Heterozygous knockout of the IRS-1 gene in mice enhances obesity-linked insulin resistance: a possible model for the development of type 2 diabetes

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Abstract

Insulin receptor substrate 1 (IRS-1) gene polymorphisms have been identified in type 2 diabetic patients; however, it is unclear how such polymorphisms contribute to the development of diabetes. Here we introduced obesity in heterozygous IRS-1 knockout (IRS-1^{+/-}) mice by gold-thioglucose (GTG) injection and studied the impact of reduced IRS-1 expression on obesity-linked insulin resistance. GTG injection resulted in ~30% weight gain in IRS-1^{+/-} and wild type (WT) mice, compared with saline-injected controls. There was no difference in insulin sensitivity between lean IRS-1^{+/-} and lean WT. Elevated fasting insulin levels but no change in fasting glucose were noted in obese IRS-1^{+/-} and WT

compared with the respective lean controls. Importantly, fasting insulin in obese IRS-1^{+/-} was 1.5-fold higher ($P < 0.05$) than in obese WT, and an insulin tolerance test showed a profound insulin resistance in obese IRS-1^{+/-} compared with obese WT. The islets of obese IRS-1^{+/-} were 1.4-fold larger than those of obese WT. The expression of insulin receptor and IRS-1 and IRS-2 was decreased in obese IRS-1^{+/-}, which could in part explain the profound insulin resistance in these mice. Our results suggest that IRS-1 is the suspected gene for type 2 diabetes and its polymorphisms could worsen insulin resistance in the presence of other additional factors, such as obesity.

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Introduction

Type 2 diabetes mellitus is characterized by insulin resistance in peripheral tissues and failure of insulin secretion from pancreatic β -cells (O'Rahilly *et al.* 1995). Part of these abnormalities seem to be genetically defined. Several candidate genes for insulin resistance have been proposed, which include the genes encoding the molecules of the insulin signaling pathway (O'Rahilly *et al.* 1995), glycolysis (Ristow *et al.* 1999) and glyconeogenesis (Ludwig *et al.* 1996). In addition, some of the genes involved in obesity, including peroxidase proliferating activator receptor- γ (Ristow *et al.* 1998) and uncoupling protein-1 (Heilbronn *et al.* 2000), may also contribute to insulin sensitivity.

Insulin receptor substrate-1 (IRS-1) is one of the major substrates of insulin receptor tyrosine kinase (Araki *et al.* 1993). After insulin stimulation, IRS-1 is immediately tyrosine phosphorylated by activated receptor tyrosine kinase, and some phosphorylated tyrosine residues of IRS-1 serve as docking sites for several src homology 2

(SH2)-containing proteins, including phosphatidylinositol 3-kinase (PI 3-kinase), SH2-containing protein tyrosine phosphatase-2 and growth factor-bound protein-2 (Myers *et al.* 1994, Argetsinger *et al.* 1995). Binding of these SH2 proteins to IRS-1 results in the activation of two major signaling pathways, the mitogen activated protein kinase pathway and the PI 3-kinase pathway, both of which mediate various biological effects of insulin.

The role of IRS-1 in insulin signaling *in vivo* has been confirmed by generating IRS-1 knockout mice by targeted disruption of the IRS-1 gene (Araki *et al.* 1994, Tamemoto *et al.* 1994). The IRS-1 homozygous knockout (IRS-1^{-/-}) mice show impaired intrauterine growth, resistance to insulin and insulin-like growth factor-I (IGF-I), and glucose intolerance upon i.p. glucose loading. On the other hand, in spite of the low expression of IRS-1 protein, IRS-1 heterozygous knockout (IRS-1^{+/-}) mice show no significant abnormalities in insulin sensitivity and glucose homeostasis.

