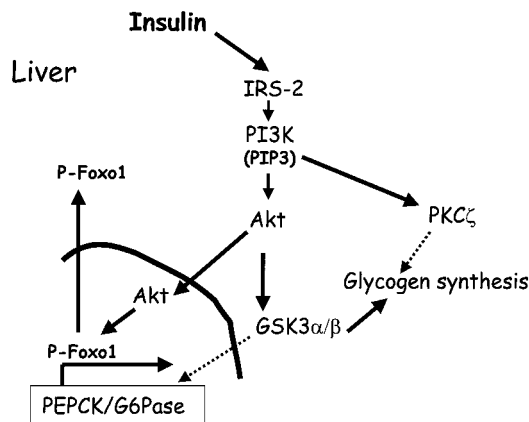


FIG. 9. Role of IRS-2 in the insulin-signaling cascade in hepatocytes. IRS-2-deficient neonatal hepatocytes showed insulin resistance in reference to IRS-2/PI 3-kinase signaling. Downstream from PI 3-kinase, insulin failed to activate Akt, GSK-3 (α and β isoforms), and PKC ζ/λ in the absence of IRS-2. At the nuclear level, the phosphorylation of Foxo1 is also impaired. Insulin upregulated GS activity and downregulated gluconeogenic gene expression in wild-type hepatocytes. The lack of IRS-2 in hepatic cells resulted in the dysregulation of GS activation and the inhibition of gluconeogenic gene expression.

The new hepatocyte cell lines (IRS-2^{+/+}, IRS-2^{+/-}, and IRS-2^{-/-}) were grown for at least 3 weeks in arginine-free medium to select cells with a functional urea cycle. Under these culture conditions, we circumvented the potential contamination of the primary cultures with nonparenchymal cells, such as fibroblasts, as assessed by negative immunofluorescence with the antivimentin antibody. In addition, all cell lines maintained the expression of albumin, a protein secreted by hepatocytes, and CPS, a urea cycle enzyme marker. Moreover, the number of IRs and their affinity were similar among the different cell types, indicating that immortalized hepatocytes are insulin target cells. In regard to insulin signaling, our experiments in immortalized neonatal hepatocytes show that the lack of IRS-2 is not compensated for by an elevation of IRS-1 protein content or an increase in tyrosine phosphorylation. Furthermore, whereas PI 3-kinase activity associated with tyrosine phosphorylated proteins was reduced by 50% in IRS-2^{-/-} hepatocyte cell lines, the IRS-1-associated PI 3-kinase was similar to the wild-type. These results are in agreement with those previously reported in liver extracts (7), thus strengthening the essential and specific role of IRS-2 in mediating downstream signaling to PI 3-kinase in the liver. Experiments performed in the muscle of IRS-2^{-/-} animals, where total PI 3-kinase activity was unaffected, support the concept of the tissue specificity of insulin action, as well as the distinct compensatory mechanisms in the different insulin-responsive tissues (7,12).

The fact that only a small number of IRS-2^{-/-} hepatocytes (<10%) translocate the PH GRP1-GFP fusion protein to the membrane in response to insulin stimulation indicates that the generation of plasma membrane PIP₃ is severely reduced in the absence of IRS-2. Consequently, the phosphorylation of Akt and GSK-3 in IRS-2^{-/-} hepatocytes upon insulin stimulation was severely reduced compared with that of wild-type. These results suggest that IRS-2-associated PI 3-kinase activity may constitute the main source of PIP₃ in hepatocytes, given that IRS-1-associated PI 3-kinase is unable to compensate this response to insulin in IRS-2^{-/-} cells. Furthermore, both Akt

and GSK-3 phosphorylation were recovered after reconstitution of IRS-2 signaling in deficient cells. Again, these data emphasize the tissue specificity of molecular defects that contribute to insulin resistance. Conversely, activation of p70s6 kinase in immortalized hepatocytes was unaffected by the lack of IRS-2. These results illustrate a diverging signaling pathway downstream from PI 3-kinase and further support the existence of alternative PI 3-kinase-independent pathways that regulate the activation of p70s6 kinase in the liver, thereby preventing inhibition of protein synthesis (37). The fact that p70s6 kinase-1 knockout mice display a diabetic phenotype suggests a mechanism of insulin resistance independent of IRS-2 (38).

Our results demonstrate that the alterations in insulin signaling related to the lack of IRS-2 in hepatocytes have important metabolic implications. Our studies reveal for the first time that insulin fails to activate GS in vitro in IRS-2^{-/-} hepatocytes, a response that can be recovered after IRS-2 reconstitution. Since PI 3-kinase activity in the liver is essential for normal function of carbohydrate and lipid metabolism in living animals (39), our results might be a direct consequence of the impaired Akt phosphorylation and GSK-3 inactivation in IRS-2^{-/-} hepatocytes. In fact, previous experiments (40) in immortalized hepatocytes from IR knockout mice show that a selective loss of IRS-2/PI 3-kinase signaling results in decreased glycogen synthesis. However, the fact that insulin-induced PKC ζ/λ activity is also suppressed in the absence of IRS-2 and further recovered after IRS-2 reconstitution indicates that this molecule could participate in the phosphorylation and inactivation of hepatic GS. Interestingly, it has been recently reported that PKC ζ phosphorylates GSK-3 α and β isoforms in L6hIR cells, and this event is an essential requirement for GS activation (41). In the light of these results, an attractive therapeutic approach to ameliorate hepatic insulin resistance in the absence of IRS-2 would be the use of novel GSK-3 inhibitors, which could improve glucose disposal through the enhancement of glycogen synthesis in the liver (42).

