Muscle-Specific Insulin Receptor Overexpression Protects Mice From Diet-Induced Glucose Intolerance but Leads to Postreceptor Insulin Resistance

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Skeletal muscle insulin resistance is a prominent early feature in the pathogenesis of type 2 diabetes. In attempt to overcome this defect, we generated mice overexpressing insulin receptors (IR) specifically in skeletal muscle (IRMOE). On normal chow, IRMOE mice have body weight similar to that of controls but an increase in lean mass and glycolytic muscle fibers and reduced fat mass. IRMOE mice also show higher basal phosphorylation of IR, IRS-1, and Akt in muscle and improved glucose tolerance compared with controls. When challenged with high-fat diet (HFD), IRMOE mice are protected from diet-induced obesity. This is associated with reduced inflammation in fat and liver, improved glucose tolerance, and improved systemic insulin sensitivity. Surprisingly, however, in both chow and HFD-fed mice, insulin-stimulated Akt phosphorylation is significantly reduced in muscle of IRMOE mice, indicating postreceptor insulin resistance. RNA sequencing reveals downregulation of several postreceptor signaling proteins that contribute to this resistance. Thus, enhancing early insulin signaling in muscle by overexpression of the IR protects mice from diet-induced obesity and its effects on glucose metabolism. However, chronic overstimulation of this pathway leads to postreceptor desensitization, indicating the critical balance between normal signaling and hyperstimulation of the insulin signaling pathway.

In humans, skeletal muscle is the major tissue responsible for insulin-stimulated glucose disposal (1). In individuals

with high genetic risk for type 2 diabetes, skeletal muscle insulin resistance is the earliest detectable defect, often preceding and predicting development of disease by many years (2,3). While muscle-specific knockout of the insulin receptor (IR) alone produces only mild changes in glucose tolerance (4), knockout of IR in muscle exacerbates glucose intolerance in mice with whole-body haploinsufficiency of IR, indicating the important role of insulin signaling in muscle in maintaining glucose and lipid homeostasis (4). Insulin signaling through its receptor also regulates muscle growth and protein turnover (5), increases muscle protein synthesis (6), and inhibits autophagy-mediated protein degradation (7). Thus, mice with muscle-specific IR deletion display reduced muscle weight and grip strength compared with littermate controls, and this is worsened when the IGF1R, which can also stimulate some of the same pathways, is concomitantly knocked out (8,9).

At the molecular level, IR signaling is mediated through two signaling cascades: one begins with tyrosine phosphorylation of insulin receptor substrates (IRS-1 to -4) and activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. The other is mediated by Shc (Src homology and collagen domain protein) and Grb2, which activate the MAPK pathway. The former plays a major role in regulation of metabolism, while the latter largely controls cell growth and proliferation (10).

In obese humans (11) and mice with genetic obesity or subjected to high-fat diet (HFD) feeding, IR expression is

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significantly downregulated in multiple tissues, including muscle (12,13), contributing to the insulin resistance in these animals. Conversely, combined overexpression of IR in muscle, brain, and pancreas has been shown to alleviate obesity and diabetic phenotypes in *db/db* mice (14). However, the specific role of IR overexpression in muscle and its contribution to the overall metabolic phenotype remains unclear.

In the current study, we have generated mice that specifically overexpress IR in skeletal muscle (IRMOE) and investigated its effects on metabolic homeostasis. We find that IRMOE mice show improved glucose tolerance compared with littermate controls even on chow diet. More striking is the finding that when challenged with HFD, IRMOE mice show dramatic improvements in glucose tolerance, fatty liver disease, and systemic insulin sensitivity. Thus, muscle-specific IR overexpression can protect mice from diet-induced obesity and its effects on glucose metabolism. At the molecular level, overexpression of IR in muscle leads to increased basal phosphorylation of IR, IRS, and Akt; however, this leads to impaired insulin-stimulated Akt phosphorylation, indicating a significant level of postreceptor insulin resistance. Since most patients with type 2 diabetes go through a hyperinsulinemic phase prior to development of hyperglycemia, identifying the postreceptor regulators of insulin signaling involved in this desensitization could provide new targets for therapy of type 2 diabetes and other insulin-resistant states.

RESEARCH DESIGN AND METHODS

Animal Care and Use

Animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee. Male mice were used for studies unless indicated. Mice transgenic for loxP-stop-loxP insulin receptor (LSL-IR) were generated through pronuclear injection of a linearized cassette carrying mouse IR isoform A cDNA driven by CAG promoter and interrupted by a floxed β -gal cDNA and a stop codon. IRMOE mice were then generated by crossing of the LSL-IR mice with mice carrying a Cre transgene driven by the human skeletal muscle actin (HSA) promoter. (HSA-Cre is also called *Acta1-Cre* [stock 006149; The Jackson Laboratory]). All mice were on C57BL/6 background and were housed at 22°C on a 12-h light/12-h dark cycle with ad libitum access to food and water. Chow diet was Mouse Diet 9F (PharmaServ), and HFD was Research Diets D12492. The caloric composition of the former was 23% protein, 21.6% fat, and 55.4% carbohydrates, while the latter contained 20% protein, 60% fat, and 20% carbohydrates. In vivo metabolic parameters were measured using Comprehensive Lab Animal Monitoring System (CLAMS), performed by Joslin Diabetes Center Animal Physiology Core, as previously described (15).

Hyperinsulinemic-Euglycemic Clamp

Surgery was performed at 5–6 days before clamp experiments to establish an indwelling catheter in jugular vein. On the day of the clamp experiment, mice were fasted overnight (~15 h), and a 2-h hyperinsulinemic-euglycemic