Previous studies from our laboratories and those of other investigators described several polymorphisms of the

IRS-1 gene in patients with type 2 diabetes (Almind *et al.* 1993, Ura *et al.* 1996). Glycine to arginine substitution at codon 971 of IRS-1 gene (G971R) is the most frequent polymorphism in any population examined. The prevalence of G971R polymorphism is significantly higher in patients with type 2 diabetes than in normal subjects, and subjects who carry IRS-1 polymorphism tend to be insulin-resistant compared with those with no such mutation (Ura *et al.* 1996). Functional analysis of mutant IRS-1 confirmed that G971R mutant showed impaired insulin-induced PI 3-kinase activation and mitogenesis in the hematopoietic 32 D cell line (Almind *et al.* 1996, Yoshimura *et al.* 1997). These *in vitro* data strongly suggest that IRS-1 abnormalities could contribute to the development of insulin resistance in human. However, it is still not clear whether or how IRS-1 abnormalities could lead to insulin resistance in humans since most IRS-1 polymorphisms exist in a heterozygous manner in type 2 diabetes, whereas the heterozygous IRS-1 knockout mice show neither impairment of glucose homeostasis nor insulin sensitivity.

It is well known that environmental factors such as obesity and high fat diet are associated with increased prevalence of type 2 diabetes. In fact, the number of type 2 diabetes patients in Asian countries including Japan has markedly increased during the last few decades due to changes in life style (King & Rewers 1993). Furthermore, Clausen *et al.* (1995) reported that obese subjects who carry IRS-1 gene polymorphism are more resistant to insulin than those who have no mutation in the IRS-1 gene, whereas non-obese carriers of the IRS-1 gene polymorphism have normal insulin sensitivity. These findings suggest that the impact of IRS-1 gene polymorphism becomes overt in the presence of obesity. In order to examine the interaction between obesity and IRS-1 gene polymorphism, we induced obesity in IRS-1^{+/-} mice by injection of gold-thioglucose (GTG). Using this model, we studied the impact of reduced IRS-1 expression on obesity-linked insulin resistance *in vivo*, and investigated the mechanisms that underlie insulin resistance in obese IRS-1^{+/-} mice.

Materials and Methods

Establishment of obese IRS-1^{+/-} mice

Animal care and experiments were carried out in accordance with institutional animal care regulations. IRS-1 heterozygous knockout mice (IRS-1^{+/-}) were generated by mating female IRS-1^{+/-} mice with male IRS-1^{+/-} mice (Araki *et al.* 1994). The genotypes of mice were examined by PCR analysis after extraction of genomic DNA from the tail. Only male mice were used in this study. Obesity was introduced by a single i.p. injection of 0.5 mg/g body weight of GTG (Sigma Chemical, St Louis, MO, USA) at 6 weeks of age. Mice were kept

on a 12 h light:12 h darkness cycle, with the light cycle between 0700 and 1900 h. The mice were allowed free access to food and water.

Assessment of plasma parameters, i.p. glucose tolerance test and i.p. insulin tolerance test

Blood samples were obtained from snipped tail ends for determination of insulin and glucose levels. Blood glucose concentration was measured by the enzymatic assay (Arkay, Kyoto, Japan). Insulin concentration was measured by ELISA (Morinaga, Yokohama, Japan). An i.p. glucose tolerance test was performed after overnight fasting by administering 2.0 g glucose/kg body weight via the peritoneal cavity in conscious mice at 40 weeks of age. The area under the curve (AUC) was calculated as the integrated blood glucose level during the tolerance test. An i.p. insulin tolerance test was performed at 35 and 45 weeks of age by administering 1.0 U insulin/kg body weight. For a more precise assessment of insulin sensitivity, we calculated the area over the curve (AOC), which represented the integrated reduced level of blood glucose during the insulin tolerance test.

Morphological analysis of the pancreas

The pancreas was obtained from mice at 50 weeks of age, fixed in paraffin, and cut into 10 sections (5 µm thick) separated by 200 µm. Sections were immunostained with anti-insulin, anti-glucagon or anti-somatostatin antibodies (Nichirei, Tokyo, Japan). Biotinylated goat anti-rabbit IgG coupled to peroxidase and alkaline phosphatase, and streptavidin-biotin complex were used as secondary antibodies. 3'3'-Diaminobenzidine or Fast Red Substrate Kit (Nichirei) was used as substrate respectively. Sections were then counterstained with hematoxylin-eosin (HE) and visualized using an IX50 microscope (Olympus, Tokyo, Japan). At least 20 islets in each section were randomly selected and analyzed. The area of each islet was measured quantitatively with an image analysis software (NIH, Bethesda, MD, USA).

