Increased Expression of the Sterol Regulatory Element-binding Protein-1 Gene in Insulin Receptor Substrate-2−/− Mouse Liver*

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Insulin receptor substrate (IRS)-2−/− mice develop diabetes because of insulin resistance in the liver and failure to undergo β-cell hyperplasia. Here we show by DNA chip microarray analysis that expression of the sterol regulatory element-binding protein (SREBP)-1 gene, a downstream target of insulin, was paradoxically increased in 16-week-old IRS-2−/− mouse liver, where insulin-mediated intracellular signaling events were substantially attenuated. The expression of SREBP-1 downstream genes, such as the spot 14, ATP citrate-lyase, and fatty acid synthase genes, was also increased. Increased liver triglyceride content in IRS-2−/− mice assures the physiological importance of SREBP-1 gene induction. IRS-2−/− mice showed leptin resistance; low dose leptin administration, enough to reduce food intake and body weight in wild-type mice, failed to do so in IRS-2−/− mice. Interestingly, high dose leptin administration reduced SREBP-1 expression in IRS-2−/− mouse liver. Thus, IRS-2 gene disruption results in leptin resistance, causing an SREBP-1 gene induction, obesity, fatty liver, and diabetes.

The pathogenesis of type 2 diabetes involves complex interactions among multiple physiological defects. Transgenic and knockout technology to create animal models of type 2 diabetes have had a major impact on assessment of the function of newly identified molecules implicated in the regulation of glucose homoeostasis in vivo (1). The insulin receptor substrate (IRS)1 proteins play a key role in signal transduction from the insulin receptor (reviewed in Refs. 2–4). These molecules are major intracellular phosphorylation targets of activated insulin receptor tyrosine kinase. The mammalian IRS protein family contains at least four members, ubiquitous IRS-1 (5) and IRS-2 (6), adipose tissue-predominant IRS-3 (7), and IRS-4, which are expressed in thymus, brain, and kidney (8). The physiological roles of each protein have been evaluated by gene targeting strategies. IRS-1−/− mice are growth-retarded and insulin-resistant (9, 10) but do not develop diabetes, because an alternate substrate IRS-2 (10) or pp190 (11) compensates for the lack of IRS-1 in liver (11) and, at least in part, in skeletal muscle (12). In addition, hyperinsulinemia associated with β-cell hyperplasia effectively counterbalanced the insulin-resistant states (13). IRS-2−/− mice, however, developed diabetes because of inadequate β-cell proliferation combined with liver-insulin resistance (14–16). Mice lacking IRS-3 or IRS-4 had milder phenotypes (17, 18).

Liver is a major target organ for insulin action, contributing to energy storage in the fed state by regulating catabolic and anabolic pathways. Liver-specific insulin receptor knockout mice exhibit dramatic insulin resistance (19). Insulin decreases gluconeogenic enzyme mRNAs (20) and increases lipogenic enzyme mRNAs. A transcription factor of sterol regulatory element-binding protein 1c (SREBP-1c) (21–23) or adipocyte differentiation and determination factor (24) plays a central role in insulin-mediated lipogenic enzyme induction. Although SREBP-1c expression is up-regulated by insulin and glucose in vitro (22, 23, 25, 26), the in vivo significance of insulin or glucose in SREBP-1c gene up-regulation is a matter of controversy. How SREBP-1c expression is regulated in vitro and in vivo and how SREBP-1c expression is involved in leptin action and insulin resistance have been the focus of intensive research.

Because the organ responsible for insulin resistance in IRS-2−/− mice is the liver (16), we focused our research on the liver and performed a global gene expression study using oligonucleotide microarrays. We discovered paradoxical SREBP-1c gene induction in insulin-resistant IRS-2−/− mice liver, even in the early euglycemic phase, indicating that neither insulin action nor hyperglycemia was responsible for SREBP-1 gene induction. We provided evidence to show that leptin resistance is causally related to SREBP-1 gene induction. The molecular mechanism, as well as biological relevance, of leptin resistance in the development of type 2 diabetes is discussed.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were from Sigma. [α-32P]dCTP was obtained from PerkinElmer Life Sciences.