clamp was conducted in awake mice with a primed and continuous infusion of human insulin (150 mU/kg body wt priming dose followed by 2.5 mU/kg/min, Novolin; Novo Nordisk, Plainsboro, NJ) (16). For maintenance of euglycemia, 20% glucose was infused at variable rates during clamps. Whole-body glucose turnover was assessed using a continuous infusion of [$3\text{-}^3\text{H}$]glucose (PerkinElmer, Waltham, MA), and 2-deoxy-D-[1- ^{14}C]glucose (2-[^{14}C]DG) was administered as a bolus (10 μCi) at 75 min after the start of clamps for measurement of insulin-stimulated glucose uptake in skeletal muscle and white adipose tissue. At the end of the clamp, mice were anesthetized and tissues were taken for biochemical analysis (16). Glucose concentrations during clamps were analyzed using 10 μL plasma by a glucose oxidase method on Analox GM9 Analyzer (Analox Instruments, Hammer-smith, London, U.K.). Plasma concentrations of [$3\text{-}^3\text{H}$]glucose, 2-[^{14}C]DG, and $^3\text{H}_2\text{O}$ were determined following deproteinization of plasma samples as previously described (16). Whole-body glucose turnover, glycolysis, and hepatic insulin action were calculated as previously described (16). For the determination of tissue 2-[^{14}C]DG-6-phosphate (2-[^{14}C]DG-6-P) content, tissue samples were homogenized, and the supernatants were subjected to an ion-exchange column for separation of 2-[^{14}C]DG-6-P from 2-[^{14}C]DG. Insulin-stimulated glucose uptake in individual tissues was assessed by determination of the tissue content of 2-[^{14}C]DG-6-P and plasma 2-[^{14}C]DG profile.

Ex Vivo Muscle Glucose Uptake

Glucose uptake was measured in isolated extensor digitorum longus (EDL) and soleus muscles ex vivo as previously described (17). Briefly, mice were fasted starting at 2100 h, and muscle was harvested the next day between 1000 and 1200 h. Isolated EDL and soleus strips were incubated in Krebs-Ringer bicarbonate buffer (KRB) containing 2 mmol/L pyruvate at 37°C at resting tension in the basal state or after stimulation with 1 mU/mL insulin (Humulin R; Lilly) for 60 min. For measurement of 2-deoxyglucose uptake, muscles were transferred to 2 mL KRB containing 1 mmol/L 2-deoxyglucose and 2-deoxy-D-[1,2- ^3H]glucose (1.5 $\mu\text{Ci}/\text{mL}$), 1 mmol/L D-mannitol, and D-[^{14}C]-mannitol (0.45 mCi/mL) at 30°C for 10 min. Muscles were continuously gassed with 95%:5% O_2 : CO_2 throughout the study. Transport was stopped by washing of the muscle in KRB at 4°C, and the muscle was blotted, trimmed, and frozen in liquid N_2 and then stored at -80°C . Muscles were weighed and processed by incubation in 250 μL of 1 N NaOH at 80°C for 10 min. Digestates were neutralized with 250 μL of 1 N HCl and centrifuged at 13,000g for 2 min for precipitation of particulates. Radioactivity in aliquots of the digested muscle was determined by liquid scintillation counting for the dual labels, and the extracellular and intracellular spaces were calculated for determination of transport activity.

Treadmill Running Capacity

We measured treadmill running capacity following a previously published protocol (18). In brief, mice were given

30 min to acclimate to the treadmill (Columbus Instruments, Columbus, OH). They then ran at 5 m/min at 0° incline for 5 min, and every 5 min the speed was increased by 5 m/min until 20 m/min was reached. Once maximum speed was reached, the slope was increased every 5 min by 5° until the mouse reached exhaustion.

Glucose and Insulin Tolerance Test

For glucose tolerance testing, mice were fasted overnight and intraperitoneally (i.p.) injected with 2 g glucose/kg body mass (chow) or 1.6 g glucose/kg body mass (HFD). For insulin tolerance testing, mice were fasted for 3 h and injected i.p. with 1.5 units insulin/kg body mass (chow) or 2 units insulin/kg body mass (HFD). Blood glucose levels were measured at 0, 15, 30, 60, and 120 min with an INFINITY glucose meter (US Diagnostics).

Glucose-Stimulated Insulin Secretion

For glucose-stimulated insulin secretion (GSIS), following an overnight fast mice were injected with 3 g/kg body wt glucose (20% dextrose) i.p. Blood (30 μ L) was drawn from a nick in the tail before and 2, 5, and 10 min after injection for analysis of glucose (by glucometer) and insulin levels (by ELISA). Mice fasting for GSIS had free access to water at all times.

β -Cell Staining

Unstained slides of pancreas were deparafinized and hydrated as follows: 7 min Xylene (twice), 5 min 100% ethanol (twice), 3 min 95% ethanol (twice), and 10 min 70% ethanol. Slides were washed twice in PBS (2 $\times$ 5 min), followed by 10 min permeabilization in 0.2% Triton X-100, and blocked in 1% BSA plus 3% goat serum for 1 h. Slides were then incubated with insulin antibody (ab7842) (1:50 dilution in blocking buffer) overnight at 4°C. The next day, slides were washed in PBS (4 $\times$ 5 min), blocked again, and incubated with goat anti-guinea pig Alexa Fluor 594 antibody (1:500) for 1 h at room temperature, followed by PBS wash again (4 $\times$ 5 min). Slides were mounted in VECTASHIELD H-1000 with coverslip.

Muscle Histology and Fiber Type Staining

Cross sections of frozen soleus muscle were used for hematoxylin-eosin (H-E) staining (Harvard Histology Core), and muscle fiber size was quantified semimanually with use of ImageJ (National Institutes of Health). Cross sections of frozen soleus muscles were also immunofluorescently costained for myosin IIa (sc-71-c; Developmental Studies Hybridoma Bank) and myosin I (BA-F8; Developmental Studies Hybridoma Bank), using the protocol previously described (15).

Succinate Dehydrogenase Activity

Cross sections of tibialis anterior (TA) muscles (middle belly) were used for succinate dehydrogenase activity staining. Briefly, frozen sections were washed in cold PBS for 5 min and then incubated in substrate solution (0.5 mol/L disodium succinate; 20 mmol/L MgCl_2 ; 0.5 $\times$ PBS, pH 7.6; and 0.5 mg/mL Nitro Blue Tetrazolium added

prior to staining) for 10–30 min depending on intensity of purple staining on tissues. All samples were incubated for the same duration and then rinsed with cold PBS, mounted in VECTASHIELD antifade mounting media, and then imaged under brightfield microscopy.

Muscle Glycogen Analysis

Whole EDL muscle was weighed and hydrolyzed in 0.25 mL of 2 mol/L HCl by heating at 95°C for 2 h (invert every 30 min). The solution was then neutralized with 0.25 mL of 2 mol/L NaOH and 10 μ L of 1 mol/L Tris (pH 7.4). The resulting free glycosyl units were assayed spectrophotometrically with a hexokinase-dependent assay kit (cat. no. TR15421; Thermo Fisher Scientific).

