Both Insulin Signaling Defects in the Liver and Obesity Contribute to Insulin Resistance and Cause Diabetes in Irs2\(^{-/-}\) Mice*

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We previously reported that insulin receptor substrate-2 (IRS-2)-deficient mice develop diabetes as a result of insulin resistance in the liver and failure of β-cell hyperplasia. In this study we introduced the IRS-2 gene specifically into the liver of Irs2\(^{-/-}\) mice with adenovirus vectors. Glucose tolerance tests revealed that the IRS-2 restoration in the liver ameliorated the hyperglycemia, but the improvement in hyperinsulinemia was only partial. Endogenous glucose production (EGP) and the rate of glucose disappearance (Rd) were measured during hyperinsulinemic-euglycemic clamp studies: EGP was increased 2-fold in the Irs2\(^{-/-}\) mice, while Rd decreased by 50%. Restoration of IRS-2 in the liver suppressed EGP to a level similar to that in wild-type mice, but Rd remained decreased in the Adeno-IRS-2-infected Irs2\(^{-/-}\) mice. Irs2\(^{-/-}\) mice also exhibit obesity and hyperleptinemia associated with impairment of hypothalamic phosphatidylinositol 3-kinase activation. Continuous intracerebroventricular leptin infusion or calorie restriction yielded Irs2\(^{-/-}\) mice whose adiposity was comparable to that of Irs2\(^{-/+}\) mice, and both the hyperglycemia and the hyperinsulinemia of these mice improved with increased Rd albeit partially. Finally combination treatment consisting of adenovirus-mediated gene transfer of IRS-2 and continuous intracerebroventricular leptin infusion completely reversed the hyperglycemia and hyperinsulinemia in Irs2\(^{-/-}\) mice. EGP and Rd also became normal in these mice as well as in mice treated by calorie restriction plus adenoviral gene transfer. We therefore concluded that a combination of increased EGP due to insulin signaling defects in the liver and reduced Rd due to obesity accounts for the systemic insulin resistance in Irs2\(^{-/-}\) mice.

Type 2 diabetes is characterized by peripheral insulin resistance and insulin secretory dysfunction (1–5). The insulin resistance in obesity (6) and type 2 diabetes is manifested by decreased insulin-stimulated glucose transport and metabolism in adipocytes and skeletal muscle and by impaired suppression of hepatic glucose output (7–10). The insulin receptor substrate (IRS)\(^{±}\) proteins play a key role in signal transduction from the insulin receptor (11, 12) and are major intracellular phosphorylation targets of activated insulin receptor tyrosine kinase (13). The mammalian IRS protein family contains at least four members: ubiquitously expressed IRS-1 (14) and IRS-2 (15), adipose tissue-predominant IRS-3 (16), and IRS-4, which is expressed in thymus, brain, and kidney (17). The physiological roles of each protein have been evaluated by gene targeting strategies. Irs1\(^{-/-}\) mice are growth-retarded and insulin-resistant (18, 19) but do not develop diabetes because an alternate substrate, IRS-2, compensates for the lack of IRS-1 in liver (19–21) and, at least in part, in skeletal muscle (22). In addition, the hyperinsulinemia associated with the β-cell hyperplasia in these mice effectively compensates for the insulin-resistant state (23). Meanwhile Irs2\(^{-/-}\) mice develop diabetes presumably due to inadequate β-cell proliferation combined with liver insulin resistance (24–27). However, it is not known whether the liver insulin resistance of Irs2\(^{-/-}\) mice is causally involved in the development of diabetes.

Another particularly noteworthy feature of Irs2\(^{-/-}\) mice is that they exhibit increased adiposity associated with hyperleptinemia (28, 29) and are even resistant to peripherally administered leptin (29). Leptin is an adipocyte-derived hormone that exerts pleiotropic effects, including profound effects on satiety, energy expenditure, and neuroendocrine function (30, 31). Leptin has a clear insulin-sensitizing effect on normal rodents when administered acutely as well as chronically (32, 33). The hypothesis that leptin resistance occurs in association with obesity was first suggested by the discovery of elevated plasma leptin levels in obese humans (34), and obesity-induced insulin resistance can be explained by leptin resistance at least in part (35).