Animal Experiments—IRS-1−/−, IRS-2−/−, and wild-type mice were prepared by heterozygote intercrosses (9, 16). Mice were housed on a 12-h light-dark cycle and were given ad libitum access to regular chow MF consisting of 25% (w/w) protein, 53% carbohydrates, 6% fat, and 8% water (Oriental Yeast Co., Ltd., Osaka, Japan). All experiments in this study were performed on male mice, except when stated specifically that female mice were used.

The abbreviations used are: IRS, insulin receptor substrate; SREBP, sterol regulatory element-binding protein.
Increased Expression of SREBP-1 Gene in IRS-2−/− Liver

FIG. 1. Increased adiposity in IRS−/− mice. A, weight of epididymal fat as a proportion of total body weight was measured in 6- and 16-week-old male wild-type (WT), IRS−/−, and IRS−/−/H11002 mice. B, left, growth curve of wild-type and IRS−/− mice. Body weight (0900–1100 h) as a proportion of total body weight was measured from 6- to 16-week-old. Right, body weight (0900–1100 h) was measured in 16-week-old male wild-type, IRS−/−, and IRS−/−/H11002 mice. C, serum leptin level was measured in 6-week-old male wild-type, IRS−/−, and IRS−/−/H11002 mice. Values are expressed as the mean ± S.E. (n = 5–7), **p < 0.01 as compared with the wild-type. N.S., difference not significant.

Body Weight, Weight of Adipose Tissue, and Serum Leptin Levels—Body weight was assessed between 0900 and 1100 h. The statistical significance of the differences in body mass between groups was determined by Student’s t test (two-tailed). The weight of the epididymal fat was measured as described previously (27). Leptin was assayed with the enzyme-linked immunosorbent assay kit (R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Liver Triglyceride and Cholesterol Content—The triglyceride and cholesterol content of the liver was measured as described previously (28).

Oligonucleotide Microarray Analysis—Total liver RNA was isolated from 16-week-old wild-type and IRS−/− mice after 15 h of fasting by TRIZOL reagent (Life Technologies, Inc.). RNA was analyzed as follows: cDNA and cRNA probe were prepared as described previously (29). Hybridization, washing, and staining of Affymetrix Genechip murine 11K probe arrays were carried out in an Affymetrix hybridization oven and fluids station according to the manufacturer’s protocol. The arrays were scanned with a Hewlett-Packard confocal laser scanner and visualized using Affymetrix Genechip 3.1 software. The fold change in expression in mutant mice versus wild-type mice was calculated using Affymetrix GeneChip 3.1 software.

Northern Blot Hybridization with cDNA Probes—Northern blot hybridization was carried out as described previously (26, 30–32). The cDNA probes for mouse SREBP-1, human SREBP-2, mouse spot 14, mouse fatty acid synthase, rat ATP citrate-lyase, mouse malic enzyme, and glucokinase were prepared by cloning reverse transcriptase-polymerase chain reaction products from mouse liver RNA into TA cloning vector pCR2.1 (Invitrogen, San Diego, CA). The corresponding bands were quantified by exposure of BAS 2000 to the filters to with BAStation software (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Leptin Administration—Wild-type and IRS−/− mice were injected intraperitoneally once a day at 2100 h with control saline or leptin (10 mg/kg/injection) (PeproTech EC Ltd., London, United Kingdom), and daily body mass and food intake were monitored at 0900 and 2100 h (27). The statistical significance of differences in body mass and food intake between groups was determined using Student’s t test (two-tailed).

Regulation of SREBP-1 Gene by Leptin—Leptin (50 mg/kg/injection) or control saline was administered to 6-week-old IRS−/− mice at 0900, 1700, and 0100 h. Body weight and food intake were monitored at 0900 and 2100 h. For the experiments of regulation of SREBP-1 gene by leptin, leptin (50 mg/kg/injection) or control saline was administered to IRS−/− mice at 0900 h, when fasting began, at 1700, and at 0100 h. Animals were sacrificed at 0900 h the next day by cervical dislocation. The liver was excised, frozen on liquid nitrogen, and maintained at −80 °C until it was processed further.