Immunoprecipitation and Western blotting

Mice were anesthetized with 100 mg/kg sodium pentobarbital injected i.p. Following the loss of pedal and corneal reflexes, saline or 5.0 U regular insulin were injected into the portal vein. The liver, gastrocnemius, and quadriceps muscles were dissected out 1 and 3 min after insulin injection, and immediately frozen in liquid nitrogen. The frozen tissue was homogenized in ice-cold lysis buffer (1% Triton X-100, 50 mM Hepes, pH 7.4, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 5 mM sodium vanadate, aprotinin (10 µg/ml), leupeptin (5 µg/ml) and polymethylsulfonyl fluoride (34 µg/ml)) using a Polytron PTA 20 S generator

operated at maximum speed for 30 s. The insoluble material was removed by centrifugation at 125 000 g, in a 70 Ti rotor (Beckman Instruments, Inc., Fullerton, CA, USA) for 60 min, and equal amounts of the supernatant were subjected to immunoprecipitation for 120 min using the indicated antibodies. Subsequently, protein A-Sepharose was added and followed by incubation for another 60 min. The samples were processed for SDS-PAGE and Western blotting as described previously (Folli *et al.* 1993).

PI 3-kinase assay

Immune-complexed PI 3-kinase activity was measured by *in vitro* phosphorylation of phosphatidylinositides (PI) as described previously (Folli *et al.* 1993). Immune complexes were incubated in 45 µl reaction buffer containing 10 µM ATP, 30 µCi [γ -³²P]ATP and 10 µg PI at room temperature under constant shaking. After 10 min, the reaction was stopped by the addition of 250 µl 1 M HCl and 80 µl CHCl₃/methanol (2:1). The samples were centrifuged, and the lower organic phase was removed and applied to a silica gel TLC plate (Merck, Darmstadt, Germany) coated with 1% potassium oxalate. TLC plates were developed in CHCl₃/CH₃OH/H₂O/NH₄OH (60:47:11:3:2), dried, and visualized by autoradiography. The ³²P-labeled PI was quantified using NIH image analysis software.

Statistical analysis

All data are expressed as means $\pm$ s.d. Differences between groups were examined for statistical significance using the non-parametric Mann-Whitney U-test. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

Results

Development of obesity following GTG administration

Body weight of both *IRS-1*^{+/-} and wild type (WT) mice increased by approximately 30% at 9 weeks after GTG administration, compared with the relative saline-injected control groups (*IRS-1*^{+/-} group: 40.2 $\pm$ 2.6 g vs control: 33.0 $\pm$ 4.0 g, *P* < 0.001, and WT group: 40.2 $\pm$ 3.4 g, vs control: 31.0 $\pm$ 4.7 g, *P* < 0.001) (Fig. 1). There were no significant differences in body weight between obese WT and obese *IRS-1*^{+/-} mice, and between lean WT and lean *IRS-1*^{+/-} mice. The increase in body weight (~30%) of the GTG-injected groups was maintained until 45 weeks of age.

Fasting blood glucose and fasting serum insulin concentrations in WT and *IRS-1*^{+/-} mice

Fasting blood glucose and serum insulin concentrations were measured at 15 weeks of age and every 15 weeks

Figure 1 Growth curves for WT and insulin receptor substrate 1 (*IRS-1*)^{+/-} mice following administration of gold-thioglucose (GTG). GTG or saline was injected into the peritoneal cavity of mice at 6 weeks of age. Body weight of GTG-injected WT (■) and *IRS-1*^{+/-} (●) mice, and saline-injected WT (□) and *IRS-1*^{+/-} (○) mice was measured until 45 weeks of age. Data are means $\pm$ s.d. of ten mice in each group. **P* < 0.001 vs lean mice group.

thereafter. There was no significant difference in fasting blood glucose levels among four groups at 15 weeks of age (Fig. 2A). Blood glucose levels did not change significantly until 45 weeks of age in all groups (data not shown). There was no significant change in fasting serum insulin concentrations between lean WT and lean *IRS-1*^{+/-} mice at 15 weeks (Fig. 2B). In contrast, the mean fasting serum insulin concentrations in obese WT mice were about three times higher than those of lean WT controls (1.62 $\pm$ 0.42 vs 0.56 $\pm$ 0.30 ng/ml, *P* < 0.01). The mean insulin concentration of obese *IRS-1*^{+/-} mice (2.56 $\pm$ 0.78 ng/ml) was also significantly higher than that of lean *IRS-1*^{+/-} mice (0.65 $\pm$ 0.57 ng/ml, *P* < 0.01). Importantly, fasting insulin concentrations in obese *IRS-1*^{+/-} mice were significantly higher (*P* < 0.05) than in obese WT mice.

