Overexpression of insulin receptor partially improves obese and diabetic phenotypes in \( db/db \) mice

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Abstract. Type 2 diabetes mellitus (T2DM) is one of the major health concern among the world. Several treatment options for T2DM are in clinical use, including injecting insulin, promoting insulin secretion by insulin secretagogues, and improving insulin sensitivity by insulin sensitizers. However, increasing the amount of insulin receptor in insulin-target tissues has not been explored. In order to test the efficacy of insulin receptor overexpression for improving glucose control, we established a transgenic mouse line expressing human insulin receptor (INSR). We analyzed, growth, energy balance, and glucose control of INSR-overexpressing \( db/db \) mice (INSR; \( db/db \)), which we produced by mating INSR transgenic mice with \( db/db \) mice, a genetic model of obesity due to insufficient leptin signaling. Compared to \( db/db \) mice, INSR; \( db/db \) mice were rescued from hyperphagia and obesity, leading to improved blood glucose levels. Unexpectedly, however, INSR; \( db/db \) mice presented with stunted growth, accompanied by decreased plasma levels of free IGF1 and IGFBP-3, indicating the down-regulation of GH/IGF1 axis. These phenotypes were observed in INSR; \( db/db \) mice but not in INSR littermates. Meanwhile, bone defects observed in \( db/db \) male mice were not rescued. Moreover, improved blood glucose was not accompanied by improved insulin sensitivity. Therefore, overexpression of insulin receptor improves obese and diabetic phenotypes in \( db/db \) mice, with consequences on growth.

Key words: Insulin receptor, Obesity, Diabetes, \( db/db \)

THERE are 347 million diabetic patients worldwide and 95 % of them are type 2 diabetes mellitus (T2DM), hallmarks of which are impaired secretion and action of insulin [1, 2]. Several treatment options for T2DM are in clinical use, including injecting insulin, promoting insulin secretion by insulin secretagogues (such as sulfonylureas and glinides), and improving insulin sensitivity by insulin sensitizers (such as biguanides and thiazolidinediones). However, increasing the amount of insulin receptor in insulin-target tissues has not been explored.

Insulin exerts its physiological effects through insulin receptors by binding to the alpha subunit of insulin receptor and stimulating the intrinsic kinase activity of the beta subunit [3-5]. However, chronic exposure of cells to insulin leads to down-regulation of insulin signaling through a net loss of insulin receptor from the plasma membrane [6], which is one of the mechanisms involved in insulin resistance. Therefore, increasing the amount of insulin receptor could alleviate insulin resistance.

Leptin is an adipokine crucial for the regulation of energy homeostasis [7]. A homozygous loss-of-function mutation in the leptin receptor (the \( db \) mutation) causes hyperphagia and obesity, leading to diabetes in mice [8, 9]. Since obesity is a major risk factor for impaired insulin sensitivity and T2DM, \( db/db \) mice are used as a genetic model of T2DM.

Abbreviations: ANOVA, analysis of variance; BMD, bone mineral density; ELISA, enzyme-linked immunosorbent assay; GH, growth hormone; IGF1, Insulin-like growth factor 1; IGFBP-3, IGF-binding protein 3; INSR, insulin receptor; IR, insulin receptor; IRS, insulin receptor substrate; T2DM, Type 2 diabetes mellitus; Tg, transgenic; WT, wild type
In this study, we sought to determine whether increasing the expression of insulin receptor ameliorated obesity and diabetes. In order to perform proof-of-concept study, we first decided to generate a transgenic (Tg) mouse line expressing human insulin receptor (INSR), so that its expression patterns among tissues can be analyzed using anti-human INSR-specific antibody. After establishing the Tg mouse line, we next used the genetic approach to address the question, because db/db mice present with severe obesity and diabetes compared to the diet-induced obese model. Although the established line expressed INSR only in skeletal muscle, pancreas, and brain, we inadvertently found that crossing them with db/db mice not only partially ameliorates obesity and diabetes, but improved blood glucose was not accompanied by improved insulin sensitivity, and affected growth of mice by down-regulating the GH-IGF-1 axis in mice.

