Anti-Insulin Receptor Antibodies Improve Hyperglycemia in a Mouse Model of Human Insulin Receptoropathy

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Loss-of-function mutations in both alleles of the human insulin receptor gene (INSR) cause extreme insulin resistance (IR) and usually death in childhood, with few effective therapeutic options. Bivalent antireceptor antibodies can elicit insulin-like signaling by mutant INSR in cultured cells, but whether this translates into meaningful metabolic benefits in vivo, wherein the dynamics of insulin signaling and receptor recycling are more complex, is unknown. To address this, we adopted a strategy to model human insulin receptoropathy in mice, using *Cre* recombinase delivered by adeno-associated virus to knockout endogenous hepatic *Insr* acutely in floxed *Insr* mice (liver insulin receptor knockout [L-IRKO] + GFP), before adenovirus-mediated add back of wild-type (WT) or mutant human *INSR*. Two murine anti-INSR monoclonal antibodies, previously shown to be surrogate agonists for mutant INSR, were then tested by intraperitoneal injections. As expected, L-IRKO + GFP mice showed glucose intolerance and severe hyperinsulinemia. This was fully corrected by add back of WT but not with either D734A or S350L mutant INSR. Antibody injection improved glucose tolerance in D734A INSR-expressing mice and reduced hyperinsulinemia in both S350L and D734A INSR-expressing animals. It did not cause hypoglycemia in WT INSR-expressing mice. Antibody treatment also downregulated both WT and mutant INSR protein, attenuating its beneficial metabolic effects. Anti-INSR antibodies thus improve IR in an acute model of insulin receptoropathy, but these findings imply a narrow therapeutic window determined by competing effects of antibodies to stimulate receptors and induce their downregulation.

Insulin exerts metabolic and growth-promoting effects that are essential for life via a homodimeric plasma membrane receptor tyrosine kinase. Insulin binding to extracellular sites induces alterations in receptor structure that promote *trans*-autophosphorylation of tyrosine residues on intracellular β subunits. This, in turn, leads to recruitment and phosphorylation of insulin receptor substrate proteins, and thence activation of a signaling network, critically including the PI3K/AKT and RAS/mitogen-activated protein kinase pathways (1).

Attenuated glucose lowering by insulin in vivo is referred to as insulin resistance (IR), and it is a core feature of the metabolic syndrome in humans. IR is closely associated with obesity, type 2 diabetes, an abnormal blood lipid profile that promotes atherosclerosis, fatty liver, and reduced fertility, but its molecular and cellular basis is not fully elucidated (2). Several severe forms of IR are known where the precise cause is established, however. The most extreme of these are caused by biallelic insulin receptor (*INSR*) mutations, and they are clinically described as Donohue syndrome or Rabson–Mendenhall syndrome (RMS) (online MIM #246200 or #262190). Death is usual within the first 3 years in Donohue syndrome, whereas in RMS, mortality in the second or third decades is common despite the use of insulin-sensitizing drugs, high-dose insulin, and experimental therapies such as recombinant human IGF-1 (3) or leptin (4). There is, thus, a major unmet need for novel approaches to circumvent the impaired function of mutant receptors. Some INSR mutations impair receptor processing and thus cell surface expression. However, many mutant INSR are well expressed at the cell

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surface but exhibit impaired insulin binding and/or impaired signal transduction (5,6). This affords the opportunity to activate the mutant receptors using surrogate ligands, and observations of the genetic spectrum of receptoropathy suggest that even modest activation is likely to elicit meaningful metabolic effects (2).

It was demonstrated in the 1980s that crosslinking of insulin receptor homodimers by bivalent antibodies could elicit signaling responses (5). In the early 1990s, the principle that insulin receptors harboring disease-causing mutations could also be partly activated by antibodies was provided for two mutations: one in a cell culture model and the other in solubilized form (7,8). With therapeutic humanized monoclonal antibodies now well established as treatments both for cancer and noncancer indications (9), interest in biological therapies targeting the INSR has been recently rekindled. Inhibitory INSR antibodies are now in phase 1 human trials (10), while stimulatory antibodies have been shown to ameliorate diabetes in rodents (11–13) and primates (14). Given the high clinical need in recessive insulin receptoropathy, we previously assessed the effect of monoclonal anti-INSR antibodies (15–19) on a series of disease-causing mutant INSRs in cell culture models, corroborating and extending prior findings by demonstrating an action of antibodies against a panel of mutant receptors (20).

Whether the stimulation of mutant receptors by anti-receptor antibodies that is observed biochemically after acute exposure of cells in culture will be sustained and metabolically beneficial in vivo has not yet been addressed. A specific concern relates to the documented effect of naturally occurring anti-INSR autoantibodies, which are partial agonists when tested acutely on cellular models, to downregulate INSR signaling when present at high concentrations in vivo, inducing acquired severe IR, known as "type B" IR (21). Such an effect has not been assessed in preclinical testing of anti-INSR antibodies reported to date (11–14), but it is a critical concern in efforts to develop safe, efficacious biological therapies targeting the INSR.

To address these issues, we have now generated a novel model of human insulin receptoropathy restricted to mouse liver that is based on adenoviral overexpression of human wild-type (WT) or mutant INSR in the liver after *cre*-mediated knockout of endogenous murine *Insr*. Using this model, we assessed the effect of two anti-human INSR monoclonal antibodies previous tested in cell culture on metabolic end points and receptor expression.