RNA Sequencing Analysis

Mouse genome (GRCm38.p6) and gene annotation files were downloaded from GENCODE (19). We aligned RNA sequencing reads to the reference genome by using STAR (20) and counted them with Subread featureCounts (21). Normalization factors were obtained by use of the weighted trimmed mean of M-values method (22). Read counts were transformed to log₂-counts per million, their mean-variance relationship was estimated, and their observational-level weights were computed with voom (23). Differential gene expression was assessed by use of linear modeling with limma (24). *P* values were corrected using the Benjamini-Hochberg false discovery rate (FDR), and FDR < 0.25 was considered statistically significant (25). The volcano plot was made by use of ggplot2 (26) and heat map by pheatmap (27). Gene sets based on canonical pathways were downloaded from the Molecular Signatures Database (MSigDB) (28), and the gene set enrichment was tested by use of the limma roast method (29).

RNA Extraction and Quantitative PCR Analysis

mRNA was extracted by homogenizing tissues in TRIzol, treating with chloroform, and precipitating in 70% ethanol. cDNA was made using High Capacity cDNA Reverse Transcription Kit (catalog 4368813; Applied Biosystems). Quantitative PCR was performed utilizing C1000 Thermal Cycler (catalog CFX384; BioRad). Primer sequences used are listed in Supplementary Table 2.

Protein Extraction, Immunoblot, and Immunoprecipitation

For immunoblots, tissues were homogenized in radioimmunoprecipitation assay buffer (EMD Millipore) with protease and phosphatase inhibitor cocktail (BioTools). Proteins were separated by use of SDS-PAGE and transferred to polyvinylidene fluoride membrane (EMD Millipore). Immunoblotting was achieved through using the indicated antibodies: phosphorylated (phospho)-IR (cat. no. 3024; Cell Signaling Technology [CST]), IR (sc-711), phospho-IRS-1 Tyr⁶⁰⁸ (09-432; Millipore), IRS-1 (611394; BD Bioscience), phospho-Akt Ser⁴⁷³ (4060; CST), phospho-Akt Thr³⁰⁸ (4056; CST), Akt (4685; CST), phospho-Shc Tyr^{239/240} (2434; CST), phospho-Erk Thr²⁰²/Thr²⁰⁴ (4370;

CST), Erk (9102; CST), IGF1R β (9750; CST), IGFBP5 (AF578), ULK1 (8054; CST), PKC δ (sc-937), TBC1D1 (4629; CST), VLDLR (sc-18824), LPL (sc-373759), tubulin (2146; CST), LC3A/B (12741; CST), phospho-mTOR (Ser²⁴⁴⁸) (2971; CST), mTOR (2972; CST), p85 (4292; CST), phospho-p85 (4228; CST), phospho-S6 (2211; CST), and S6 (2317; CST). We performed quantification of immunoblots using ImageJ.

Statistical Analyses

All data are presented as mean $\pm$ SD. Student *t* test was performed for comparison of two groups, and ANOVA was performed for comparison of three or more groups for determination of significance.

Data and Resource Availability

The resources generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request. The RNA sequencing data generated and/or analyzed during the current study are deposited in National Center for Biotechnology Information's Gene Expression Omnibus (GEO) under accession number GSE149662.

RESULTS

Muscle-Specific IR Overexpression Changes Body Composition of the Mice

To create mice with muscle-specific IR overexpression (IRMOE), we generated a construct containing the cDNA of mouse IR (isoform A) driven by the CAG promoter but in which the IR coding sequence and the promoter were interrupted by β -gal cDNA and a stop codon flanked by *loxP* sites (Fig. 1A and Supplementary Fig. 1A). The linearized construct was released from the plasmid backbone by digestion with SbfI and SmaI (Supplementary Fig. 1A), and a transgenic mouse line (LSL-IR) harboring this cassette was created through pronuclear injection. IRMOE mice were then generated by breeding of LSL-IR mice with mice that express a Cre recombinase transgene under the control of HSA promoter (30) (Fig. 1A). The LSL-IR transgenic mice and HSA-Cre-only littermates were used as controls.

Compared with controls, IRMOE mice had 6- to 10-fold increases in IR expression in skeletal muscle at both the mRNA (Fig. 1B) and protein (Fig. 1C) levels. This was true in muscles of different fiber type composition including quadriceps, TA, EDL, and soleus. Consistent with the specificity of the HSA promoter (9,30), there were no significant differences in IR mRNA or protein levels between control and IRMOE mice in heart or other tissues (Supplementary Fig. 1B and C and data not shown). On chow diet, there was no difference in body weight between control and IRMOE mice for at least the first 5 months of life (Fig. 1D). However, DEXA imaging revealed that overexpression of IR in muscle resulted in a significant increase in percent lean body mass (Fig. 1E) with a reciprocal significant decrease in percent fat mass (Fig. 1F). Consistent with this, direct

assessment of tissue weights of 5-month-old male mice at the time of sacrifice showed that IRMOE mice had significant increases in weight of most muscles, with the greatest effects on EDL, soleus, and TA muscles, which had 10–30% increases compared with controls (Fig. 1G and H). There was also a trend toward increase in the weight of the quadriceps. This increase in muscle mass was accompanied by a significant decrease in weight of the epididymal (eWAT) and subcutaneous white adipose tissue but no change in weight of the interscapular brown fat pad (Fig. 1I). Histological examination of the soleus muscle revealed that the increased muscle weight was associated with a 30% increase in muscle fiber size (Fig. 1J and K). As a result of the increased muscle mass, IRMOE mice showed significantly increased grip strength (Fig. 1L). However, there was no change in capacity for treadmill running (Fig. 1M).