In the present study, since we assumed that the insulin

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* The abbreviations used are: IRS, insulin receptor substrate; PI, phosphatidylinositol; PEPCK, phosphoenolpyruvate carboxykinase; ITT, insulin tolerance test; GTT, glucose tolerance test; GIR, glucose infusion rate; EGP, endogenous glucose production; Rd, rate of glucose disappearance; STAT, signal transducers and activators of transcription.
resistance in Irs2−/− mice was caused by the insulin signaling defects in the liver, we investigated the effect of IRRS-2 restoration in the liver by adenovirus-mediated gene transfer on their development of insulin resistance and diabetes. Although the diabetes was prevented by reduction of endogenous glucose production, the hyperinsulinemia was not completely ameliorated, suggesting that there must be additional causes of the insulin resistance in Adeno-IRS-2-infected Irs2−/− mice. We next evaluated the involvement of obesity in the insulin resistance of Irs2−/− mice and found that continuous intracerebroventricular infusion of leptin yielded lean Irs2−/− mice whose adiposity was almost the same as that of wild-type (Irs2+/+) mice. Both the hyperglycemia and the hyperinsulinemia improved in these mice with elevated glucose utilization albeit partially. Caloric restriction also increased glucose utilization but did not affect glucose production in Irs2−/− mice. Finally we found that combination treatment consisting of both adenovirus gene transfer of IRS-2 and continuous intracerebroventricular leptin infusion completely abolished the hyperglycemia and hyperinsulinemia of the Irs2−/− mice. Glucose production and utilization also became normal in the mice as well as mice treated with caloric restriction plus adenoviral gene transfer. We concluded that both the insulin signaling defects in the liver and obesity associated with leptin resistance contribute to the development of insulin resistance in Irs2−/− mice.

EXPERIMENTAL PROCEDURES

Animals—Mice lacking IRS-2 were generated as described elsewhere (26), and both Irs2−/+ mice and Irs2−/− mice were used in the experiments described below. Irs2−/− mice were originally maintained on the C57BL/6 and CBA hybrid background and backcrossed onto C57BL/6 strain for at least three generations. The mice were housed on a 12-h light-dark cycle and given ad libitum access to regular chow except as indicated. All experiments in this study were performed on 12–16-week-old male mice. The animal care and procedures of the experiments were approved by the Animal Care Committee of University of Tokyo.

Generation of Adenoviruses—cDNA of mouse IRS-2 including the regulatory subunit were purchased from Upstate Biotechnology. Monoclonal anti-FLAG was purchased from Sigma. Rabbit polyclonal antibodies against Akt, phospho-Akt (Ser-473), STAT3, and phospho-STAT3 (Tyr-705) were purchased from Cell Signaling Technology (Beverly, MA).

PI 3-Kinase Assay—PI 3-kinase activity was determined in immunoprecipitates with the antibody indicated as described elsewhere (22, 36). The liver was removed 75 s after injection of 5 units of insulin into the inferior cava and then homogenized in ice-cold buffer A. The hypothalamus was removed 10 min after injection of 0.2 unit of insulin into the inferior cava and removed 30 min after leptin injection (10 mg/kg of body weight intraperitoneally or 1.0 μg intracerebroventricularly). To the immunoprecipitates in 50 μl of kinase buffer (25 mm Tris-HCl (pH 7.4), 0.5 mm EGTA, 100 mm NaCl, 1 mg/ml phosphatidylinositol, 5.0 μl of reaction buffer (200 μM ATP, 1 μg/ml PLIP, 10 μM MgCl2) was added. The reaction was stopped after 20 min at room temperature, and the lipids were extracted with chloroform and subjected to thin-layer chromatography. The amount of hot ATP incorporated into the lipid substrates was quantified with an image analyzer (BA 2000, Fujin Film, Tokyo, Japan) and expressed as the intensity of phosphostimulated luminescence. Phosphatidylinositol was purchased from Sigma. [γ-32P]ATP was purchased from PerkinElmer Life Sciences.