RESEARCH DESIGN AND METHODS

Mice

All mouse experiments were approved under the UK Home Office Animals (Scientific Procedures) Act 1986 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Board. $Insr^{loxP/loxP}$ mice were described previously (22), as was use of adeno-associated virus (AAV) to deliver *Cre* to generate liver insulin receptor knockout (L-IRKO) mice (23). $Insr^{loxP/loxP}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were fed regular laboratory SAFE105 chow diet (Safe Diets, Augy, France) throughout the study. Male mice were injected via the tail vein at 8 weeks of age with 10^{11} copies per mouse of AAV serotype 8 containing a hybrid promoter based on the thyroid hormone–binding globulin promoter and macroglobulin/bikunin enhancer. This permitted liver-specific expression of iCre or EGFP to generate L-IRKO or liver WT (L-WT) mice, respectively. At 10 weeks of age, mice were injected via the tail vein with 5×10^9 infectious units per mouse of adenovirus (AdV) serotype 5 containing the liver albumin promoter driving liver-specific expression of either COOH-terminal myc-tagged human insulin receptor (WT or one of S350L and D734A mutants) or EGFP. Plasma ALT and AST levels were measured before and after AAV administration (week 9) and after AdV administration (week 11) in a pilot study. AST levels did not change, while ALT levels in the L-IRKO + WT mice at week 11 were elevated at threefold higher than the upper limit of normal for C57BL/6J mice, indicating mild liver inflammation that would be clinically insignificant (Supplementary Fig. 1).

For antibody studies, the mice were treated twice over 1 week with 10 mg/kg antibody via intraperitoneal injection, with the first dose given the day after AdV was administered. The antibodies used have been extensively studied and are available from various vendors. Antibodies were highly purified from mycoplasma-free hybridoma culture supernatants as a paid service by BioServUK, and they were diluted in PBS prior to administration. Blood glucose 60 min after administration of antibody to ad libitum fed animals, revealed no change in blood glucose concentration. Experiments were performed a week after AdV injection. Mice were euthanized by cervical dislocation at the conclusion of the oral glucose tolerance test (OGTT), and tissues were harvested and snap-frozen immediately in liquid nitrogen before storage at −80°C until further processing. Body weights of mice were measured throughout the study, and no significant change was observed among different genotypes or treatment groups (Supplementary Fig. 2).

Metabolic Measurements

For OGTTs, mice were fasted for 5 h prior to oral gavage of glucose at 2 g/kg body weight. Blood glucose measurements were made using a blood glucose analyzer (Alpha-TRAK) at 0, 15, 30, 60, 90, 120 min. For plasma insulin analysis, tail blood was collected at 0 and 15 min into glass microhematocrit capillary tubes with sodium heparin (Hirshmann-Laborgeräte, Eberstadt, Germany). Insulin concentrations were determined by a sandwich immunoassay providing an electrochemiluminescent readout (Meso Scale Discovery, Rockville, MD).

Liver Protein Extraction and Western Blotting

Liver tissues were homogenized in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham,

MA) containing protease and phosphatase inhibitors (Roche, Penzberg, Germany) using Matrix D ceramic beads and a FastPrep-24 benchtop homogenizer (MP Biomedicals, Santa Ana, CA). Lysates were cleared of insoluble debris by centrifugation prior to determination of protein concentration by bicinchoninic acid (BCA) assay (Bio-Rad, Hercules, CA). Lysates were electrophoresed through either 4–12% NuPAGE or 8% E-PAGE gels (Thermo Fisher Scientific) and were transferred to nitrocellulose by iBlot 2 dry blotting system (Thermo Fisher Scientific). The following antibodies were used for immunoblotting at a dilution of 1:1,000: INSR (3025), Myc-tag (2276), Beta-actin (4967) from Cell Signaling Technology (Danvers, MA). Anti-GFP antibody (ab290; Abcam, Cambridge, U.K.) was used at 1:2,000. Horseradish peroxidase–conjugated secondary antibodies and Immobilon Western Chemiluminescent horseradish peroxidase substrate (Millipore, Burlington, MA) were used to detect protein-antibody complexes, and grayscale 16-bit TIFs captured with an ImageQuant LAS4000 camera system (GE Healthcare Lifesciences, Marlborough, MA). Pixel density of grayscale 16-bit TIFs was determined in ImageJ 1.52b (National Institutes of Health). The rectangle tool was used to select lanes, and the line tool was used to enclose the peak of interest and subtract the background. The magic-wand tool was used to select the peak area and obtain the raw densitometry value. Normalized values for INSR were scaled to the mean expression of INSR in L-WT tissues and Myc-tagged INSR values were scaled to the mean expression of INSR-myc in control antibody-treated L-IRKO + WT animals.

Liver mRNA Isolation and Quantitative PCR

Total RNA was isolated from liver tissues using the Directzol RNA extraction kit from Zymo Research (Irvine, CA). cDNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega Corporation, Fitchburg, WI). Relative expression of genes of interest was quantified by real-time PCR using TaqMan gene expression assays (Mm02619580_g1 ACTB, Mm01211877_m1 Mm Insr, Hs00961560_m1 Hs INSR) and the QuantStudio 7 Flex Real-Time PCR system (Thermo Fisher Scientific). The results were analyzed by the comparative Ct method. Validation experiments were performed both to confirm species specificity of TaqMan gene expression assays and that their relative amplification efficiencies permitted analysis by the comparative Ct method.