Insulin tolerance and glucose tolerance tests

To assess glucose intolerance in *IRS-1*^{+/-} mice, glucose (2.0 g/kg body weight) was injected i.p. at 40 weeks of age. Obese *IRS-1*^{+/-} and obese WT mice showed a significant elevation in blood glucose concentrations after glucose loading compared with the respective lean controls (*P* < 0.01) (Fig. 3). Furthermore, blood glucose levels in obese *IRS-1*^{+/-} mice at 60 min after glucose challenge were higher than in obese WT mice. However, the AUC of obese WT mice (47 408 $\pm$ 3492) was not significantly different from that of obese *IRS-1*^{+/-} mice (45 982 $\pm$ 2579).

Figure 2 Fasting blood glucose concentrations (A) and serum insulin concentrations (B) of mice at 15 weeks of age. After overnight fasting, blood samples were collected from the tail and blood glucose and serum insulin concentrations were determined as described in Materials and Methods. Data are means $\pm$ S.D. of ten mice in each group. * P <0.01 vs lean WT, # P <0.01 vs lean IRS-1^{+/-}, * P <0.05.

Figure 3 I.p. glucose tolerance test in lean WT (□), lean IRS-1^{+/-} (○), obese WT (■), and obese IRS-1^{+/-} (●) mice. After overnight fasting, glucose (2.0 g/kg body weight) was injected into the peritoneal cavity of 40-week-old mice. Blood samples were collected from the tail at the indicated time points and glucose concentrations were measured. Data are means $\pm$ S.D. of five mice in each group.

We also assessed insulin sensitivity at 35 and 45 weeks of age by an i.p. insulin tolerance test. At 35 weeks of age, both obese groups showed a significant reduction in glucose-lowering effect of insulin compared with their lean controls (data not shown). Glucose levels after insulin challenge tended to be higher in obese IRS-1^{+/-} mice

Figure 4 Insulin tolerance test in lean WT (□), lean IRS-1^{+/-} (○), obese WT (■), and obese IRS-1^{+/-} (●) mice. After overnight fasting, insulin (1.0 U/kg body weight) was injected into the peritoneal cavity of 45-week-old mice. Blood samples were collected from the tail at the indicated time and glucose concentrations were measured. Data are means $\pm$ S.D. of five mice in each group.

than in obese WT mice, albeit statistically insignificant (data not shown). An i.p. insulin tolerance test at 45 weeks of age showed that both obese WT and obese IRS-1^{+/-} mice exhibited a significant reduction in the glucose-lowering effect of insulin compared with their lean controls at all times after insulin challenge (Fig. 4). Importantly, the glucose-lowering effect of insulin in obese IRS-1^{+/-} mice was markedly diminished

compared with obese WT mice. For a more precise assessment of the insulin-lowering effect, we calculated the AOC. The AOC of lean WT mice (1692 ± 201) was not significantly different from that of lean *IRS-1*^{+/-} mice (1476 ± 201). However, the AOC of obese *IRS-1*^{+/-} mice (315 ± 319) was significantly lower than that of obese WT mice (692 ± 333).

Morphological analysis of the pancreas

Islets in the pancreas section of obese WT and obese *IRS-1*^{+/-} mice were significantly larger than those in their lean mice controls (Figs 5 and 6A). Interestingly, the mean islet size of obese *IRS-1*^{+/-} mice was 36% larger than that of obese WT mice ($P < 0.05$), whereas there was no difference in islet size between lean WT and lean *IRS-1*^{+/-} mice (Figs 5 and 6A). Next we analyzed sections immunostained for insulin, glucagon and somatostatin. There was no obvious degranulation of β -cells in anti-insulin antibody stained sections from all groups. Furthermore, the relative number of β -cells in the islets was higher in obese *IRS-1*^{+/-} mice than in obese WT mice (Figs 5 and 6B). In contrast, there was no significant difference in the number of non- β -cells between the two obese groups (Figs 5 and 6C).