RNA Preparation and Northern Blot Analysis—Total RNA was prepared from the liver with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Northern blot analysis was performed according to the standard protocol using 20 μg of total RNA. Total RNA from the liver was loaded onto a 1.0% agarose gel and then transferred to a nylon membrane (Hybond N+, Amersham Biosciences). The cDNA probes for mouse phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase were prepared by cloning reverse transcription-polymerase chain reaction products from mouse liver mRNA into TA cloning vectors (Invitrogen). The polymerase chain reaction primers used to generate these probes were as follows: PEPCK: forward primer, 5′-GGAGAACAAAGGAGTGGAAAG-3′, and reverse primer, 5′-GAGGA-ACCTGCGCACATTGTTT-3′; and glucose-6-phosphatase: forward primer, 5′-TCTCCGGGCCTGTCGGTGGA-3′, and reverse primer, 5′-CTCTGC-AAATCCAGCGGAGGC-3′. The corresponding bands were quantified by exposure of BAS 2000 to the filters and analysis of the images with BAS Station software.

Insulin Tolerance Test (ITT)—Insulin tolerance was tested in fed mice. The animals were intraperitoneally injected with 1.0 milliunits/kg (body weight) human insulin (Humulin R, Lilly). Blood samples were drawn from the tail vein at the times indicated, and glucose was measured with an automatic blood glucose meter (Glutest Pro, Sanwa Chemical, Nagoya, Japan).

Glucose Tolerance Test (GTT)—Before the study, mice were fasted for 16 h starting at 19:00, and at the end of the fast they were loaded with 1.0 mg/kg (body weight) glucose orally. Blood samples were collected at different times, and glucose was measured with an automatic blood glucose meter immediately. The blood was collected in heparinized tubes, and the plasma was stored at −20°C. Insulin levels were determined with an insulin immunomunassay kit (BIO-TRAK, Amersham Biosciences) using rat insulin as a standard (26).

Hyperinsulinemic-Euglycemic Clamp Study—Four days before the study, a catheter that consisted of a silicon part (Phicon Tube, Fuji Systems, Tokyo, Japan) and a polyethylene part (PE-50, BD Biosciences) was inserted into the right jugular vein for infusion under general anesthesia with sodium pentobarbital. Studies were performed on mice under conscious and unstressed conditions after a 6-h fast. A primed continuous infusion of insulin (Humulin R, Lilly) was given (5.0 milliunits/kg·min−1), and the glucose blood concentration was monitored at 5 min and maintained at ~ 120 mg/dl with 100 units of regular insulin/kg/min infused in heparinized tubes, and the plasma was stored at −20°C. Insulin levels were determined with an insulin immunomunassay kit (BIO-TRAK, Amersham Biosciences) using rat insulin as a standard (26).

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stainless steel cannula was inserted into the right lateral cerebral ventricle (1.0 mm posterior, 1.0 mm lateral, and 2.0 mm (Irs2−/−) or 1.5 mm (Irs2+/+) ventral to the bregma). To prevent the cannula from being blocked by blood clots, a stainless steel stylet was inserted into each cannula until used. The animals were allowed to recover for 1 week after the operation (41). One week after insertion of the cannula, DURECT miniosmotic pumps (DURECT Corp., Cupertino, CA) were connected to the intracerebroventricular cannula in the mice, and the pumps delivered a constant infusion of 1.0 ml/h for 3 days. Mice were anesthetized, and 5 units of insulin were injected into the inferior vena cava. After 75 s, the liver was removed and homogenized in ice-cold buffer A. The liver lysates were immunoprecipitated (IP) with antibodies against IRS-2, FLAG peptide, or IRS-1 and subjected to SDS-PAGE. c–e, insulin signaling in the Irs2−/− mouse liver after adenoviral gene transfer. Mice were anesthetized, and 5 units of insulin were injected into the inferior vena cava. After 75 s, the liver was removed and homogenized in ice-cold buffer A, c, tyrosine-phosphorylated IRS proteins in the liver in response to insulin. The liver lysates were immunoprecipitated with the antibodies indicated (PY20 anti-phosphotyrosine, anti-IRS-2, or anti-IRS-1 antibodies), and the immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with anti-phosphotyrosine antibody. d, insulin-induced PI 3-kinase activity associated with IRS-2 protein in the liver. The liver lysates were immunoprecipitated with anti-IRS-2 and subjected to PI 3-kinase assay as described under “Experimental Procedures.” e, phosphorylation of Akt in the liver. The liver lysates were subjected to Western blotting with anti-phosphorylated Akt (α p-Akt) or anti-Akt (α Akt) antibodies.