Statistical Analysis

Mice were randomly assigned to viral injection schedules and antibody treatment groups. Investigators were blind to the assignment at the time of administering treatments and performing experiments. All data presented are the mean ± SD with the exception of the OGTT histograms which are the mean ± SEM. Statistical analysis were performed using GraphPad Prism 8 for macOS version 8.3.0 (GraphPad Software, San Diego, CA). Protein abundance, mRNA expression, fasting blood glucose and insulin, and area under the curve (AUC) (blood glucose mmol/L × 120 min were computed using the trapezoid rule) and analyzed by one-way ANOVA followed by Tukey multiple comparisons test. Blood glucose levels during OGTT were analyzed by two-way (repeated measures) ANOVA followed by Tukey multiple comparisons test. A probability level of 5% ($P < 0.05$) was considered statistically significant.

Data and Resource Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

RESULTS

Creation of a Flexible Murine Model of Human Insulin Receptoropathy

We first set out to generate a humanized mouse model of insulin receptoropathy (Fig. 1*A*). This was necessary as the previously described anti-human INSR monoclonal antibodies to be tested (83-7 and 83-14, which bind distinct epitopes on the receptor α subunit) were raised in mice and do not bind rodent receptor (16). Eight-week-old $Insr^{loxP/loxP}$ mice were injected via the tail vein with an AAV containing *Cre* recombinase under control of a liver-specific promoter (thyroid hormone–binding globulin). This deleted endogenous hepatic *Insr*, generating L-IRKO mice. As described previously (23), this approach avoids the compensatory, secondary responses seen on congenital liver Insr knockout (22). WT or mutant human INSR, or GFP alone, were then expressed in knockout liver by injection via the tail vein at 10 weeks of age of AdV containing transgenes under the control of the liver-specific albumin promoter, to create a series of add-back models of human receptoropathy, denoted here by L-IRKO + WT, L-IRKO + (*INSR* mutation) and L-IRKO + GFP, respectively. All *INSR* constructs included COOH-terminal myc-tags to aid detection of transgene expression. L-WT mice with unperturbed liver *Insr* expression were generated by injecting $Insr^{loxP/loxP}$ mice with AAV encoding GFP at 8 weeks of age, followed by AdV encoding GFP at 10 weeks of age, and served as additional controls (Fig. 1*A*).

Two INSR mutations, D734A and S350L, were selected for study based on prior evaluation in cell signaling assays (20). Both D734A (24) and S350L (25) mutations produce receptors that are normally processed and expressed at the cell surface but demonstrate severely reduced insulin binding and autophosphorylation. Indeed, the INSR D734A mutation lies in the αCT segment of the extracellular domain of the INSR, which is a critical structural components of insulin-binding site 1, initially identified in biochemical studies (26). Importantly, both mutant INSRs have been shown to be activatable by anti-INSR antibodies (20,27).

The INSR D734A mutant was used first to evaluate the add-back approach. Western blots of liver lysates confirmed efficient deletion of endogenous *Insr* in mice administered