Expression of insulin receptor and *IRS-1* and *IRS-2* in liver and skeletal muscles

To elucidate the molecular mechanisms of enhanced insulin resistance in obese *IRS-1*^{+/-} mice, we examined the expression levels of insulin receptor and *IRS-1* and *IRS-2* proteins in the liver and muscles. Quantitative analysis showed no differences in the expression of insulin receptor between lean WT and lean *IRS-1*^{+/-} mice, in both the liver and muscles (Fig. 7A and B). Furthermore, the expression of the insulin receptor in the liver and muscles of both obese groups was significantly lower than in their lean controls (by 55% in WT and by 83% in *IRS-1*^{+/-} in the liver, 44% in WT and 62% in *IRS-1*^{+/-} in muscles). There was also no difference in insulin receptor expression in the muscles between obese WT and obese *IRS-1*^{+/-} mice (Fig. 7B). Interestingly, insulin receptor expression in the liver of obese *IRS-1*^{+/-} mice was 39% of that of obese WT mice (Fig. 7A).

As expected, *IRS-1* protein content in lean *IRS-1*^{+/-} was approximately half of that in lean WT mice in the liver and muscles (59 and 44% respectively) (Fig. 7C and D). Compared with their lean controls, *IRS-1* protein content was significantly reduced by 83% in obese WT and by 74% in obese *IRS-1*^{+/-} in the liver (Fig. 7C), and reduced by 28% in obese WT and by 32% in obese *IRS-1*^{+/-} in muscles (Fig. 7D). Importantly, *IRS-1* content in the muscles of obese *IRS-1*^{+/-} was 45% of that in obese WT mice, but there was no difference in *IRS-1* levels in the liver between the two obese groups.

Interestingly, the expression of *IRS-2* protein in lean *IRS-1*^{+/-} mice was significantly increased in both the liver and muscles compared with lean WT mice (Fig. 7E and F). *IRS-2* expression in the liver was significantly reduced in obese WT and in obese *IRS-1*^{+/-} mice, compared with their lean controls (by 58 and by 66% respectively) (Fig. 7E). Similarly, *IRS-2* protein level in the muscles of obese groups tended to be lower in WT (by 23%) and in *IRS-1*^{+/-} (by 52%) than in their lean controls (Fig. 7F). There were no differences in *IRS-2* levels in both the liver and muscles between obese *IRS-1*^{+/-} mice and obese WT mice.

PI 3-kinase activity

Activation of PI 3-kinase is crucial for the metabolic action of insulin (Hara *et al.* 1994). Therefore, we examined the expression of p85 subunit of PI 3-kinase and PI 3-kinase activity associated with the immunoprecipitant using anti-phosphotyrosine antibody. In contrast to insulin receptor or *IRS* proteins, there was no change in expression level of p85 subunit of PI 3-kinase among the four groups (Fig. 8A and B). There was also no difference in insulin-stimulated PI 3-kinase activity between lean WT and lean *IRS-1*^{+/-} mice in both the liver (Fig. 8C) and muscles (Fig. 8D). The two obese groups showed a significant reduction in insulin-stimulated PI 3-kinase activity compared with their lean controls in the liver (diminished by 46 and by 60% in obese WT and obese *IRS-1*^{+/-} mice respectively) (Fig. 8C) and in muscles (by 27 and by 55% in WT and *IRS-1*^{+/-} respectively) (Fig. 8D). PI 3-kinase activity in the muscles of obese *IRS-1*^{+/-} mice was significantly lower than that in obese WT (by 36%). PI 3-kinase activity in the liver of obese *IRS-1*^{+/-} mice tended to be lower than that in obese WT, albeit statistically insignificant ($P = 0.07$).