insulin resistance in the liver and failure of β-cell hyperplasia (26). In the present study we investigated whether restoration of IRS-2 to the liver by adenovirus-mediated gene transfer would improve the insulin resistance and diabetes of Irs2−/− mice. The expression of exogenous IRS-2 protein in the liver was confirmed by immunohistochemistry and Western blot analysis, and almost the same level of IRS-2 was detected in the liver of Irs2−/− mice infected with Adeno-IRS-2 as in Irs2−/− mice (Fig. 1, a and b). The IRS-2 protein was recognized by an anti-FLAG antibody, confirming that the protein had been introduced exogenously. Infection with the Adeno-IRS-2 and Adeno-LacZ vectors did not affect expression of IRS-1 in the liver (Fig. 1b).

FIG. 1. Effect of IRS-2 restoration by adenovirus-mediated gene transfer on insulin signaling in Irs2−/− mouse liver. Male Irs2−/− mice at the age of 12–16 weeks were injected with the adenoviruses as described under “Experimental Procedures.” a, expression of IRS-2 in the Irs2−/− mouse liver after adenoviral gene transfer. Representative images of immunohistochemistry (IHC) using antibodies against IRS-2 (α IRS-2) or FLAG peptide (α FLAG) are presented. b, Western blot analysis. Liver lysates were immunoprecipitated (IP) with antibodies against IRS-2, FLAG peptide, or IRS-1 (α IRS-1) and subjected to SDS-PAGE. c, insulin signaling in the Irs2−/− mouse liver after adenoviral gene transfer. Mice were anesthetized, and 5 units of insulin were injected into the inferior vena cava. After 75 s, the liver was removed and homogenized in ice-cold buffer A, c, tyrosine-phosphorylated IRS proteins in the liver in response to insulin. The liver lysates were immunoprecipitated with the antibodies indicated (PY20 anti-phosphotyrosine, anti-IRS-2, or anti-IRS-1 antibodies), and the immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with anti-phosphotyrosine antibody. d, insulin-induced PI 3-kinase activity associated with IRS-2 protein in the liver. The liver lysates were immunoprecipitated anti-IRS-2 and subjected to PI 3-kinase assay as described under “Experimental Procedures.” e, phosphorylation of Akt in the liver. The liver lysates were subjected to Western blotting with anti-phosphorylated Akt (α p-Akt) or anti-Akt (α Akt) antibodies.

All values are reported as means ± S.E. Statistics were calculated using one-way analysis of variance; Scheffé’s post hoc test was used to determine significance. Where paired data were analyzed, a t test was used.