RESULTS

IRS−/− Mice Showed Increased Adiposity—During the course of experiment, we found that the amount of body fat per body weight was increased in IRS−/− mice (Fig. 1A). The whole growth curve of wild-type and IRS−/− mice from 6 to 16 weeks old revealed that IRS−/− mice had gained more body weight after 8 weeks of age before the development of diabetes (Fig. 1B). The proportion of the weight of epididymal fat to total body weight was significantly increased in IRS−/− mice at the age of 6 weeks before IRS−/− mice had more body weight gain than wild-type mice (Fig. 1A). Moreover, the serum leptin level was increased in IRS−/− mice (Fig. 1C), suggesting that these mice were leptin-resistant. By contrast, epididymal fat weight as a proportion of body weight and the serum leptin level of IRS−/− mice did not differ significantly from the values in wild-type mice (Fig. 1, A and C).

Expression of SREBP-1 and Downstream Target Genes Were Enhanced in IRS−/− Mice According to the Results of DNA Chip Analysis—To understand the molecular mechanism of the development of diabetes in IRS−/− mice, we performed an integrated gene expression study using liver samples. Total RNA was isolated from 16-week-old wild-type, IRS−/−, and IRS−/− mice and analyzed by murine 11K oligonucleotide microarray (Affymetrix). Among the −11K genes and expressed sequence tags analyzed, there was detected an increased expression of the two copies of the SREBP-1 gene in the liver from 16-week-old IRS−/− mice in the fasted states (Fig. 2A). This result was surprising, because insulin reportedly up-regulates SREBP-1 gene expression whereas insulin-mediated intracellular signaling events, including phosphatidylinositol 3-kinase activation, are decreased substantially in IRS−/− mice (33). We also found up-regulated expression of several SREBP-1c target genes, including the spot 14, ATP citrate-lyase, fatty acid synthase, and malic enzyme gene, all of which are involved in fatty acid synthesis (Fig. 2B). All these findings were confirmed by Northern blot analysis. An enhanced expression of SREBP-1 with an unaltered expression of SREBP-2 in the fasted state (Fig. 2C), and the enhanced expression of its target genes, including the spot 14, ATP citrate-lyase, fatty acid synthase, and malic enzyme gene, in the fasted state was observed in IRS−/− mice (Fig. 2D). In addition, RNase protection assay revealed that SREBP-1c expression was up-regulated specifically in IRS−/− mice (data not shown). Consistent with these findings, the triglyceride content of IRS−/−/H11002 mouse liver was elevated significantly (Fig. 2E), confirming the physiological significance of an enhanced SREBP-1c expression in IRS−/− mice. By contrast, the cholesterol content of IRS−/−/H11002 mouse liver was slightly reduced, consistent with unaltered SREBP-1a and SREBP-2 expression (21).

SREBP-1 Expression Was Increased in Euglycemic 6-Week-old IRS−/− Mice—Because SREBP-1 expression is reportedly induced by glucose itself (25, 26), we assessed the involvement of hyperglycemia in SREBP-1 gene induction in IRS−/−
mice. First, we examined the expression of SREBP-1 in the liver of 6-week-old IRS-2−/− mice having normal glucose tolerance and found an elevated expression of SREBP-1 gene (Fig. 3A). Moreover, a comparable increment in SREBP-1 expression was detected in female IRS-2−/− mice, which have better glucose tolerance than male mice (Fig. 3B). Thus, hyperglycemia may not be the cause of the SREBP-1 gene induction.