Materials and Methods

Animals

All animal care and experimental procedures were approved by the Institutional Animal Care and Experimentation Committee at Gunma University, Japan. Mice were housed in individual cages in a temperature-controlled facility with a 12-h light/dark cycle. Mice were allowed free access to water and were given a standard laboratory chow diet (CLEA Rodent diet CE-2; CLEA Japan, Tokyo, Japan). db/+ mice were purchased from CLEA Japan. To generate INSR-Tg mice to achieve general overexpression of INSR, an expression cassette consisting of the cytomegalovirus intermediate early enhancer, the chicken beta-actin promoter, the rabbit beta-globin intron (the CAG promoter), and INSR cDNA was injected into oocytes from C57BL6 mice. INSR-Tg mice (in a B6 background) and db/+ mice (in a BKS background) were mated to generate parent mice (db/+ or INSR; db/+ in a B6 x BKS background), and these parents were mated to generate experimental groups (wild type, INSR heterozygote (het), db/db, and INSR(het); db/db). Body weight, food intake, and perigonadal white adipose tissue weight of male and female mice were measured as described previously [10]. Mice were euthanized under anesthesia. Plasma insulin levels were measured via mouse ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan). Plasma free IGF1 levels were measured via mouse free IGF1 ELISA kit (ALPCO Diagnostics, Salem, New Hampshire, USA). Plasma IGF-binding protein 3 (IGFBP-3) levels were measured with mouse ELISA kits (Abcam, Tokyo, Japan). Tests of glucose tolerance and insulin tolerance were performed as previously described [11] at 17 weeks of age and 8 and 16 weeks of age, respectively. In brief, for the glucose tolerance test (GTT), mice were subjected to an overnight fast followed by i.p. glucose injection (1.0 g/kg). For the insulin tolerance test (ITT), human insulin (Eli Lilly, Kobe, Japan) was injected (0.75 U/kg i.p.) to randomly fed mice.

PCR genotyping

PCR genotyping for the db locus was carried out as described previously using genomic DNA extracted from tails of mice [9]. The presence of the INSR transgene was detected with the following primers: sense-1048F, 5’-TGAGTGTCCCTCCGGGTACACG-3’; antisense-1404R, 5’-TTGTCCAAGGCATAGAAGGAG-3’. Amplicons were 357 base pairs in size and generated with KOD FX NEO enzyme (Toyobo, Osaka, Japan).

Immunoprecipitation and western blotting

To detect INSR in tissues, the protein was first immunoprecipitated with anti-human insulin receptor alpha antibody (83-7) (ab36550, Abcam), which does not react with murine insulin receptor, and then probed with anti-insulin receptor beta (C-19) antibody (sc-711, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), which reacts with insulin receptors of both mouse and human origin. To assess the insulin signaling of the liver and soleus, 7-week-old male mice were fasted for 24 h and received intraperitoneal injection (5 U/kg) of human insulin (Eli Lilly, Kobe, Japan). The liver and soleus were harvested 15 min later, and samples were subjected to western blotting using the following antibodies: insulin receptor beta (sc-711, Santa Cruz Biotechnologies), alpha-tubulin (sc-5286, Santa Cruz Biotechnologies), phospho-AKT (Ser473) (#9271, Cell Signaling, Tokyo, Japan), AKT (#9272, Cell Signaling), phospho-insulin receptor substrate 1 (IRS-1) (Ser636/639) (#2388, Cell Signaling), and IRS-1 (D23G12) (#3407, Cell Signaling) [11]. Signal intensity was measured with ImageQuantTL (GE Healthcare UK Ltd., Buckinghamshire, England).

Islet histology

After 17-week-old mice were euthanized, their pan-
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creata were fixed in 4% paraformaldehyde and embedded in paraffin. Tissues were sectioned and stained with anti-glucagon (G2654, Sigma, USA) and anti-insulin antibodies (A0564, Dako, USA). We visualized immune complexes with fluorescein isothiocyanate- or Cy3-conjugated secondary antibodies [12].

Mouse computed tomography (CT)

Six-week-old male mice were anesthetized and whole-body scanned with an X-ray CT scanner for experimental animals (LaTheta LCT-200, Hitachi Aloka Medical, Tokyo, Japan). Morphometric analyses of tibia and perioral soft-tissue thickness measurements were performed with the software accompanying the LCT-200.

Statistical analyses

In order to assess the effect of genetic differences on biological phenotypes at each time point, ANOVA was performed at each time point with a post-hoc Bonferroni correction for between-group comparisons in Excel. For western-blot densitometries, one-way ANOVA with post-hoc Tukey’s tests were performed with SPSS.

Results

INSR protein is expressed predominantly in skeletal muscle, pancreas, and brain of INSR-Tg mice

We characterized the tissue distribution of human INSR protein in INSR-Tg mice via immunoprecipitation and western blotting using human-specific anti-insulin receptor antibody. Human INSR was expressed in skeletal muscle, pancreas, and brain (and heart, to a lesser extent), while it was absent from liver, white adipose tissue, kidney, lung, and spleen (Fig. 1A). Human INSR was broadly expressed within the central nervous system, and pituitary to a lesser extent, with a scant expression in the perioral soft tissue (Fig. 1B).