RESULTS

Expression of IRS-2 in Irs2−/− Mouse Liver by Adenovirus-mediated Gene Transfer—The results of our previous study suggested that Irs2−/− mice develop diabetes because of insulin resistance in the liver and failure of β-cell hyperplasia (26). In the present study we investigated whether restoration of IRS-2 to the liver by adenovirus-mediated gene transfer would improve the insulin resistance and diabetes of Irs2−/− mice. The expression of exogenous IRS-2 protein in the liver was confirmed by immunohistochemistry and Western blot analysis, and almost the same level of IRS-2 was detected in the liver of Irs2−/− mice infected with Adeno-IRS-2 as in Irs2−/− mice (Fig. 1, a and b). The IRS-2 protein was recognized by an anti-FLAG antibody, confirming that the protein had been introduced exogenously. Infection with the Adeno-IRS-2 and Adeno-LacZ vectors did not affect expression of IRS-1 in the liver (Fig. 1b).
Fig. 2. Effect of IRS-2 restoration in the liver on glucose metabolism in Irs2<sup>−/−</sup> mice. 
a. ITT of mice infected with the adenoviruses indicated. ITT was performed on the groups of mice indicated: Irs2<sup>−/−</sup> (white circles, n = 32), Irs2<sup>−/−</sup> + Adeno-LacZ (black squares, n = 16), and Irs2<sup>−/−</sup> + Adeno-IRS-2 (gray squares, n = 18). 
b. GTT was performed in mice infected with the adenoviruses indicated. The groups of mice indicated were orally loaded with a 1 mg/g of body weight dose of glucose: Irs2<sup>−/−</sup> (white circles, n = 32), Irs2<sup>−/−</sup> + Adeno-LacZ (black squares, n = 16), and Irs2<sup>−/−</sup> + Adeno-IRS-2 (gray squares, n = 18). 
c. Plasma insulin concentrations of the mice during the GTT. Plasma was collected from the groups of mice indicated: Irs2<sup>−/−</sup> (white circles, n = 6), Irs2<sup>−/−</sup> + Adeno-LacZ (black squares, n = 8), and Irs2<sup>−/−</sup> + Adeno-IRS-2 (gray squares, n = 9). 
d–f, effect of IRS-2 restoration in the liver on glucose kinetics during hyperinsulinemic-euglycemic clamp studies. Four days after surgical implantation of intravenous catheters and adenovirus infection, clamp studies were performed as described under “Experimental Procedures.” d, GIRs in Adeno-IRS-2-infected Irs2<sup>−/−</sup> mice were significantly higher than that in Adeno-LacZ-infected mice. e, EGP was decreased by IRS-2 restoration in the liver of Irs2<sup>−/−</sup> mice. f, Rd values were not significantly affected. clamp studies were performed on the groups of mice indicated: Irs2<sup>−/−</sup> (n = 6), Irs2<sup>−/−</sup> + Adeno-LacZ (n = 6), and Irs2<sup>−/−</sup> + Adeno-IRS-2 (n = 4). Plasma insulin concentrations during the clamp studies were as follows: 9.4 ± 1.1 ng/ml (Irs2<sup>+/+</sup>), 9.9 ± 1.0 ng/ml (Irs2<sup>−/−</sup> + Adeno-LacZ), and 9.2 ± 0.4 ng/ml (Irs2<sup>−/−</sup> + Adeno-IRS-2). 
g, suppression of PEPCK and glucose-6-phosphatase (G6Pase) mRNA in the liver after the clamp studies. β-Actin expression is shown as an internal control. Each bar represents means ± S.E. of four independent experiments. Values are means ± S.E. #, p < 0.05 versus Irs2<sup>+/+</sup>. *, p < 0.05 versus Irs2<sup>−/−</sup> + Adeno-LacZ. N.S., difference not significant.
No exogenous IRS-2 expression in other tissues of the infected animals, such as skeletal muscle, white adipose tissue, or islets, was detected by immunoblotting with an anti-FLAG antibody (data not shown).

After insulin injection into the inferior vena cava the exogenously introduced IRS-2 was tyrosine-phosphorylated in the liver as much as endogenous IRS-2 in Irs2$^{+/+}$ mice (Fig. 1c). Interestingly insulin-induced tyrosine phosphorylation of IRS-2 in other tissues, such as skeletal muscle, white adipose tissue, or islets, was not detected by immunoblotting with an anti-FLAG antibody.
IRS-1 was enhanced in the liver of Irs2<sup>−/−</sup> mice, as IRS-2 phosphorylation is increased in the liver of Irs1<sup>−/−</sup> mice as reported previously (20, 21), and the enhancement was antagonized by restoration of IRS-2 (Fig. 1c). IRS-2-associated PI3-kinase activity and phosphorylation of Akt induced by insulin injection were restored to almost the same level as in Irs2<sup>−/−</sup> mouse liver (Fig. 1, d and e). These results suggested that the exogenously introduced IRS-2 was functioning in the insulin signaling pathway. We used the ITT first to investigate whether insulin resistance was ameliorated in Adeno-IRS-2-infected Irs2<sup>−/−</sup> mice. The glucose-lowering effect of insulin injected into the Adeno-IRS-2-infected Irs2<sup>−/−</sup> mice had significantly improved, whereas the Adeno-LacZ-infected Irs2<sup>−/−</sup> mice remained insulin-resistant (Fig. 2a). We noted that the blood glucose levels of the Irs2<sup>−/−</sup> mice infected with Adeno-IRS-2 rose at 100 min, whereas the Irs2<sup>+/−</sup> mice remained hypoglycemic. The GTT showed that the hyperglycemia was ameliorated in Adeno-IRS-2-infected Irs2<sup>−/−</sup> mice.
2-infected Irs2−/− mice but not in the Adeno-LacZ-infected Irs2−/− mice (Fig. 2b). These results suggested that insulin resistance in the liver was required for diabetes to develop in Irs2−/− mice. However, the fasting blood glucose values of the Adeno-IRS-2-infected Irs2−/− mice remained significantly higher, and the GTT also revealed that Adeno-IRS-2-infected Irs2−/− mice remained hyperinsulinemic, suggesting that the insulin resistance in Irs2−/− mice improved only partially as a result of restoration of IRS-2 in the liver (Fig. 2c). In addition, hyperinsulinemic-euglycemic clamp studies revealed that restoration of IRS-2 in the liver reduced EGP in Irs2−/− mice to the same level as that in wild-type mice (Fig. 2d). Corresponding to the reduction of EGP in Adeno-IRS-2-infected Irs2−/− mice during clamp studies, suppression of PEPCK and glucose-6-phosphatase in the liver of the mice improved, whereas it was impaired in the mice treated with control adenovirus (Fig. 2g). The low GIRs in the Irs2−/− mice, however, were only partially ameliorated (Fig. 2d). The values of Rd in the Irs2−/− mice were significantly lower than in wild-type mice, and they were unaffected by IRS-2 restoration in the liver (Fig. 2f). These findings prompted us to attempt to identify other factors involved in the insulin resistance of Irs2−/− mice.