IR Overexpression in Muscle Improves Energy Expenditure and Glucose Tolerance and Makes Muscle More Glycolytic

Although IRMOE mice had no difference in body weight compared with controls when on normal chow diet (Fig. 1D), they had a 20% increase in food consumption compared with their littermate controls (Fig. 2A). This is likely due to decreased plasma leptin levels in both fed and fasted states in IRMOE mice (Fig. 2B), reflecting the decrease in adipose tissue mass. Compensating for this, IRMOE mice had a 10–15% increase in energy expenditure as measured by O₂ consumption (Fig. 2C and Supplementary Fig. 2A) and CO₂ production rates (Fig. 2D and Supplementary Fig. 2B), allowing them to maintain body weight similar to that of control littermates. The increased O₂ consumption was more apparent during the light cycle and occurred with no change in spontaneous activity (Supplementary Fig. 2C) or change in respiratory exchange ratio (RER) (Supplementary Fig. 2D). IRMOE mice also showed a trend toward lower fasting glucose levels at 2.5 months of age, and this became significant at 4 and 7 months of age (Fig. 2E). Although IRMOE mice did not differ from control mice in glucose tolerance at 2.5 months of age (Supplementary Fig. 3A), by 4 months of age, they displayed improved glucose tolerance compared with controls (Fig. 2F). This occurred with no significant change in plasma insulin levels or whole-body insulin sensitivity as assessed by i.p. insulin tolerance testing (Supplementary Fig. 3B and C). However, despite having β -cell mass and morphology similar to those of controls (Fig. 2G and Supplementary Fig. 3D), IRMOE mice displayed enhanced first-phase insulin secretion in response to glucose (Fig. 2H), indicating improved β -cell function.

Western blot analysis of insulin signaling proteins in skeletal muscle in the basal state, i.e., after a 2-h fast, revealed a 5- to 10-fold increase in IR and IR precursor proteins in IRMOE mice, and this was accompanied by a parallel increase in IR Tyr phosphorylation (Fig. 2I). There was also a twofold increase in basal phosphorylation of IRS-1 on Tyr⁶⁰⁸. Further downstream, Akt phosphorylation was increased fourfold compared with that of controls (Fig. 2I).

Figure 1—Muscle-specific IR overexpression changes mouse body composition. **A**: Schematic of the breeding strategy used to generate the IRMOE mice. **B**: IR expression measured by qPCR in quadriceps (Quad), TA, EDL, and soleus muscles of 5-month-old control (Ctl) and IRMOE mice fed a chow diet ($n = 5$). **C**: IR protein levels measured by Western blotting of soleus muscles from 5-month-old male mice ($n = 4$). **D**: Body weight of control and IRMOE mice from 2.5 to 5 months of age fed a chow diet ($n = 5$). **E**: Lean mass (as a percentage of total) measured by DEXA scan in chow-fed control and IRMOE mice ($n = 5$). **F**: Fat mass (as a percentage of total) measured by DEXA scan in chow-fed control and IRMOE mice ($n = 5$). **G** and **H**: Muscle weight, as percentage of body weight, in 5-month-old chow-fed control and IRMOE mice ($n = 5$). **I**: Fat weight as percentage of body weight in 5-month-old chow-fed control and IRMOE mice ($n = 5$). sWAT, subcutaneous white adipose tissue; BAT, brown adipose tissue. **J**: H-E staining of soleus muscle cross section in 3-month-old mice. Scale bar = 100 μ m. **K**: Quantification of muscle fiber size as shown in **J**. $n \geq 190$ fibers from three mice per group. Grip strength (**L**) and time to exhaustion in treadmill running (**M**) in 4-month-old control and IRMOE mice ($n = 5$). Data represent mean $\pm$ SD. Student t test, * $P < 0.05$.

Figure 2—IR overexpression in muscle improves energy expenditure and glucose tolerance and makes muscle more glycolytic. **A**: Cumulative food intake in control (Ctl) and IRMOE mice fed with chow diet during 48 h feeding followed by 24 h fasting (n = 5). **B**: Plasma leptin levels in control and IRMOE mice under fed and fasting conditions on chow diet at 5 months of age (n = 5–7). **C**: Area under the curve (AUC) for O_2 consumption rate in 4-month-old control and IRMOE mice fed with chow diet (n = 5). **D**: Area under the curve for CO_2 production in control and IRMOE mice fed with chow diet (n = 5). **E**: Fasting blood glucose levels of control and IRMOE mice fed a chow diet (n = 5–8). m, months old. **F**: Glucose tolerance test in 4.5-month-old control and IRMOE mice on chow diet (n = 5). **G**: β -Cell mass in 3.5-month-old Ctl and IRMOE mice on chow diet (n = 3). **H**: GSIS in 3.5-month-old female control and IRMOE mice on chow diet (n = 7). **I**: Western blot showing basal insulin signaling in quadriceps muscles from control and IRMOE mice after a 2-h fast starting at 11:00 A.M., 4.5 h into the light cycle (n = 4). **J**: SDH staining in TA muscles from 3-month-old control and IRMOE mice. Scale bar = 200 μm (n = 3). **K**: Myosin heavy-chain staining in soleus muscle cross sections from 3-month-old control and IRMOE mice. Scale bar = 100 μm (n = 3–4). **L**: Quantification of soleus muscle fiber types shown in K. Data represent mean $\pm$ SD. Student *t* test, **P* < 0.05. BW, body weight; hr, hours; p-, phosphorylated.

Interestingly, this occurred despite a $\sim 50\%$ decrease in total Akt protein levels. IRS-1 protein levels were more variable but also decreased $\sim 40\%$ on average. This downregulation of Akt, and to some extent IRS-1, proteins suggests some form of negative feedback response to the increase of IR levels. There were no significant changes in Erk, mTOR phosphorylation, or Erk, mTOR protein, levels.

IR overexpression also caused the muscle to be more glycolytic, as demonstrated by reduced succinate dehydrogenase (SDH) activity/immunostaining of the TA muscle (Fig. 2J). This is consistent with, and reciprocal with, previous studies showing that loss of IR in skeletal muscle results in decreased Akt activity and increased SDH activity (9,31). There was also a change in soleus muscle in

IRMOE mice to a more glycolytic phenotype as demonstrated by a 30% decrease in slow oxidative type I myosin fibers and a 60% increase in fast glycolytic type IIb/IIx myosin fibers (32) (Fig. 2K and L).