INSR; db/db mice are leaner than db/db mice

After confirming the expression patterns of human INSR in INSR-Tg mice, we crossed these mice with db/db mice to test if the additional expression of human INSR ameliorates obese phenotypes of db/db mice. The ratio of genotypes of the pups was similar to that anticipated based on the Mendelian genetics (data not shown). The body weight and food intake of these mice were monitored from 3 to 16 weeks of age. INSR overexpression rescued the obese phenotype of db/db mice (Fig. 2A). The amount of perigonadal white adipose tissue at 17 weeks of age was significantly decreased in INSR; db/db mice compared to db/db littermates (Fig. 2B). The hyperphagia observed in db/db mice was also rescued in INSR; db/db mice (Fig. 2C). However, when re-feed intake was assessed at 8 weeks of age after 24-h fasting, reduced re-feeding intake of db/db mice was not rescued in INSR; db/db mice. Overall, increased INSR expression in certain insulin-targeted organs prevented the hyperphagia and obesity caused by leptin signaling deficiency in db/db mice.

INSR overexpression in db/db mice caused stunted growth by affecting GH/IGF1 axis

Although INSR; db/db mice were leaner than db/db mice, they were also shorter (Fig. 3A and B). The linear growth is controlled by growth hormone (GH) secreted from the pituitary, which controls the production of IGF1 and IGFBP-3 from the liver [13]. However, serum GH level fluctuates dramatically within a day due to the well-known pulsatile release pattern, which makes it difficult to assess the difference in GH secretion among groups. Therefore, we measured plasma free IGF1 and IGFBP-3 levels in our mice as surrogate markers for the tone of the GH-IGF-1 axis in these mice. Both plasma free IGF1 and IGFBP-3 levels were significantly decreased in INSR; db/db mice (Fig. 3C and D).

INSR overexpression in db/db mice does not rescue bone defects observed in male db/db mice

Leptin signaling can also affect bone morphology [14, 15]. Therefore, we measured cortical and total
Fig. 2  Effects of INSR overexpression on body weight, adiposity, and food intake in 
2db/db mice.
(A) Change in body weight from 3 to 16 weeks of age (n= 8 ~ 10).  (B) Perigonadal white adipose tissue (WAT) weight of 17-week-old mice (n= 8 ~ 10).  (C) Cumulative food intake from 3 to 16 weeks of age (n= 8 ~ 10).  (D) Food intake after 24 hour fasting in 8-week-old mice (n=4 ~ 6).  The following color represents the genotype of mice: blue (wild type (WT)), red (INSR), green (db/db), purple (INSR; db/db).  Data were analyzed by one-way ANOVA with post-hoc Bonferroni correction. Following abbreviations indicate significant differences (p < 0.05) between groups: a (different from WT and INSR), b (different from WT, INSR, and INSR; db/db), c (different from WT), d (different from INSR). hIR, human INSR.

Fig. 3  INSR overexpression in db/db mice causes stunted growth by affecting plasma free IGF1 and IGFBP-3 levels.  (A) Representative images of male mice studied.  (B) Body lengths of mice at 17 weeks of age (n = 8 ~ 10).  (C) Plasma free IGF1 levels of 17-week-old mice (n = 8 ~ 10).  (D) IGFBP-3 levels of 17-week-old mice (n = 8 ~ 10).  Data were analyzed with one-way ANOVA with post-hoc Bonferroni correction. *p < 0.05. hIR, human INSR.
bone mineral density, second moments of area, and polar second moments of the inertia of tibia. Bone phenotypes caused by leptin signaling deficiency in \(db/db\) mice were not rescued in \(INSR; db/db\) mice (Fig. 4A-D). Therefore, despite the effect on growth observed in \(INSR; db/db\) mice, these mice were not rescued from the bone defects caused by the lack of leptin signaling.

**INSR; db/db mice have better glucose control than \(db/db\) mice**

The hyperglycemia observed in \(db/db\) mice was dramatically improved in \(INSR; db/db\) mice (Fig. 5A), accompanied by improved hyperinsulinemia (Fig. 5B). These mice also showed better glucose tolerance at 17 weeks of age (Fig. 5C). We also analyzed these mice at 7 weeks of age. The significant hyperglycemia and hyperinsulinemia observed in \(db/db\) mice upon intraperitoneal glucose injection were partially ameliorated in \(INSR; db/db\) mice (Fig. 5D-F), consistent with improved blood glucose levels observed in \(INSR; db/db\) mice. Qualitative analysis of pancreatic islets at 17 weeks of age indicated that islet hypertrophy in \(db/db\) male mice were partially rescued in \(INSR; db/db\) mice (Fig. 5G). Therefore, increased INSR expression in certain insulin-targeted organs improved glucose metabolism in \(db/db\) mice.