Effect of Obesity: Intracerebroventricular Administration of Leptin and Caloric Restriction of Irs2−/− Mice—We previously reported that Irs2−/− mice exhibit leptin resistance and hyperleptinemia in addition to increased adiposity (29). Since the insulin signaling pathway in the hypothalamus, including PI 3-kinase, is now considered to be important to the mechanisms of leptin signal transduction (42–45), we attempted to demonstrate an association between IRS proteins and PI 3-kinase p85 regulatory subunit in the hypothalamus. Western blot analysis revealed an association between IRS-2 and p85 in the wild-type hypothalamus and compensatory enhancement of the IRS-1 association with p85 in Irs2−/− hypothalamus (Fig. 3a). The PI 3-kinase activation by insulin was blunted in Irs2−/− hypothalamus (Fig. 3b). Consistent with the leptin resistance in Irs2−/− mice, both STAT3 phosphorylation and PI 3-kinase activation were impaired in the hypothalamus after intraperitoneal leptin injection (Fig. 3, c and d). Interestingly intracerebroventricular injection of leptin (1.0 μg) induced STAT3 phosphorylation in the Irs2−/− hypothalamus as well as in the wild type (Fig. 3e), but it did not activate PI 3-kinase in the Irs2−/− hypothalamus (Fig. 3f). These results suggested an important role of IRS-2 for PI 3-kinase activation, not for STAT3 phosphorylation, in the hypothalamus.

Next we investigated whether the obesity associated with leptin resistance contributes to insulin resistance. Intracerebroventricular leptin infusion at a rate of 300 ng/h for 3 days yielded Irs2−/− mice whose body weight and adiposity were almost the same as those of control Irs2−/− mice and overcame their leptin resistance (Fig. 4, a and b). The results of ITT revealed that the intracerebroventricular leptin infusions significantly ameliorated the insulin resistance of the Irs2−/− mice (Fig. 4c). This insulin-sensitizing effect of intracerebroventricular leptin infusion was observed in both wild-type and Irs2−/− mice, and the GTT showed partial improvement of hyperglycemia and hyperinsulinemia in the leptin-treated Irs2−/− mice, whereas there was no significant difference between leptin- and saline-treated wild-type mice (Fig. 4, d and e). The GIR in the leptin-treated Irs2−/− mice during the clamp studies partially improved (Fig. 5a) with increased Rd, an index of glucose utilization in the whole body (Fig. 5c), but EGP
did not decrease despite amelioration of the obesity (Fig. 5b). Interestingly, while EGP was unchanged, suppression of PEPCK and glucose-6-phosphatase mRNA was enhanced by intracerebroventricular leptin infusion at the end of the hyperinsulinemic clamp studies even in the liver of Irs2−/− mice (Fig. 5d). Four weeks of caloric restriction also yielded lean Irs2−/− mice whose adiposity was comparable to that of wild-type mice, and the results of the clamp studies were quite similar to those in the leptin-treated Irs2−/− mice (Fig. 6, a–c). These findings indicated that obesity-induced insulin resistance as well as insulin signaling defects in the liver contributes to the insulin resistance in Irs2−/− mice.