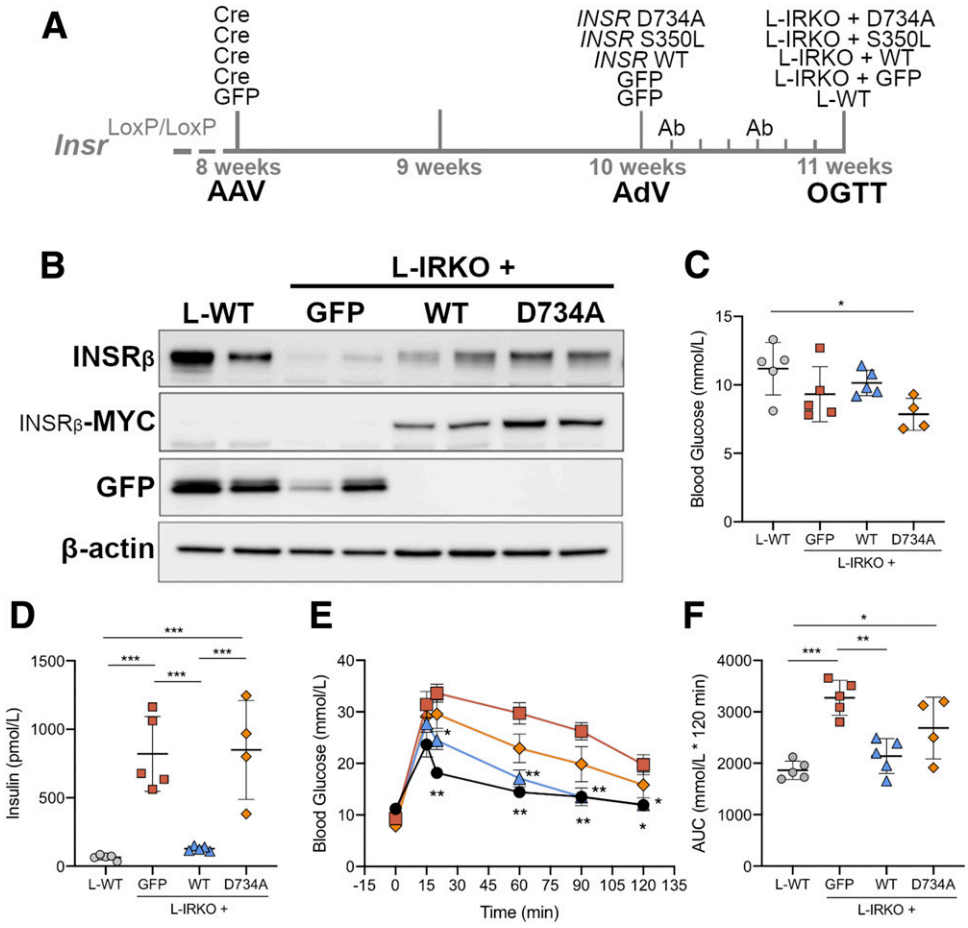

Figure 1—Hyperglycemia and IR due to liver insulin receptor deletion are rescued by WT but not mutant human INSR. *A*: Schematic representation of the generation of the insulin receptoropathy model. *B*: Western blot of liver lysates from mice on completion of OGTT and probed for insulin receptor β subunit ($INSR_β$), MYC, GFP, or β-actin. *C* and *D*: Blood glucose (*C*) and insulin (*D*) concentrations in mice after 5 h fasting. *E*: Results of OGTT (2 g/kg glucose) after 5 h fasting, L-IRKO + GFP (squares), L-IRKO + D734A (diamonds), L-IRKO + WT (upward triangles), L-WT (circles). *F*: OGTT AUC. L-WT mice = AAV-GFP/AdV-GFP (i.e., GFP control only), L-IRKO + GFP mice = AAV-iCre/AdV-GFP (i.e., liver *Insr* knockout only), L-IRKO + WT = AAV-iCre/AdV-Hs*INSR*-WT-myc (i.e., L-IRKO with WT INSR add back), L-IRKO + D734A = AAV-iCre/AdV-Hs*INSR*-D734A-myc (i.e., L-IRKO with D734A INSR add back). Data in *C*, *D*, and *F* are shown as mean ± SD, with statistical significance tested by one-way ANOVA with Tukey multiple comparison test. $n = 5$ per group, except L-IRKO + D734A ($n = 4$). Data in *E* are mean ± SEM, with statistical significance of difference from L-IRKO + GFP tested by two-way repeated measures ANOVA with Tukey multiple comparisons test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Ab, antibody.

AAV-Cre, expression of GFP in mice administered the control virus, and expression of myc-tagged INSR in mice administered the AdV encoding human *INSR* transgenes (Fig. 1*B*). Blood glucose concentrations following 5 h fasting were the same across all groups except L-IRKO + D734A, which demonstrated decreased fasting blood glucose (Fig. 1*C*), presumably due to the action of increased insulin concentration on unaffected peripheral tissues (Fig. 1*D*). L-IRKO + GFP mice demonstrated a marked increase of fasting blood insulin concentration compared with L-WT mice ($P < 0.001$), and this was rescued upon expression of human WT INSR in the liver ($P < 0.001$) (L-IRKO + WT) (Fig. 1*D*). Likewise, mice expressing the INSR D734A mutant demonstrated significant elevation of blood insulin concentration compared with L-WT and L-IRKO + WT (both $P < 0.001$) (Fig. 1*D*). L-IRKO + GFP mice were more glucose intolerant than L-WT mice (Fig. 1*E*) with increased glucose excursion during 120 min OGTT, assessed as the AUC (Fig. 1*F*). Add back of human WT INSR but not mutant D734A INSR restored glucose tolerance (Fig. 1*F*). These findings confirmed that an add-back model of human insulin receptoropathy was capable of discriminating clearly between WT and mutant INSR.

Antibody Treatment Downregulates WT Human INSR Expression With a Minimal Effect on Glucose Homeostasis

The primary aim of this study was to assess whether dysfunctional, mutant INSR can be activated by anti-INSR antibodies. However, such bivalent antibodies can also bind and activate WT human INSR (16). Naturally occurring polyclonal anti-INSR antibodies induce hypoglycemia at low titers and extreme IR at high titers in humans (21).

On the other hand, monoclonal human anti-INSR antibodies have been suggested as a therapeutic strategy in common forms of diabetes, without evaluation of effects on receptor expression (11–14). Understanding the balance between surrogate agonism and receptor downregulation is likely to be a critical consideration in the development of antibody therapeutics for receptoropathy. The effects of anti-INSR antibodies were thus first assessed in mice with WT INSR added back. Antibody was administered at a dose of 10 mg/kg, delivered by intraperitoneal injection at 1 and 4 days after adenoviral injection, before metabolic evaluation 7 days after adenoviral injection (Fig. 1*A*). The antibody dose and treatment schedule were based on previous studies of agonistic INSR antibodies (11–13). Control antibody-treated animals demonstrated a similar pattern of glucose tolerance and circulating insulin concentrations to those in initial characterization studies (Supplementary Fig. 3). Anti-INSR antibodies caused significant ($P < 0.01$) downregulation of myc-tagged INSR protein expression (Fig. 2*A* and *B*) with no change in mRNA expression of human *INSR* transgene (Fig. 2*C*) among treatment groups, indicating that the decrease in myc-tagged INSR protein levels was not due to failed liver transduction with the transgene. No further decrease in the very low level of residual endogenous *Insr* was seen (Fig. 2*D*). Treatment of L-IRKO + WT mice with anti-INSR antibodies did not alter glucose tolerance (Fig. 2*E* and *F*), but fasting blood glucose concentration was mildly decreased in 83-14–treated mice compared with control and 83-7–treated mice ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 2*G*). Fasting blood insulin concentration was unaffected by either antibody treatment (Fig. 2*H*).