IR Overexpression Leads to Postreceptor Insulin Resistance

To examine how IR overexpression affects insulin signaling, we performed vena cava injection of insulin in chow-fed control and IRMOE mice after overnight fasting. Consistent with the data above, at the protein level, there was a ninefold increase of both the mature, processed IR and IR precursor protein compared with in controls (Fig. 3A and B). Insulin-stimulated IR autophosphorylation was increased by twofold in IRMOE muscles, even with normalization for the higher level of IR protein (Fig. 3A and C), indicating a more efficient receptor activation at the higher level of receptor expression. Consistent with this, IRS-1 Tyr phosphorylation was also elevated by twofold by insulin in IRMOE muscle, despite a 40% decrease in total IRS-1 protein levels (Fig. 3A–C). Although total protein levels of the PI3K regulatory subunit p85 were not different between muscles of control and IRMOE mice, levels of phospho-p85 (Y458) were significantly elevated in IRMOE muscle when stimulated with insulin, while in muscles from control mice, phospho-p85^{Y458} was undetectable under both basal and insulin-stimulated conditions (Fig. 3A). As seen in the absence of insulin injection (Fig. 2J), total Akt protein level was also decreased by 30% in IRMOE muscle. In contrast to IR and IRS-1, however, this was accompanied by an 80% and 40% decrease in levels of phospho-Akt at Thr³⁰⁸ and Ser⁴⁷³, respectively, resulting in a 70% and 15% decrease in the corresponding ratios of phospho-Akt to total Akt (Fig. 3A–C), consistent with a significant level of postreceptor insulin resistance. By contrast, there was no significant change in either phospho-Erk or Erk protein levels in the IRMOE mice. Phospho- and total protein levels of mTOR and S6 were also not different between control and IRMOE mice regardless of insulin stimulation (Supplementary Fig. 4I).

We performed a 2-h hyperinsulinemic-euglycemic clamp in awake IRMOE mice and controls using an insulin infusion rate of 2.5 mU/kg/min to assess insulin sensitivity and glucose metabolism in individual organs. Consistent with postreceptor insulin resistance, the clamp study revealed that both the glucose infusion rate and whole-body glucose turnover, which measures rate of insulin-stimulated glucose disposal, were significantly decreased in IRMOE mice compared with controls—by 24% and 19%, respectively (Fig. 3D and E). However, when glucose uptake in individual muscles was analyzed, only EDL showed significantly lower insulin-stimulated glucose uptake in IRMOE mice, while in other muscles and other tissues, glucose uptake was similar to controls (Supplementary Fig. 4A–H). Analysis of [³H]glucose uptake *ex vivo* (4) revealed that both EDL and soleus muscles from IRMOE mice had lower basal glucose uptake, but in

response to insulin, maximum glucose uptake was similar (Fig. 3F and G). Thus, insulin stimulated a larger fold increase in glucose uptake in IRMOE muscle than in control. The decrease in basal glucose uptake in EDL and soleus muscles from IRMOE mice is likely due to higher glycogen content in the IRMOE muscles (Fig. 3H), as previous studies have shown that high glycogen content can decrease muscle's ability to take up glucose, reflecting feedback by the high level of stored energy (33).

Another interesting finding in the hyperinsulinemic-euglycemic clamp was that basal hepatic glucose production (HGP) was significantly decreased in IRMOE mice compared with controls (Fig. 3I). This explains, at least in part, the lower fasting blood glucose levels observed in IRMOE mice as they age (Fig. 2E). However, during the clamp, HGP and percent suppression of HGP by insulin were not different between control and IRMOE mice, suggesting similar hepatic insulin sensitivity (Fig. 3I and J). Given that genes responsible for gluconeogenesis were not differentially expressed in livers between control and IRMOE mice (Supplementary Fig. 4J), we hypothesized that there might be a decrease in plasma amino acid levels in IRMOE mice under a fasting condition, thus providing less substrates for gluconeogenesis. To our surprise, however, although IRMOE mice had a marked reduction in muscle autophagy flux under fasting conditions, as measured by lower levels of Δ -LC3II (Fig. 3K and L), levels of plasma amino acids were similar, if not higher, in IRMOE mice compared with control (Supplementary Fig. 4K).

RNA Sequencing Analysis Reveals Complex Regulation of Muscle Gene Expression in Response to IR Overexpression in Skeletal Muscle

To determine how IR overexpression improves hyperglycemia while leading to postreceptor insulin resistance, we performed RNA sequencing analysis on quadriceps muscles from control and IRMOE mice after a 24-h fast, *i.e.*, the basal state. Out of 13,225 mRNAs detected, 921 were significantly downregulated, and 934 were significantly upregulated in IRMOE muscle, with a range of -9.3 - to 8.8 -fold (FDR < 0.25). Reactome pathway analysis revealed that the top upregulated pathway in IRMOE muscle was Shc-related events (Supplementary Fig. 5A), while Notch transcription pathway was the highest ranking downregulated pathway (Supplementary Fig. 5B).

Figure 4A and B show the genes up- and downregulated in IRMOE muscle in both volcano plot and heat map forms, and a complete list of RNA sequencing analysis can be found in Supplementary Table 1. The heat map revealed that many components of the IR signaling pathway, such as *Irs1*, *Irs2*, and *Sos2*, were downregulated in IRMOE muscle, while many negative regulators of IR signaling and glucose uptake, such as *Prkcd* (PKC δ), *Grb14*, *Tbc1d1*, and *Socs7*, were upregulated. Western blot analysis confirmed upregulation of PKC δ and TBC1D1 at the protein level as well (Fig. 4C). Previous studies have shown that PKC δ levels in muscle are increased with aging and that

Figure 3—IR overexpression leads to postreceptor insulin resistance. **A**: Western blot showing insulin signaling in TA muscles under basal conditions and after vena cava injection of insulin ($n = 3$). **B**: Quantification of total protein levels in **A**. **C**: Ratios of phosphorylated and total protein after vena cava injection in **A**. **D**: Glucose infusion rate (GIR) during hyperinsulinemic-euglycemic clamps of 7-month-old mice on chow diet ($n = 8$). **E**: Whole-body glucose turnover during hyperinsulinemic-euglycemic clamps. **F**: Ex vivo glucose uptake in EDL muscles from 7-month-old female mice on chow diet ($n = 6-8$). **G**: Ex vivo glucose uptake in soleus muscles from 7-month-old female mice on chow diet ($n = 6-7$). **H**: Glycogen content in EDL muscles from fed ($n = 4-6$) and overnight fasted ($n = 6-7$) mice on chow diet. **I**: Basal and clamped HGP during hyperinsulinemic-euglycemic clamps ($n = 8$). **J**: Percent suppression of HGP during the clamp. **K**: Western blot of TA muscles in 4-month-old mice fasted for 24 h $\pm$ injection of leupeptin (40 μ g/g) ($n = 3-4$). **L**: Quantification of autophagy flux in **K**; flux = LC3II (+ leupeptin) / tubulin - LC3II (- leupeptin) / tubulin. Data represent mean $\pm$ SD. Student t test, * $P < 0.05$, ** $P < 0.01$. p-, phosphorylated.