Combination Therapy Consisting of Adenovirus-mediated Gene Transfer and Reducing Adiposity—Combination therapy was performed to determine whether the insulin resistance in Irs2−/− mice was explained by a combination of insulin signaling defects in the liver and obesity-induced insulin resistance, and the adiposity of the Irs2−/− mice after Adeno-IRS-2 gene transfer and intracerebroventricular leptin combination therapy was found to be comparable to that of the Adeno-LacZ/saline group of Irs2−/+ mice and the Adeno-LacZ/leptin group of Irs2−/− mice (Fig. 7a). GTT showed that combination therapy completely improved the hyperglycemia of Irs2−/− mice as expected (Fig. 7b), but importantly it also almost completely abolished the hyperinsulinemia of the Irs2−/− mice (Fig. 7c). The ITT results corroborated the practically complete abolition of the insulin resistance in Irs2−/− mice after combination therapy (Fig. 7d), consistent with the results of hyperinsulinemic-euglycemic clamp studies showing conversion to completely normal GIR, EGP, and Rd (Fig. 7, e–g). Clamp studies also revealed the complete amelioration of GIR, EGP, and Rd in the Irs2−/− mice treated by Adeno-IRS-2 gene transfer plus caloric restriction (Fig. 8, a–c). These findings indicated that a combination of increased EGP due to insulin signaling defects in the liver and reduced Rd due to obesity accounts for the systemic insulin resistance in Irs2−/− mice.

DISCUSSION

The development of type 2 diabetes is thought to be linked to insulin resistance coupled with inadequate insulin secretion by pancreatic β-cells (46, 47). We previously demonstrated that Irs2−/− mice develop diabetes due to insulin signaling defects in the liver and failure of β-cells to undergo hyperplasia (24, 26). To determine the role of insulin resistance in the liver in the development of diabetes, we investigated the effect of IRS-2 restoration in the liver by adenovirus-mediated gene transfer. Although the development of diabetes was prevented, the hyperinsulinemia was not completely ameliorated, suggesting partial improvement of the insulin resistance in Irs2−/− mice. In fact, hyperinsulinemic-euglycemic clamp studies showed increased EGP and diminished Rd in Irs2−/− mice with the clamp in place, and restoration of IRS-2 into the liver normalized only the increased EGP. These findings prompted us to consider additional causes of the insulin resistance in Adeno-IRS-2-infected Irs2−/− mice.

Next we demonstrated the involvement of obesity associated with lepin resistance in the development of insulin resistance in Irs2−/− mice. We and others have previously reported that Irs2−/− mice exhibit lepin resistance associated with increased adiposity (28, 29) that leads to increased induction of sterol-regulatory element-binding protein-1 gene and liver steatosis (29). Obesity can be caused by defects in insulin signaling in the hypothalamus based on the following observations. (i) Both neuron-specific disruption of the insulin receptor gene (known as NIKRO mice) and inhibition of hypothalamic insulin receptor gene expression with antisense oligonucleotides lead to hyperleptinemia and systemic insulin resistance (42, 44), and

![Image](http://www.jbc.org/)

**FIG. 6.** Effect of caloric restriction in Irs2−/− mice on glucose kinetics during hyperinsulinemic-euglycemic clamp studies. Food restriction (3.0 g of chow/day) for 4 weeks yielded lean Irs2−/− mice whose adiposity was almost comparable to that in wild-type mice, and hyperinsulinemic-euglycemic clamp studies were performed on those mice as described under "Experimental Procedures." a, GIR in the Irs2−/− food-restricted mice was significantly higher than that in the control Irs2−/− mice. b, EGP was not affected by food restriction. c, Rd was significantly increased by food restriction. These findings were similar to the results of intracerebroventricular lepin infusion experiments. Clamp studies were performed on the groups of mice indicated: Irs2−/− (n = 6), Irs2−/− + food restriction (n = 5), and Irs2−/− + food restriction (n = 4). Values are means ± S.E. *, p < 0.05 versus Irs2−/−. N.S., difference not significant.
Insulin Resistance in Irs2−/− Mice

Peripheral leptin administration was hardly sufficient to overcome the marked peripheral leptin resistance in Irs2−/− mice (29), and continuous intracerebroventricular infusion yielded Irs2−/− mice whose adiposity was almost comparable to that of Irs2−/− mice probably by stimu-

![Graphs and images showing weight, glucose tolerance, insulin production, and glucose disappearance in Irs2−/− mice with different treatments.](image-url)
In vivo leptin administration in wild-type or Irs2 knockout mice under hyperinsulinemic-euglycemic clamp conditions revealed that both insulin signaling defects in the liver and obesity are necessary and sufficient for the development of insulin resistance in Irs2 knockout mice. The complete abolition of insulin resistance after the combination therapy also suggests that the lack of IRS-2 in skeletal muscle and adipose tissue had a minimal impact on glucose homeostasis, consistent with our previous observation that the insulin-stimulated PI 3-kinase activity in the skeletal muscle of young Irs2−/− mice is comparable to that in wild-type mice (26).