The development of fasting hyperglycemia in type 2 diabetes is believed to be secondary to increased HGP, mainly through an increase in gluconeogenic pathways. The importance of the liver in the maintenance of normoglycemia has been studied through different approaches. In mice overexpressing PEPCK or G6Pase, insulin is unable to suppress HGP despite the presence of hyperinsulinemia (43,44). These findings prompted us to investigate whether the alterations of IRS-2/PI 3-kinase signaling in IRS-2^{-/-} hepatocytes could account for dysregulation in gluconeogenic gene expression. We found that insulin failed to inhibit dexamethasone/cAMP-induced PEPCK and G6Pase mRNA in primary hepatocytes of IRS-2^{-/-} mice, but not in wild-type or heterozygous mice. Moreover, IRS-2 reconstitution of deficient hepatocytes led to a full recovery of insulin inhibition in gluconeogenic gene expression. Thus, our in vitro results indicate that the metabolic changes in hepatic function in IRS-2-deficient animals (45) could be the result of alterations in insulin signaling that produce a dysregulation of gene expression. Previous experiments (46) performed in liver-specific IR knockout mice showed a substantial increase in gluconeogenic gene expression accompanied by an unexpected

increase in IRS-2 protein content. More recently, it has been reported (47) that PEPCK overexpression in the liver results in a selective downregulation of IRS-2 expression and a failure of insulin to suppress HGP. Taken together, all of these data strongly suggest that IRS-2, PI 3-kinase, and Akt signaling drive insulin-negative regulation of gluconeogenic gene expression. In addition, GSK-3 may also regulate PEPCK and G6Pase gene expression in neonatal hepatocytes through the phosphorylation and 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 (48).

The possibility of controlling hepatic glucose utilization and production as a treatment of type 2 diabetes has been explored by Ferre et al. (49) by overexpressing glucokinase in the liver of diabetic mice. In the liver of these animals, glycolysis and glycogen synthesis were induced while gluconeogenesis was blocked, resulting in the normalization of blood glucose levels. Therefore, this approach could be of potential interest in the treatment of the hepatic insulin resistance that is developed in type 2 diabetes and is associated with the lack of IRS-2, and it might circumvent the requirement of β -cell compensatory mechanisms. In a study related to therapies for ameliorating hepatic insulin resistance, Nakae et al. (35) found that the dominant-negative Foxo1 mutant provides a useful reagent for inhibiting gluconeogenesis in experimental systems. In fact, they recently demonstrated that Foxo1 haploinsufficiency corrects hepatic insulin resistance in IR^{+/-} diabetic mice (50). Foxo1 is phosphorylated in an insulin-responsive manner by PIP3-dependent kinases, such as Akt, and then excluded from the nucleus (51–53). Studies in hepatoma cells (54,55) suggest that Foxo1 and -3 regulate the transcription of reporter genes containing insulin response elements from the PEPCK and G6Pase promoters. More recently, insulin signaling via Akt to Foxo1 has been shown to inhibit the expression of the peroxisome proliferator-activated receptor- γ coactivator (PGC-1) (56), which is induced in the liver in the fasting state and in various mouse models with deficient insulin action. It is also required for the expression of PEPCK and G6Pase (57). In the current study, we demonstrate that phosphorylation of Foxo1 by Akt relies on IRS-2 signaling in neonatal hepatocytes and that the Δ 256Foxo1 dominant-negative mutant rescues the insulin resistance observed in IRS-2-deficient hepatocytes, thus restoring the inhibition of PEPCK and G6Pase expression induced by dexamethasone/cAMP in an insulin-independent manner. Whether Δ 256Foxo1 directly blocks access to Foxo sites in the PEPCK promoter or functions indirectly by blocking the expression of coactivators such as PGC-1, which are required for PEPCK expression, remains to be established. These results reinforce the hypothesis that dominant-negative Foxo1 could be of potential benefit in reducing glucose production in diabetic patients. However, further effects of this therapy on liver function need to be investigated extensively.

In conclusion, our results demonstrate the essential role of IRS-2 in insulin-regulated glycogen synthesis and in the expression of gluconeogenic enzymes involved in HGP, as summarized in Fig. 9. The current studies, taken together

with the phenotype of the IRS-2 knockout model, implicate IRS-2 as the principal mediator of insulin action in liver and suggest that impaired IRS-2 signaling may represent a major component of liver insulin resistance.

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