muscle-specific deletion of PKC δ can improve whole-body insulin sensitivity and glucose tolerance (18). Tbc1d1 is a Rab-GTPase-activating protein abundant in skeletal muscle and involved in glucose uptake (34). Silencing of *Tbc1d1* in L6 muscle cells increases basal and insulin-stimulated GLUT4 translocation (35), whereas overexpression of *Tbc1d1* in 3T3-L1 adipocytes inhibits this response (34,36). *Pi3kr1*, which encodes the p85 regulatory subunit of PI3K, has also been shown to negatively affect insulin signaling (37); yet, despite elevated mRNA levels in IRMOE mice,

protein expression of *Pi3kr1* was not different between genotypes (Figs. 3A and 4C). Aside from the IR (*Insr*) itself, also among the most highly and significantly upregulated genes in muscles from IRMOE mice were IGF-binding protein 5 (*Igfbp5*), glucokinase (*Gck*), membrane palmitoylated protein 3 (*Mpp3*), transmembrane protein 37 (*Tmem37*), N(G), N(G)-dimethylarginine dimethylaminohydrolase 1 (*Ddadh1*), and MAPK kinase 6 (*Map2k6*). *Igfbp5* showed an ~ 3.5 fold increase at the mRNA level (Fig. 4A), and this was paralleled by a fourfold increase in IGFBP5 protein by

Western blotting (Fig. 4C). IGFBP5 is known to be highly expressed in skeletal muscle and can be secreted and bind to IGFs in circulation (38). Glucokinase mRNA is also upregulated in muscles of IRMOE mice and is likely responsible for increased glycogen content in these muscles, as glucokinase converts glucose to glucose-6-phosphate, a precursor for glycogenesis. Moreover, mice overexpressing glucokinase in muscle have been shown to have lower glycemia and insulinemia and are more sensitive to low-dose insulin-stimulated skeletal muscle glucose disposal (39). The upregulation of *Map2k6* (which encodes MAPKK6) likely serves as part of a negative-feedback response to counter

insulin signaling, as MAPKK6 phosphorylates and activates p38 MAP kinase in response to inflammatory cytokines or environmental stress, and overexpression of MAPKK6 has been shown to inhibit insulin signaling (40) and diminish insulin stimulation of Glut4-mediated glucose transport, while increasing Glut1-mediated basal glucose transport (41).

In addition to the downregulation of multiple genes encoding proteins downstream in the insulin signaling cascade noted above, the most significantly downregulated genes in IRMOE muscle were *Slc7a2*, *Slc15a2*, *Igf1r*, and *Ulk1* (Fig. 4A and B). The solute carrier SLC7A2 is responsible for absorption of cationic amino acids such as

Figure 4—RNA sequencing analysis reveals a complex regulation of muscle gene expression in response to IR overexpression in skeletal muscle. **A:** Volcano plot of RNA sequencing results from quadriceps muscle of 5-month-old control and IRMOE mice after 24-h fast ($n = 5$). **B:** Heat map of RNA sequencing results from quadriceps muscle of 5-month-old control and IRMOE mice after 24-h fast, showing top up- and downregulated genes in IR signaling pathway ($n = 5$). **C:** Western blot of quadriceps muscles from 5-month-old control and IRMOE mice after 24-h fast ($n = 6$). CPM, counts per million.

arginine, lysine, and ornithine (42), whereas SLC15A2 is responsible for uptake of small peptides (43). Both are likely downregulated due to the reduced energy demands of IRMOE muscle during starvation. Ulk1 is a key kinase involved in regulation of autophagy, and downregulation of Ulk1 in IRMOE muscle would contribute to the decrease in autophagy flux noted above. Downregulation of IGF1R transcripts in IRMOE muscle may also serve as a negative feedback to prevent overactivation of IR signaling. At the protein level, this is reflected as downregulation in IGF1R precursor (Fig. 4C). Interestingly, there was no downregulation of the mature IGF-1 receptor, suggesting additional changes in IGF1R processing and/or turnover.

IR Overexpression in Muscle Protects Mice from Diet-Induced Obesity and Glucose Intolerance

Although IRMOE mice showed no difference in body weight compared with controls on standard chow (Fig. 1D), when challenged with a HFD (60%) for 17 weeks, IRMOE mice gained less weight than controls (Fig. 5A). This was due primarily to a reduced increase in adipose tissue mass in the IRMOE mice with no change in lean mass (Fig. 5B and C). The reduced fat mass was accompanied by lower plasma leptin levels (Fig. 5D), and this led to increased food intake, even after adjustment for body weight (Fig. 5E). Metabolic cage assessment revealed that the ability to maintain the lower body weight was due to increased metabolic activity as measured by 13–16% increases in O_2 consumption and CO_2 production (Fig. 5F and Supplementary Fig. 6A). This occurred without alterations in spontaneous activity levels (Supplementary Fig. 6B).

Assessment of insulin signaling following vena cava injection of insulin in HFD-fed control and IRMOE mice revealed persistent postreceptor insulin resistance in IRMOE muscle as reflected by lower insulin-stimulated Akt-Ser⁴⁷³ phosphorylation (Fig. 5G and H), while Akt-Thr³⁰⁸ phosphorylation was not significantly different between control and IRMOE groups due to variation (Fig. 5G). As in chow-fed mice, IRS-1 and Akt protein levels were downregulated in the IRMOE mice, albeit to a lesser extent, reflecting some negative feedback to prevent overactivation of the insulin signaling pathway (Fig. 5G and H). Importantly, however, the Shc-Erk branch of the IR signaling was spared from any negative regulation, and as a result, insulin-stimulated phosphorylation of Shc and Erk in muscles of IRMOE mice was increased in proportion to the increase in receptor phosphorylation (Fig. 5G). Despite the presence of postreceptor insulin resistance in muscles from IRMOE mice, expression of genes responsible for fatty acid oxidation, lipid synthesis, and inflammation was not different between control and IRMOE mice on chow diet (Supplementary Fig. 6C). With HFD feeding, muscles from IRMOE mice even had lower expression of FSP27, a marker for lipid accumulation, and reduced expression of CCL2, an inflammatory cytokine (Fig. 5J). IL6 was known to stimulate glucose uptake in multiple cell types in vitro (44), and its expression was significantly elevated in muscles from IRMOE mice compared with control (Fig. 5J).