Discussion

In the present study, we induced hypothalamic obesity in *IRS-1*^{+/-} mice and examined the impact of decreased *IRS-1* expression on the obesity-linked insulin resistance *in vivo*. After i.p. administration, GTG is mainly transported to cells of the ventromedial hypothalamus and causes necrotic lesions in that area. In rodents, the GTG-induced hypothalamic lesion causes insulin resistance and obesity, which is mainly due to the associated hyperphagia (Le Marchand *et al.* 1978). The first events after hypothalamic lesion is hyperphagia, and insulin resistance is considered to be a secondary event due to weight gain and hyperinsulinemia, although the exact cause of hyperinsulinemia in GTG-induced obesity is unclear at present (Tsao *et al.* 2000).

Administration of GTG in *IRS-1*^{+/-} mice resulted in increased food consumption and subsequent increase

Figure 6 Islet size and number of β -cells and non- β -cells in the pancreatic islets. Islet size (A), and number of β -cells (B) and non- β -cells (C) per islet were quantified as described in Materials and Methods. Data are means $\pm$ S.D. of five mice in each group. * $P < 0.05$, ** $P < 0.001$ vs lean WT, ## $P < 0.01$ vs lean *IRS-1*^{+/-}, † $P < 0.05$.

in body weight, which was somewhat similar to that seen in GTG-injected WT mice. Our results also showed no significant difference in abdominal fat weight between obese *IRS-1*^{+/-} and obese WT mice (data not shown). These findings suggest that heterozygous disruption of the *IRS-1* gene does not have a major impact on hypothalamic lesion-induced hyperphagia and increased fat mass. Not only obese *IRS-1*^{+/-} mice but also obese WT mice showed impaired response to blood glucose upon insulin challenge, demonstrating that insulin resistance is accompanied by hypothalamic obesity in these mice. Interestingly, although *IRS-1*^{+/-} and WT mice acquire a similar increase of body weight, obese *IRS-1*^{+/-} mice showed more profound insulin resistance than obese WT mice during the insulin tolerance test.

Despite the profound insulin resistance in obese *IRS-1*^{+/-} mice, there was no difference in fasting blood glucose levels between obese *IRS-1*^{+/-} and obese WT mice. This may be due to compensatory changes manifested by increased insulin secretion by increased numbers of pancreatic β -cells in obese *IRS-1*^{+/-} mice. It is also of note that there was no significant difference in fasting blood glucose, fasting insulin levels, and islet size between

lean *IRS-1*^{+/-} and lean WT mice. These results indicate that the impact of heterozygous knockout of the *IRS-1* gene on glucose homeostasis and insulin sensitivity becomes overt only after induction of obesity. Indeed, the additive effect of heterozygous disruption of the *IRS-1* gene on insulin resistance and pathogenesis of diabetes is supported by the results that double-heterozygous knockout of the *IRS-1* gene and insulin receptor gene in the mice is more diabetogenic than single hetero-knockout of each gene (Kido *et al.* 2000). In the present study, we observed upregulation of *IRS-2* protein in both the liver and muscles in lean *IRS-1*^{+/-} mice. This could in part explain the normal glucose tolerance and insulin sensitivity in lean *IRS-1*^{+/-} mice.

Abnormalities of early steps of insulin signaling pathway are known to cause insulin resistance *in vivo* and *in vitro*. In animal models of insulin resistance as well as in diabetic subjects, decreased binding of insulin to the insulin receptor, decreased receptor kinase activity, and decreased expression levels of insulin receptor and *IRS-1* and *IRS-2* have been demonstrated in the liver and skeletal muscles (Kahn *et al.* 1973, Soli *et al.* 1975, Folli *et al.* 1993). To determine the mechanism that underlies the profound

Figure 5 Morphological analysis of the pancreas obtained from 50-week-old mice. Hematoxylin-eosin (HE) stained (A–D) sections of the pancreas obtained from representative lean WT (A), lean *IRS-1*^{+/-} (B), obese WT (C) and obese *IRS-1*^{+/-} (D) mice. The samples were fixed, sectioned and stained with HE. Photographs show the typical islets of each group (magnification $\times 40$). Immunohistochemical analysis of islets of obese WT and obese *IRS-1*^{+/-} mice (E–H). Pancreases were obtained from 50-week-old mice, and sections were immunostained with anti-insulin (E, F) or anti-glucagon and anti-somatostatin cocktail antibodies (G, H). Photographs show the typical islets of each group (magnification $\times 100$).