As the anti-INSR antibodies used do not bind mouse Insr, off-target metabolic effects were not anticipated. To confirm this, anti-INSR antibodies were administered to L-WT mice (Supplementary Fig. 4). As expected, no effect on endogenous liver Insr protein expression (Supplementary Fig. 4*A* and *B*), mRNA expression (Supplementary Fig. 4*C*), glucose tolerance (Supplementary Fig. 4*D* and *E*), fasting blood glucose concentration (Supplementary Fig. 4*F*), or fasting blood insulin concentration (Supplementary Fig. 4*G*) was seen. L-IRKO + GFP mice, with severely reduced liver Insr expression (Supplementary Fig. 4*A*, *H*, and *I*), also showed no change in any metabolic assessment (Supplementary Fig. 4*J*–*M*). Furthermore, insulin signaling in other tissues was unaffected by either AAV/AdV administration or antibody treatment (Supplementary Fig. 5).

Antibody Treatment Improves Glucose Tolerance and Hyperinsulinemia in Receptoropathy Models but Downregulates INSR Protein Expression

In L-IRKO + GFP mice with add back of INSR D734A, treatment with 83-7 and 83-14 antibodies downregulated myc-tagged INSR protein levels compared with control-treated animals ($P < 0.0001$) (Fig. 3*A* and *B*). This was not accompanied by any change in either human *INSR* transgene mRNA (Fig. 3*C*) or endogenous mouse *Insr* mRNA (Fig. 3*D*). Despite this, treatment with 83-7 and 83-14 did improve glucose tolerance (Fig. 3*E* and *F*). This was not accompanied by any change in fasting blood glucose concentrations (Fig. 3*G*). However, antibody 83-14 significantly ($P < 0.05$) reduced fasting insulin concentrations in L-IRKO + D734A animals (Fig. 3*H*).

In L-IRKO + GFP mice with add back of S350L mutant human INSR, treatment with 83-7 and 83-14 also reduced myc-tagged INSR protein levels (Fig. 4*A* and *B*). As in L-IRKO + WT and L-IRKO + D734A mice, this was not due to failed liver transduction with human *INSR*, as quantitative PCR demonstrated stable human *INSR* transgene mRNA across all treatment conditions (Fig. 4*C*) and effective deletion of endogenous mouse *Insr* (Fig. 4*D*). Animals treated with 83-7 and 83-14 showed only a trend to improved glucose tolerance (Fig. 4*E* and *F*), and neither antibody lowered fasting blood glucose concentrations (Fig. 4*G*). Treatment of L-IRKO + S350L mice with anti-INSR antibody 83-7 did reduce fasting blood insulin concentration compared with control and 83-14–treated animals (both $P < 0.05$), indirectly demonstrating hypoglycemic action of antibody (Fig. 4*H*). Collectively these findings demonstrate that anti-INSR monoclonal antibodies improve glucose tolerance and reduce fasting hyperinsulinemia in mice expressing human INSR mutations that cause recessive insulin receptoropathy. They also show that the magnitude of the improvement seen is likely attenuated by downregulation of INSR expression.

DISCUSSION

Extreme congenital IR was first clinically described as Donohue syndrome, and the less severe RMS, decades before the insulin receptor was identified, and thus long before the genetic cause, namely biallelic *INSR* mutations, was established (28,29). Both syndromes feature extreme metabolic derangement, characterized by high blood glucose concentration that is unresponsive or minimally responsive to insulin therapy. They also feature severely impaired linear growth and underdevelopment of insulin-responsive tissues, such as skeletal muscle and adipose tissue. Less intuitively, marked overgrowth of other tissues and organs, including skin, kidneys, liver, gonads, and colonic mucosa, is also seen and may pose clinical challenges (2). Overgrowth is thought to be driven by compensatory elevation of blood insulin concentration, which can act on the trophic IGF-1 receptor, which is structurally similar to the INSR (2).

The clinical course of recessive insulin receptoropathy is bleak, with death common between infancy, at which stage it often occurs during viral infection, and early adolescence, when it is more likely due to complications of uncontrolled diabetes, such as ketoacidosis or microvascular damage. Pharmacotherapy relies on case reports and case series only and commonly includes insulin-sensitizing drugs, such as metformin, and high-dose insulin. In the