Along with that, HFD-fed IRMOE mice showed marked improvement in glucose tolerance and insulin sensitivity compared with their littermate controls (Fig. 6A and B). This was accompanied by significantly lower plasma insulin levels in both fed and fasted states (Fig. 6C). In CLAMS metabolic cage assessment, IRMOE mice showed a 13% increase in O_2 consumption (Fig. 5F) and 16% increase in CO_2 production (Supplementary Fig. 5A), resulting in a higher RER throughout most of the day, indicating a trend toward more carbohydrate than fat usage in these mice (Fig. 6D). Interestingly, plasma free fatty acids (FFA) and triglyceride (TG) levels were also significantly elevated in IRMOE mice compared with control mice on HFD (Fig. 6E and F), while liver TG levels were significantly lower in IRMOE mice (Fig. 6G). The elevation in plasma TG level is likely due to the decreased muscle expression of VLDLR and LPL (Fig. 6H–K), which would result in reduced uptake and hydrolysis of TG-rich particles. The increase in plasma FFA levels in IRMOE mice reflects the changes in fatty acid turnover in white adipose tissue (Fig. 7A–C and discussed below). The fact that increased plasma FFA did not lead to elevation in liver TG is likely due to the much lower plasma insulin levels in IRMOE mice compared with control (Fig. 6C), since insulin is a major driver of TG synthesis in the liver.

IR Overexpression in Muscle Improves White Fat and Liver Health in Diet-Induced Obesity

In diabetes and metabolic syndrome, there is cross talk between muscle, fat, and liver that serves important roles in regulating metabolism. H-E staining of eWAT of control mice after 17 weeks of HFD feeding revealed high levels of macrophage infiltration with increased crown-like structures indicating tissue inflammation, whereas adipose tissue of IRMOE mice challenged with HFD showed almost no inflammatory changes (Fig. 7A). Consistent with this, mRNA levels of F4/80, a marker for macrophages, were significantly lower in eWAT from IRMOE mice than in controls, as were other markers of inflammation, including monocyte chemoattractant protein-1 (MCP-1/CCL2) and TNF α (Fig. 7B), while mRNA levels of fatty acid oxidation genes, such as LCAD/MCAD in eWAT, were not different between control and IRMOE mice. On the other hand, there was an increase in mRNAs of genes involved in lipid synthesis (*Scd1*, *Dgat1*) and genes involved in lipolysis (*Hsl*, *Atgl*) in the adipose tissue of IRMOE mice, suggesting increased lipid turnover (Fig. 7C).

Liver from IRMOE mice also showed changes in histology and gene expression indicating reduced inflammation and an improved metabolic profile. Consistent with decreased TG content (Fig. 6G), H-E staining of IRMOE mice on HFD revealed less steatosis and lipid accumulation in hepatocytes compared with control (Fig. 7D). There was also decreased expression of FSP27/CIDEA in liver measured by qPCR (Fig. 7E). Livers of IRMOE mice also had lower mRNA expression of CCL2, TNF α , Col1A, and TGF β 1, reflecting reduced inflammation and fibrosis (Fig. 7E). No differences in lipid synthesis or fatty acid

Figure 5—IR overexpression in muscle protects mice from diet-induced obesity. **A**: Body weight (BW) of control (Ctl) and IRMOE mice over the course of 17 weeks of HFD feeding ($n = 7-9$). **B**: Fat mass and **C**: lean mass measured by DEXA scan after 11 weeks on HFD ($n = 6$). **D**: Plasma leptin levels of control and IRMOE mice after 13 weeks on HFD ($n = 7-9$). **E**: 24-h food intake normalized to body weight in control and IRMOE mice after 11 weeks on HFD ($n = 5-6$). **F**: O_2 consumption rate in control and IRMOE mice after 11 weeks on HFD ($n = 5-6$). **G**: Western blot of TA muscles from control and IRMOE mice after 17 weeks on HFD and vena cava injection of saline or insulin ($n = 4$ per condition). **H**: Quantification of total Akt, IRS-1 protein levels, and phosphorylated vs. total protein in **G**. **I**: Fatty acid oxidation gene expression measured by qPCR in quadriceps muscles of control and IRMOE mice after 17 weeks' HFD feeding ($n = 7-9$). **J**: Myokine and lipid metabolism gene expression measured by qPCR in quadriceps muscles of control and IRMOE mice after 17 weeks HFD feeding ($n = 7-9$). Data represent mean $\pm$ SD., Student t test, * $P < 0.05$, ** $P < 0.01$. hr, hour; LE, long exposure; p-, phosphorylated.

oxidation gene expression were observed between the two genotypes (Fig. 7F). Interestingly, despite having lower fasting blood glucose (Fig. 6A), IRMOE mice on HFD had 43% and 47% higher expression of G6Pase and PEPCK, respectively, in their livers after overnight fasting (Fig. 6C).

Unlike the postreceptor insulin resistance in muscle, eWAT from IRMOE mice showed no change in IR phosphorylation but had significantly increased insulin-

stimulated Akt and Erk phosphorylation compared with control (Fig. 7G), whereas in liver, insulin-stimulated Akt and Erk phosphorylation was similar between genotypes (Fig. 7H). However, basal Akt phosphorylation was lower in both eWAT and liver from IRMOE mice (Fig. 7G and H), resulting in larger fold increases of insulin-stimulated Akt phosphorylation (Fig. 6B), contributing to improved systemic insulin sensitivity in IRMOE mice.