**Leptin Lowered SREBP-1 Expression in IRS-2−/− Mouse Liver**—Leptin resistance is known to be associated with an increased expression of SREBP-1 gene (34). To assess the leptin action in IRS-2−/− mice, we administered the low dose leptin to euglycemic 6-week-old IRS-2−/− mice with increased adiposity and hyperleptinemia (see Fig. 1). The daily intraperitoneal administration of leptin (10 mg/kg body weight per day) was sufficient to reduce food intake and body weight in the wild-type but failed to do so in IRS-2−/− mice (Fig. 4A). Thus, IRS-2−/− mice were leptin-resistant even in the euglycemic stage. Next we examined the effects of higher dose leptin administration. We found that administration of a 50 mg/kg body weight dose to 6-week-old IRS-2−/− mice three days a time resulted in a significant decrease in food intake and body weight gain (Fig. 4B). Moreover, administration of the same amount of leptin into the fasted IRS-2−/− mice resulted in the amelioration of SREBP-1 overexpression (Fig. 4C). Thus, a high dose of leptin administration into leptin-resistant IRS-2−/− mice lowered the increased SREBP-1 expression in the liver.

**DISCUSSION**

We have demonstrated an induction of SREBP-1 gene expression in the liver of diabetic insulin-resistant IRS-2−/− mice. This paradoxical finding prompted us to explore factors other than insulin that induced SREBP-1 gene expression in IRS-2−/− mice. We showed that leptin resistance contributes, at least in part, to the up-regulation of the SREBP-1 gene by demonstrating that high dose leptin administration not only reduced food intake and body weight but also ameliorated SREBP-1 overexpression in IRS-2−/− mice. Analogous situations were reported in ob/ob mice, lipostropic mice (35, 36), and leptin-unresponsive obese Zucker diabetic fatty fa/fa rats (34), suggesting that leptin resistance is linked causally to SREBP-1 gene induction in liver. We observed further an up-regulated expression of SREBP-1 gene in goldthioglyco-
treated leptin-resistant mice, indicating that leptin resistance in hypothalamus is involved directly in SREBP-1 gene induction in liver.

How does leptin resistance contribute to increased SREBP-1c gene expression? As leptin reportedly increases glucose uptake and glucose turnover in peripheral tissues, thus decreasing glucose influx into the liver (37), leptin resistance may provoke an increased glucose influx into liver. Because glucose influx into hepatocytes is one of the most important up-regulators of SREBP-1 gene (25, 26), an enhanced liver glucose uptake in leptin-resistant IRS-2−/− mice causes up-regulation of SREBP-1 gene expression, thus causing fatty liver. Our hypothesis that leptin resistance is the cause of SREBP-1 gene induction may explain why fatty liver is commonly observed among insulin-resistant over-nourished obese type 2 diabetes patients with relatively preserved insulin secretion function.

SREBP-1 gene up-regulation may possibly contribute to ameliorating insulin resistance through bypassing the insulin requirement for energy storage by inducing lipogenic and glucokinase genes (22, 23, 31). Increased triglyceride content in liver. Our hypothesis that leptin resistance is the cause of fatty liver development may be a kind of adaptive response in leptin-resistant over-nourished state, with liver taking up excess energy from blood flow instead of peripheral tissues.

How does leptin resistance in IRS-2−/− mice develop? Our in vitro study showed that lack of IRS-2 had little effect on insulin-stimulated glucose uptake in the isolated adipocytes of IRS-2−/− mice.3 The almost normal insulin function in IRS-2−/− mice, the 6-week-old IRS-2−/− with leptin resistance in the hypothalamus (38, 39), the obese mice showed that lack of IRS-2 had little effect on insulin-stimulated glucose uptake in the isolated adipocytes of IRS-2−/− mice. Thus, lack of IRS-2 in mice adipocytes may cause an increased glucose and lipid uptake by these cells in the presence of hyperinsulinemia, which was provoked by liver-insulin resistance in IRS-2−/− mice, and thus result in the enlargement of adipocytes. In accordance with the observation that adipocyte hypertrophy is associated with leptin resistance in the hypothalamus (38, 39), the obese IRS-2−/− mice showed leptin resistance before developing diabetes (see Fig. 1 and Fig. 4). Alternatively, lack of IRS-2 may influence directly leptin signaling pathways in the hypothalamus, because leptin-stimulated phosphorylation of signal transducers and activators of transcription 3 in the hypothalamus is attenuated in IRS-2−/− mice (40), and because neuron-specific insulin receptor knockout mice showed leptin resistance (41).

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