Moreover we have reported that IRS-1 is essential for insulin action, including PI 3-kinase activation, glucose uptake, and antilipolytic activity in primary adipocytes, and that IRS-3, not IRS-2, is the major tyrosine-phosphorylated protein in Irs1−/− mouse adipocytes (48, 49). Consistent with this, the phenotypes of lipoatrophic Irs1−/−Irs3−/− mice and plump Irs2−/−Irs3−/− mice are strikingly different, suggesting that IRS-2 plays a minor role in lipid storage in adipose tissue (50, 51). Increased lipolysis and decreased peripheral glucose utilization in Irs2−/− mice under hyperinsulinemic-euglycemic clamp conditions have been reported by others (52), suggesting the presence of “insulin resistance” in adipose tissue and skeletal muscle. The results of our previous studies suggest that adipocyte hypertrophy, not IRS-2 deficiency in adipose tissue, may cause the increased lipolysis in Irs2−/− mice. Similarly the decreased glucose utilization in Irs2−/− mice, which was also confirmed in our clamp studies, may result from the obesity associated with leptin resistance. In fact, intracerebroventricular leptin infusion directly into the hypothalamus has been reported to potentiate the effects of insulin on glucose uptake in muscle and adipose tissue (53–55). When Irs2−/− mice were food-restricted to determine the extent to which reduction of body weight contributed to the improvement in insulin resistance, partial improvement of GIR and Rd was observed, but EGP was unaffected, and the results were similar to those in leptin-treated Irs2−/− mice. Thus, reduced adiposity can explain most of the improvement in insulin resistance in leptin-treated Irs2−/− mice.

We did not observe any alteration of EGP by intracerebroventricular leptin administration in wild-type or Irs2−/− mice during the clamp studies, and the effect of leptin on EGP has been a matter of controversy. Burcelin et al. (56) reported that EGP was augmented by intravenous leptin infusion in ob/ob mice, while Barzilai et al. (33) reported marked suppression of EGP by subcutaneous leptin administration in rats during hyperinsulinemic-euglycemic clamp studies. Our results for PEPCK mRNA expression in the liver were consistent with the report by Burcelin et al. (56) showing a reduction of hepatic PEPCK activity after leptin infusion. Perfusion of isolated rat liver with leptin plus insulin has also been reported to additively reduce PEPCK activity (57). The effect of leptin on PEPCK regulation may provide an explanation for the partial improvement in PEPCK suppression in the liver of Irs2−/− mice treated with adenovirus-mediated gene transfer of IRS-2 (Fig. 2g).

Assessment of the involvement of β-cell components in the development of diabetes in Irs2−/− mice has shown that although their β-cells fail to undergo hyperplasia, glucose-induced insulin secretion and insulin content on a per cell basis
are well preserved in our Ir2−/− mice (26). In fact, the Ir2−/− mice remain hyperinsulinemic even after they develop diabetes at 30 weeks of age, suggesting that β-cell function is well preserved despite lacking the ability to undergo hyperplasia (26, 58). The finding that restoration of IRS-2 in the liver was prevented from the postprandial hyperglycemia in Ir2−/− mice demonstrated the importance of insulin resistance in the development of diabetes when insulin-secretory function is relatively well preserved. The complete abolition of hyperglycaemia after the combination therapy also suggests that lack of IRS-2 in β-cells may not be sufficient for diabetes to develop in the absence of insulin-resistant conditions, including obesity and insulin signaling defects in the liver.

In conclusion, the insulin signaling defects in the liver, but not in skeletal muscle or adipose tissue, play a major role in the development of diabetes in Ir2−/− mice. The combination of insulin signaling defects in the liver and obesity associated with leptin resistance is responsible for the development of insulin resistance in Ir2−/− mice.

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