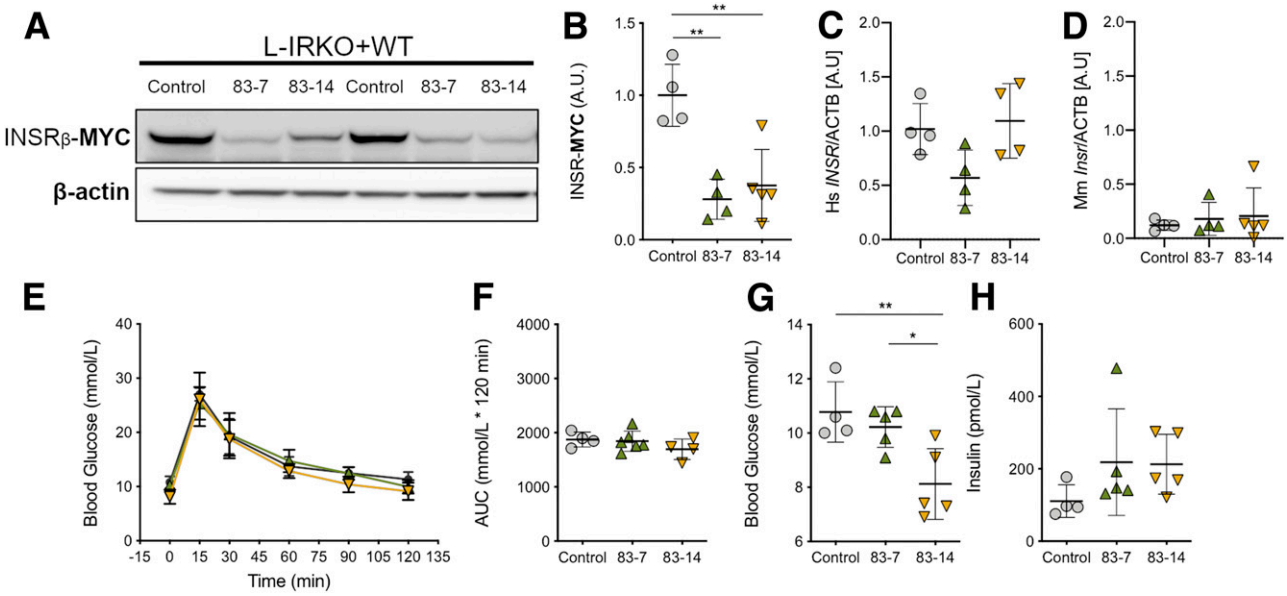

Figure 2—Antibody treatment downregulates WT human INSR expression in mouse liver with minimal effect on glucose homeostasis. L-IRKO + WT mice were dosed twice over 1 week with 10 mg/kg control ($n = 4$) or anti-INSR antibodies 83-7 ($n = 5$) or 83-14 ($n = 5$) as indicated. *A*: Western blot of liver lysates from L-IRKO + WT mice at the completion of OGTT, probing for MYC-tagged β subunit or β-actin as indicated. *B–D*: Quantification of Myc-tagged human INSR protein (*B*), human *INSR* mRNA (*C*), and endogenous *Insr* mRNA (*D*) in livers from the same experiment. mRNA was quantified by quantitative PCR. *E*: OGTT (2 g glucose/kg) after a 5-h fast in antibody-treated L-IRKO + WT mice. *F*: AUC for blood glucose during OGTT in antibody-treated L-IRKO + WT mice. *G*: Blood glucose concentrations in antibody-treated L-IRKO + WT mice after a 5-h fast. *H*: Insulin concentrations in antibody-treated L-IRKO + WT mice after a 5-h fast. All data (except *E*) are shown as mean ± SD, with statistical significance tested by one-way ANOVA with Tukey multiple comparison test. $^*P < 0.05$, $^{**}P < 0.01$. Data in *E* are mean ± SEM. Circles are control antibody. Upward triangles are 83-7 antibody. Downward triangles are 83-14 antibody. Lack of statistical significance was determined by two-way repeated measures ANOVA with Tukey multiple comparisons test.

most severe cases, recombinant human IGF-1 is often used based on reports of its acute hypoglycemic effects in Donohue syndrome and on some evidence that it may improve longevity in recessive receptoropathy (3). Nevertheless, the lack of placebo-controlled studies, the likelihood of reporting bias in the existing case literature, and the underlying variability in the natural history of recessive receptoropathies are all reasons for caution. Furthermore, tissue overgrowth, for example, of liver, kidneys, heart, skin, and ovaries, is a prominent feature of severe receptoropathy, and it is most likely mediated by IGF-1 receptors, which can be stimulated by high insulin concentrations. There is, thus, a major unmet therapeutic need for novel insulin-mimetic agents, ideally with no action on the IGF-1 receptor. Genetic considerations suggest that only a small degree of activation of nonfunctional receptors may be required to achieve major clinical benefits: Donohue syndrome is caused by complete or near complete loss of receptor function, while RMS, with a better prognosis, features around 10–20% receptor function. Autosomal-dominant insulin receptoropathy, which usually presents only around puberty, features no more than 25% receptor function, while the lack of one INSR allele (50% function) has not been associated with IR. This suggests a steep relationship between INSR function and prognosis between 0 and 25% receptor function.

We previously demonstrated the ability of bivalent, specific anti-INSR antibodies to act as surrogate ligands on a series of mutant INSR in cell culture models (20), and we now report their evaluation in vivo in a novel mouse model of human insulin receptoropathy. The humanized mouse model of insulin receptoropathy was generated by using sequential viral infection to knockout endogenous *Insr* and, then, to re-express human *INSR*. This enabled changes in metabolic outcomes upon antibody treatment to be attributed to action on re-expressed human mutant INSR as the monoclonal anti-INSR antibodies tested do not bind rodent Insr (16). Use of a viral strategy made liver the most tractable organ to target and also had the benefit that liver parenchyma is particularly accessible to the antibody due to the fenestration of hepatic capillaries. This approach also avoided the compensatory responses reported in congenital liver Insr deficiency (22), while offering flexibility to study various mutant human INSR transgenes without generating distinct genetically modified mouse lines. On the other hand, technical success relies on efficient administration of viral vectors by skilled operators, and AdV vectors limit the duration of transgene expression, constraining the time window for study.