**Systemic insulin sensitivity is not improved in \(INSR; db/db\) mice despite improved glucose tolerance**

We performed insulin tolerance tests at 8 and 16 weeks of age. We detected no improvement in systemic insulin sensitivity in \(INSR; db/db\) mice compared to \(db/db\) mice (Fig. 6A-D). \(INSR-Tg\) mice also did not show any higher sensitivity to exogenous insulin compared to wild-type mice.

We also tested insulin signaling in the liver and soleus via intraperitoneal injection of insulin to 7-week-old male mice. Consistent with the results in Figure 1A, INSR expression was increased in the soleus of \(INSR\) mice and \(INSR; db/db\) mice, but not in the liver of either strain (Fig. 7A-B). The decreased AKT phosphorylation observed in the soleus of \(db/db\) mice was improved in \(INSR; db/db\) mice, but it was accompanied by increased IRS-1 serine phosphorylation (Fig. 7B).

**Discussion**

In this study, we overexpressed human INSR in several organs and found that it rescued the obese phenotype and blood glucose levels of \(db/db\) mice without significant improvements in systemic insulin sensitivity. It was accompanied by growth defect through the effect on the GH/IGF-1 axis, which may have confounded the study.

Generally, GH coordinately regulates the expression of IGF1 and IGFBP-3 [13]. IGF1 and IGFBP-3 works as effectors toward peripheral tissues to cause growth. Plasma IGF1 exists either free or in an IGFBP-bound form; free IGF1 exerts biological activity. IGFBP-3 is the most abundant IGFBP and accounts for 80% of all IGF binding [16]. Considering that human INSR was detected both in the hypothalamus and the pituitary, it is possible that increased INSR proteins in these tissues may have caused the down-regulation of the GH-IGF-1 axis at the levels of hypothalamus and/or
INSR overexpression improves blood glucose levels in db/db mice. (A) Random blood glucose levels at 3-16 weeks of age. (B) Random plasma insulin levels at 17 weeks of age (n = 8-10). (C) Intraperitoneal glucose tolerance tests at 17 weeks of age (n = 8-10). (D) Fasting blood glucose at 7 weeks of age (male, n = 9-15; female, 7-11). (E) Blood glucose levels 15 min after intraperitoneal glucose injection at 7 weeks of age (male, n = 9-15; female, 7-11). (F) Plasma insulin levels 15 min after intraperitoneal glucose injection at 7 weeks of age. (G) Representative islet histology at 17 weeks of age. Magnification (100-fold) of insulin (green) and glucagon (red) double stains is shown. For A-F, the following color represents the genotype of mice: blue (wild type (WT)), red (hIR), green (hIR, db/db), purple (hIR, db/db). Data were analyzed by one-way ANOVA with post-hoc Bonferroni correction. Following abbreviations indicate significant differences (p < 0.05) between groups: a (different from WT and INSR), b (different from WT, INSR, and INSR; db/db), c (different from WT). hIR, human INSR.
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Fig. 6 INSR overexpression does not improve systemic insulin sensitivity in db/db mice despite improved glucose tolerance. (A-B) Insulin tolerance tests (A) and area under the curve data (B) at 8 weeks of age (n = 8 ~ 10). (C-D) Insulin tolerance tests (C) and area under the curve data (D) at 16 weeks of age (n = 8 ~ 10). The following color represents the genotype of mice: blue (wild type (WT)), red (INSR), green (db/db), purple (INSR; db/db). Data were analyzed by one-way ANOVA with post-hoc Bonferroni correction. Following abbreviations indicate significant differences (p < 0.05) between groups: a (different from WT and INSR), c (different from WT). Abbreviation: A.U., arbitrary unit; hIR, human INSR.