Figure 7 Expression levels of insulin receptor and IRS-1 and IRS-2 in the liver and muscles. Equal amounts of protein obtained from the liver (left panels) and muscles (right panels) were separated by SDS-PAGE in duplicate and immunoblotted with anti-insulin receptor (IR) (A, B), anti-IRS-1 (C, D) or anti-IRS-2 (E, F) antibody. Representative results of Western blot analysis are shown above each bar plot, and quantitative analysis of the signals by NIH image analysis software is shown below the blots. The expression levels are relative to those of lean WT and expressed as means $\pm$ S.D. of five independent experiments (total ten animals). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs lean WT, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs lean *IRS-1*^{+/-}, † $P < 0.05$, †† $P < 0.01$ vs lean WT, ‡ $P < 0.05$.

insulin resistance observed in obese *IRS-1*^{+/-} mice, we examined the expression of insulin receptor and IRS-1 and IRS-2 in the liver and striated muscles.

As shown in other obese animal models (Folli *et al.* 1993, Saad *et al.* 1993, Kerouz *et al.* 1997), the expression levels of insulin receptor and IRS-1 and IRS-2 in the liver and muscles of our obese mice groups were lower than in the respective lean controls. We found that, in addition to the decrease in IRS-1 expression, insulin receptor in the liver in obese *IRS-1*^{+/-} mice was significantly lower than in obese WT. It is also of note that IRS-2 expression in the liver and muscles was upregulated in lean *IRS-1*^{+/-} mice, and this upregulation was blunted in obese *IRS-1*^{+/-} mice. Based on the results of knockout mice of either the IRS-1 or IRS-2 gene, several investigators proposed that IRS-1 is an important substrate for the metabolic actions of insulin in the muscles while IRS-2 is

necessary for insulin action in the liver (Araki *et al.* 1994, Tamemoto *et al.* 1994, Withers *et al.* 1998). The importance of the insulin receptor in the muscles and liver has also been confirmed by muscle- and liver-specific disruption of the insulin receptor in mice (Brüning *et al.* 1998, Wojtaszewski *et al.* 1999, Michael *et al.* 2000). Therefore, the decrease in insulin receptor and IRS-1 in both tissues and blunted upregulation of IRS-2 protein could explain the profound insulin resistance in obese *IRS-1*^{+/-} mice.

We also studied the PI 3-kinase activity, a key signal involved in insulin-induced glucose uptake in peripheral tissues and regulation of glyconeogenesis in the liver (Agati *et al.* 1998). Our results showed that PI 3-kinase activity in the liver and muscles was significantly reduced in obese mice. This reduction could be explained by the low expression of insulin receptor and IRS-1 and IRS-2 in both tissues. We also found a significant reduction in

Figure 8 Expression of p85 α phosphatidylinositol (PI) 3-kinase and phosphotyrosine (PY)-associated PI 3-kinase activities in the liver and muscles. Equal amounts of protein obtained from the liver (A) and muscles (B) were separated by SDS-PAGE in duplicate and immunoblotted with anti-p85 α antibody. Animals were injected with 5 U of regular human insulin or saline in the portal vein. Liver and muscles were removed 1 and 3 min later. Equal amounts of protein were subjected to immunoprecipitation with PY antibody followed by PI 3-kinase assays as described in Materials and Methods. ³²P incorporation into PI 3 phosphate (PIP3) in immunoprecipitates of the liver (C) and muscles (D) was quantified using NIH image analysis software. Expression levels are relative to those of lean WT mice under insulin stimulation and expressed as means $\pm$ S.D. of five independent experiments (total ten animals). * $P < 0.05$ vs lean WT, # $P < 0.05$, ## $P < 0.01$ vs lean *IRS-1*^{+/-}, † $P < 0.05$.

insulin-stimulated PI 3-kinase activity in the muscles of obese *IRS-1*^{+/-} mice compared with obese WT mice. This could be, at least in part, due to reduced expression of *IRS-1*.