Encouragingly, both monoclonal anti-INSR antibodies tested (83-7 and 83-14) did improve glucose tolerance in L-IRKO + D734A mice, while 83-14 treatment also lowered

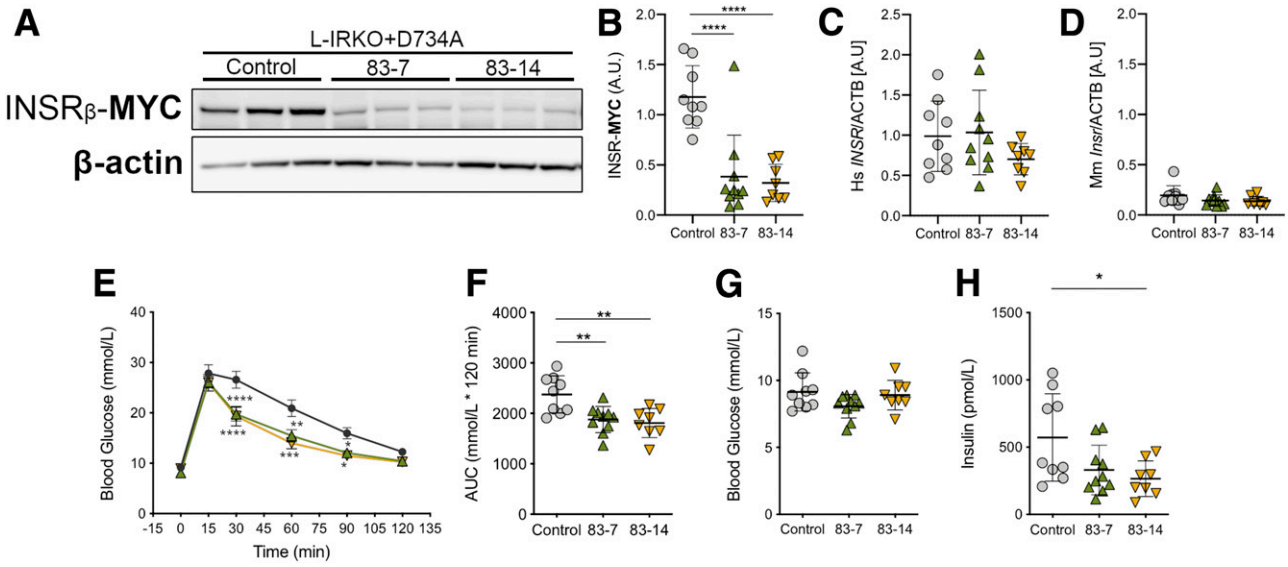

Figure 3—Antibody treatment improves glucose tolerance and hyperinsulinemia in INSR D734A add-back mice but downregulates INSR protein expression. L-IRKO + D734A mice were treated twice over 1 week with 10 mg/kg control ($n = 9$) or anti-INSR antibodies 83-7 ($n = 10$) or 83-14 ($n = 8$). *A*: Western blot of liver lysates from L-IRKO + D734A mice at completion of OGTT, probed for the proteins as indicated. *B–D*: Quantification of Myc-tagged human INSR protein (*B*), human *INSR* mRNA (*C*), and endogenous *Insr* mRNA (*D*) in livers from the same experiment. mRNA was quantified by quantitative PCR. *E*: OGTT (2 g glucose/kg) after a 5-h fast in antibody-treated L-IRKO + D734A mice. *F*: AUC for blood glucose during OGTT in antibody-treated L-IRKO + D734A mice. *G*: Blood glucose concentrations in antibody-treated L-IRKO + D734A mice after a 5-h fast. *H*: Insulin concentrations in antibody-treated L-IRKO + D734A mice after a 5-h fast. All data (except *E*) are shown as mean ± SD, with statistical significance tested by one-way ANOVA with Tukey multiple comparison test. Data in *E* are mean ± SEM. Circles are control antibody. Upward triangles are 83-7 antibody. Downward triangles are 83-14 antibody. Statistical significance was tested by two-way repeated measures ANOVA with Tukey multiple comparisons test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

fasting blood insulin concentration in these mice (Fig. 3*H*). Also, 83-7 lowered fasting blood insulin concentration in L-IRKO + S350L mice (Fig. 4*H*). Collectively, these observations demonstrate that anti-INSR antibodies can improve glucose tolerance and reduce fasting hyperinsulinemia in mice expressing human INSR that cause severe disease in humans, adding to evidence that antibody-based surrogate agonism may be of metabolic benefit in vivo. The effects observed in this acute receptoropathy model were modest and not fully consistent between mutants, antibodies, or indices of IR. However, several factors may have adversely affected the potential for antibodies to ameliorate the condition. First, overexpression of human INSR added back may have attenuated the degree of IR that mutants confered compared with humans with endogenous expression of the same mutations, reducing the dynamic range of IR of the model. This may explain the relatively mild IR seen with S350L at baseline (Fig. 4), despite this mutation being found to cause RMS in several unrelated families. While future calibration of the viral models described against mice with endogenous expression of mutant receptors would be of great interest, the need to study human INSR rather than mouse Insr makes this a challenging technical proposition.