Fig. 7 The effects of INSR overexpression on insulin signaling in liver and soleus of db/db mice. (A-B) Western blots of insulin signaling in liver (A) and soleus (B) of male mice 15 min after intraperitoneal insulin injection. Data were analyzed with one-way ANOVA with post-hoc Tukey’s test. The marks (“x” or “y”) indicate genotypes belonging to the same group according to Tukey’s test. *p < 0.05. Abbreviations: A.U., arbitrary unit; IR, insulin receptor; IRS, insulin receptor substrate; hIR, human INSR.
pituitary. IGF-1 has 48% amino acid sequence identity with proinsulin, and although IGF I was only 6 percent as potent as insulin in the production of hypoglycemia on a molar basis when administered to healthy human, IGF-1 does bind to insulin receptors with low affinity [17]. Therefore, the binding of circulating IGF-1 to additionally available receptors (either INSR receptors or hybrid INSR/IGF-1 receptors) on top of endogenous IGF-1 receptors in the pituitary, may provide additional feed-back signaling to tone down the GH-IGF-1 axis. Importantly, the reduction in IGF1 and IGFBP-3 levels were observed in INSR; db/db mice, indicating that both INSR overexpression and contribute to the down-regulation of the GH-IGF-1 signaling in INSR; db/db mice.

Hyperphagia of db/db mice was rescued in INSR; db/db mice, evidenced by reduced cumulative food intake (Fig. 2C). However, there was no rescue in re-feeding intake after 24-h fasting (Fig. 2D). Because db/db mice are significantly heavier and has significantly higher fasting blood glucose level (Figs. 2A and 5D), db/db mice may not feel as hungry as WT or INSR-Tg mice after 24-h fasting. Although INSR; db/db mice are significantly heavier than WT and INSR-Tg mice only in female but tend to have higher fasting blood glucose levels in both male and female, the higher fasting blood glucose levels may have caused the lack of rescue in the reduced re-feeding intake.

Improvements in hyperphagia and obesity caused by deficient leptin signaling were accompanied by improved blood glucose and glucose tolerance in INSR; db/db mice; yet, systemic insulin sensitivity measured via insulin tolerance testing was not improved in these mice. The reversal of hyperglycemia may have mitigated beta-cell compensation failure and decrease in basal hepatic glucose output. Liver (which does not express INSR) contributes more to systemic glucose metabolism than skeletal muscle (which express INSR) in mice; the lack of INSR expression in liver may have led to the lack of improvement in systemic insulin sensitivity in INSR; db/db mice. Indeed, muscle-specific insulin receptor knock-out mice has been reported to exhibit normal blood glucose and normal insulin level [18]. Another possibility is the contribution of IRS-1 to insulin sensitivity. Western blotting revealed that phosphorylation of IRS-1 serine residues was significantly increased in the soleus of these mice. Serine phosphorylation of IRS-1 is part of a negative feedback mechanism for insulin signaling; hyperactivation of insulin signaling due to the increased expression of insulin receptors and/or effects from increased IGF1 signaling may have contributed to the down-regulation of insulin signaling in skeletal muscle. This hyperactivity may have also contributed to a mild (but significant) increase in AKT phosphorylation in the soleus of INSR; db/db mice, despite several fold-increased insulin receptor expression. Increased IRS-1 protein levels in the liver of db/db and INSR; db/db mice is also consistent with the insulin resistance observed in these mice, because insulin signaling promotes the degradation of IRS-1 protein through the ubiquitin-proteasome system via serine phosphorylation of IRS-1, a target of rapamycin (mTOR) and ribosomal p70 S6K [19-23]. Why glucose tolerance was ameliorated without improvement of insulin sensitivity? One possibility is that insulin-independent glucose-lowering factors, e.g. GLP-1 or FGF21, may have contributed to the better glucose control. Another possibility is that glucose-sensing via glucokinase signaling may have enhanced in INSR; db/db mice. In any case, the detailed molecular mechanism by which better glucose control was achieved in INSR; db/db mice needs to be elucidated in the future.

The major limitation of the transgenic mice established in the current study is that human insulin receptor was not expressed in the liver or white adipose tissue where it is normally expressed. We could establish only one Tg line, so we could not identify which organ(s) are responsible for the phenotypes observed in INSR; db/db mice, including benefits and side effects. Although the INSR transgene was designed to be expressed globally by using the CAG promoter, the transgene expression was limited to the certain tissues. We speculate that human INSR expression in the liver could have caused lethal hypoglycemia, which led to the lack of its expression in the liver of the established line. To address these issues, overexpression of INSR either in tissue-specific fashion and/or in tissues were it is normally expressed are necessary.

In summary, overexpression of insulin receptor improves obese and diabetic phenotypes in db/db mice, with consequences on growth.
Author Contribution

Study design and manuscript preparation: TS. Execution of the study and data analysis: TS, MK, SS, SM, OK, YVS, and YT. Discussion: MK and TY. Coordination of research: TK.

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Conflict of Interest

The authors have nothing to disclose.

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References