However, an important question remains unanswered: what are the mechanisms that regulate the expression of these molecules? It is known that chronic insulin treatment downregulates the expression and/or the phosphorylation of insulin signaling molecules, including insulin receptor and *IRS-1* and *IRS-2* (Friedman *et al.* 1997, Kerouz *et al.* 1997). In our model, the expression levels of insulin receptor and *IRS-1* were low while serum insulin levels were high in obese *IRS-1*^{+/-} and obese WT. At present, it is not clear how insulin receptor is downregulated under a chronic hyperinsulinemic state. On the other hand,

previous studies proposed that downregulation of *IRS-1* protein in the presence of hyperinsulinemia is due to the degradation of *IRS-1* protein (Araki *et al.* 1995). It is also reported that *IRS-2* expression is negatively regulated by insulin by suppressing gene transcription and enhancing protein degradation (Rui *et al.* 2001, Zhang *et al.* 2001). Interestingly, *IRS-2* protein, which is upregulated to supernormal levels in lean *IRS-1*^{+/-} mice, was reduced in obese *IRS-1*^{+/-} mice, to levels as low as those seen in obese WT mice. It is possible that *IRS-2* is upregulated in lean *IRS-1*^{+/-} by reduction of the insulin signal through *IRS-1*, and chronic hyperinsulinemia could cause a downregulation of *IRS-2* protein in both obese mice. It is also reported that factors produced by adipocytes, such as adipocytokines and free fatty acids, can modulate the

insulin signaling pathway (Hotamisligil *et al.* 1994, Dresner *et al.* 1999, Griffin *et al.* 1999). Further studies are therefore necessary to determine the precise mechanism(s) involved in the regulation of these proteins in our mouse models.

Although insulin resistance in obese IRS-1^{+/-} mice was more severe than in obese WT mice, there was no significant difference in fasting glucose levels between these two groups of mice. This is probably due to the compensation by increased insulin secretion from hypertrophied pancreatic islets. The latter were larger in both groups of obese mice compared with their lean controls. Furthermore, the mean islet size of obese IRS-1^{+/-} mice was significantly larger than that of obese WT mice, and this enlargement was, at least in part, due to an increased number of pancreatic β -cells. It is known that vagal hyperactivity induced by ventromedial hypothalamic lesions stimulates proliferation of pancreatic β -cells through a cholinergic receptor mechanism (Kiba *et al.* 1996). On the other hand, as shown in Fig. 6, the increase in islet size (which was larger than that of the β -cell number per islet) suggested possible β -cell hypertrophy in obese IRS-1^{+/-} mice. We examined the size of β -cells in some islets by microscopy, and found that obese IRS-1^{+/-} mice tended to have larger β -cells compared with obese WT mice, albeit statistically insignificant (data not shown). Thus, we could not exclude a possibility of β -cell hypertrophy in obese IRS-1^{+/-} mice. Considered together, the enlargement of each islet might be the result of both an increased number of β -cells and β -cell hypertrophy. It is known that other obese models such as ob/ob mice (Tomita *et al.* 1992) and lean insulin-resistant models such as IRS-1^{-/-} mice (Brüning *et al.* 1997) also show an increase in islet mass. In our study, the size of islets paralleled the degree of insulin resistance in each mouse group. The regulation of pancreatic β -cell growth is not fully understood at present. It has been reported that the IGF-I signal mediated by IRS-2 is important for pancreatic β -cell growth (Schuppert *et al.* 1998), and recent studies proposed the involvement of Akt, a serine/threonine kinase activated by insulin or IGF-I stimulation, in β cell growth (Tuttle *et al.* 2001). Further studies are necessary to investigate the insulin signaling pathway including Akt activity in β -cells in our model.

In conclusion, we have demonstrated in the present study that GTG administration induced obesity and insulin resistance in both IRS-1^{+/-} and WT mice. Importantly, obese IRS-1^{+/-} mice were more insulin-resistant and had larger islets than obese WT mice, although insulin sensitivity of lean IRS-1^{+/-} mice was similar to that of lean WT mice. Our results suggest that IRS-1 gene polymorphisms might be associated with worsening of insulin sensitivity in humans in the presence of additional factor(s), such as obesity. These findings widen the scope of our understanding of the mechanism involved in the development of type 2 diabetes mellitus in obese subjects.

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