A second potential reason why the metabolic effects of antibodies were not larger has more profound implications for INSR surrogate agonist-based strategies for treating IR. Antibody treatment downregulated receptor expression across all INSR species studied, as expected from the known coupling of receptor activation to internalization. Following internalization by endocytosis, receptors are trafficked through the endosomal/lysosomal pathway and either recycled to the cell surface in the unliganded state or degraded (30). The mechanisms governing internalization, trafficking, and the balance of subsequent recycling and degradation in response to stimulation are poorly understood, but the potential importance of this in the context of anti-INSR antibodies is known from studies of type B IR (21). This is a naturally occurring, acquired form of insulin receptoropathy driven by anti-INSR antibodies. It is well known that low titers of such antibodies can produce clinically important hypoglycemia, but that when antibody titers rise, severe receptor desensitization and fulminant IR occurs that may be life threatening (21). This harmful effect of high antibody levels will likely narrow the therapeutic window for agonistic anti-INSR antibodies in recessive receptoropathy unless ways of uncoupling partial agonist and receptor desensitizing effects are devised, perhaps by selectively modulating receptor recycling and degradation rates. Interestingly, studies in the 1980s suggested that lysosomes may not be critical for receptor desensitization (31), suggesting that other processes, such as proteasomal degradation, warrant study in this context.

In summary, we report a novel approach to modeling recessive human insulin receptor defects in the mouse using sequential virally mediated knockout of endogenous

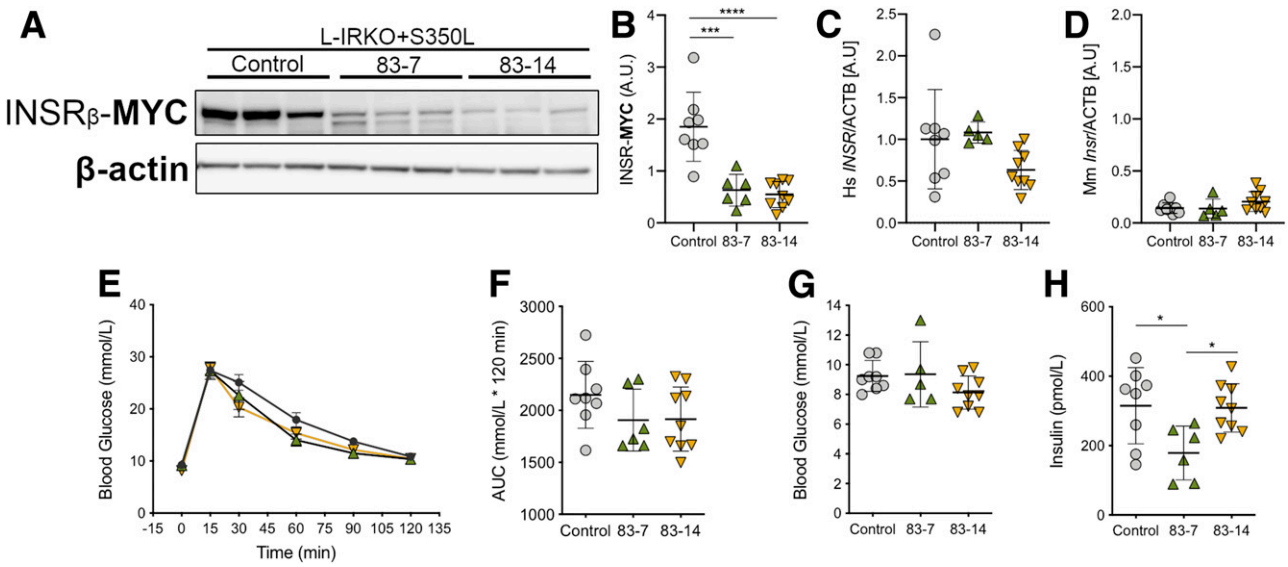

Figure 4—Antibody treatment reduces fasting hyperinsulinemia in INSR S350L add-back mice but downregulates INSR protein expression. L-IRKO + S350L mice were treated twice over 1 week with 10 mg/kg control ($n = 8$) or anti-INSR antibodies 83-7 ($n = 6$) or 83-14 ($n = 9$). *A*: Western blot of liver lysates from mice at completion of OGTT were probed for the proteins indicated. *B–D*: Quantification of Myc-tagged human INSR protein (*B*), human *INSR* mRNA (*C*), and endogenous *Insr* mRNA (*D*) in livers from the same experiment. mRNA was quantified by quantitative PCR. *E*: OGTT (2 g glucose/kg) after a 5-h fast in antibody-treated L-IRKO + S350L mice. *F*: AUC for blood glucose during OGTT in antibody-treated L-IRKO + S350L mice. *G*: Blood glucose concentrations in antibody-treated L-IRKO + S350L mice after a 5-h fast. *H*: Insulin concentrations in antibody-treated L-IRKO + S350Lmice after a 5-h fast. All data (except *E*) are shown as mean ± SD, with statistical significance tested by one-way ANOVA with Tukey multiple comparison test. Data in *E* are mean ± SEM. Circles are control antibody. Upward triangles are 83-7 antibody. Downward triangles are 83-14 antibody. Lack of statistical significance was determined by two-way repeated measures ANOVA with Tukey multiple comparisons test. $^*P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

and re-expression of human insulin receptor. This yielded mice with acute IR due to two previously studied INSR mutations that have been shown in cell models to exhibit activation by anti-INSR antibodies. Injection of well-characterized monoclonal anti-INSR antibodies improved IR in both models; however, the magnitude of the effect is likely to have been limited by downregulation of receptor. Our findings confirm the potential utility of surrogate agonist strategies for treating lethal insulin receptoropathy but caution that receptor downregulation may attenuate the benefits realized unless this can concomitantly be reduced.

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