Figure 6—IR overexpression in muscle improves glucose tolerance and insulin sensitivity on HFD. **A:** Glucose tolerance tests in control (Ctl) and IRMOE mice after 15 weeks on HFD ($n = 7-9$). **B:** Insulin tolerance test in control and IRMOE mice after 13 weeks on HFD ($n = 7-9$). **C:** Plasma insulin levels in control and IRMOE mice after 13 weeks (fed) and 16 weeks (fast) on HFD ($n = 7-9$). **D:** RER in control and IRMOE mice after 11 weeks on HFD ($n = 5-6$). **E:** Plasma FFA levels in control and IRMOE mice after 13 weeks (fed) and 16 weeks (fast) on HFD ($n = 7-9$). **F:** Fasting plasma TG levels in control and IRMOE mice after 16 weeks on HFD ($n = 7-9$). **G:** Fasting liver TG levels in control and IRMOE mice after 17 weeks on HFD ($n = 7-9$). **H:** VLDLR and LPL mRNA expression measured by qPCR in quadriceps muscles of control and IRMOE mice after 17 weeks' HFD feeding ($n = 7-9$). **I:** Western blot analysis of VLDLR and LPL in quadriceps of control and IRMOE mice after 17 weeks' HFD feeding. VLDLR(1)/(2) are two isoforms of VLDLR. ($n = 6$). **J:** Quantification of VLDLR total protein level and different isoform protein levels shown in **I**. **K:** Quantification of LPL protein level shown in **I**. Data represent mean $\pm$ SD. Student t test, $*P < 0.05$, $**P < 0.01$. GTT, glucose tolerance test; ITT, insulin tolerance test.

DISCUSSION

Skeletal muscle insulin resistance is an important and early component in the pathogenesis of type 2 diabetes and metabolic syndrome. This defect is detectable years before diagnosis of the disease (2), and in offspring of parents with diabetes, skeletal muscle insulin resistance is a strong

predictor for disease development (45). Our study shows that overexpression of IR specifically in skeletal muscle protects against systemic glucose intolerance and development of diabetes in mice. This beneficial effect is even more prominent when mice are fed with HFD, which normally leads to downregulation of IR expression in

Figure 7—IR overexpression in muscle improves white fat and liver health in diet-induced obesity. **A**: H-E staining of eWAT in control (Ctl) and IRMOE mice after 17 weeks on HFD. Scale bar = 200 μ mol/L ($n = 7$ –9). Inflammatory (**B**) and lipid metabolism (**C**) gene expression measured by qPCR in eWAT from control and IRMOE mice after 17 weeks on HFD ($n = 7$ –9). **D**: H-E staining of liver in control and IRMOE mice after 17 weeks on HFD. Scale bar = 200 μ mol/L ($n = 7$ –9). Inflammatory (**E**) and lipid metabolism (**F**) gene expression measured by qPCR in liver from control and IRMOE mice after 17 weeks on HFD ($n = 7$ –9). **G**: Western blot of eWAT from control and IRMOE mice after 17 weeks on HFD and vena cava injection ($n = 3$ per group). **H**: Western blot of liver from control and IRMOE mice after 17 weeks on HFD and vena cava injection ($n = 3$ per group). Data represent mean $\pm$ SD. Student t test, $*P < 0.05$. LE, long exposure; p, phosphorylated.

muscle and systemic insulin resistance (12). This also occurs despite some level of postreceptor insulin resistance at the level of Akt in muscle of IRMOE mice.

The role of muscle insulin signaling in whole-body glucose homeostasis is important but complex. Although deletion of IR, or even combined deletion of IR and IGF1R, in skeletal muscle alone does not result in diabetes or marked impairment of glucose tolerance in B6/J mice, commonly used to model type 2 diabetes (4,9), deletion of IR in muscle

does markedly exacerbate glucose intolerance in mice with mild systemic insulin resistance, for example, those with heterozygous whole-body IR knockout (4). Likewise, inhibition of muscle insulin signaling by overexpression of a dominant-negative form of the human IGF1R results in early-onset type 2 diabetes in 129S6 mice (46). This effect, however, is strain dependent and is not observed in B6/J mice (9), suggesting that differences in genetic background can affect the postreceptor response to altered IR levels.

The current study and studies in mice with combined IR/IGF1R deletion in skeletal muscle (MIGIRKO) suggest that at least one point of postreceptor modification is at the level of TBC1D1, an Rab-GAP protein involved in retention of GLUT4-containing vesicles inside the cell (47). Normally, GLUT4 translocation to the plasma membrane requires Akt-mediated phosphorylation and inhibition of TBC1D1 with simultaneous Akt-mediated phosphorylation and activation of TBC1D4 (also known as AS160), which promotes translocation (47). But in mice with muscle-specific knockout of both IR and IGF1R, levels of TBC1D1 are low, thus allowing for increased constitutive glucose uptake. Indeed, these MIGIRKO mice not only display normal whole-body glucose tolerance when fed with HFD but also have fasting hypoglycemia reflecting increased basal glucose uptake (9). Mirroring this scenario, muscles from IRMOE mice have significantly increased TBC1D1 expression at both the mRNA and protein level, which would lead to decreased GLUT4 translocation to plasma membrane, as evidenced by the decreased basal glucose uptake observed in muscles from IRMOE mice *ex vivo* and the reduced glucose turnover rate in the euglycemia clamp studies *in vivo*. Taken together, the current and previous studies establish a role of IR/IGF1R in muscle to suppress basal glucose transport, especially in the fasted state.

One unexpected finding of this study is the development of postreceptor insulin resistance in muscle of IRMOE mice, as evidenced by lower insulin-stimulated Akt phosphorylation compared with control. This could represent some compensatory response to enhanced insulin secretion from β -cells, thus preventing excessive clearance of glucose through increased muscle glucose uptake. This also suggests the existence of some previously unrecognized negative-feedback system that prevents overactivation of postreceptor IR signaling. Indeed, our mRNA sequencing data show that in response to IR overexpression, there are multiple layers of negative feedback at the gene expression level, including downregulation of mediators of IR signaling, such as IRS-1 and IRS-2, and upregulation of expressions of negative regulators of IR signaling, like SHIP2, Grb14, and PKC δ . In addition, although Akt mRNA level is not significantly reduced, Akt protein level is decreased $\sim 40\%$, indicating postreceptor adaptations in protein synthesis or turnover. Together, these factors contribute to the stepwise dampening of phosphorylation signals relayed from IR to Akt in response to insulin stimulation.

Our data further demonstrate the important role of insulin/IGF-1 signaling on muscle growth. Thus, IRMOE mice have increases in muscle fiber size, changes in fiber type, and increased grip strength. This also mirrors the changes observed in mice with muscle-specific IR and IR/IGF1R knockout, where there is significant reduction in muscle fiber size, an increased percentage of oxidative muscle fibers, and decreased grip strength (8,48). Mechanistically, O'Neill et al. (8,48) have shown that IR/IGF1R signaling regulates muscle protein homeostasis through FoxO transcription factors. Thus, combined deletion of

FoxO1, FoxO3, and FoxO4 in muscle reverses the increased autophagy and muscle atrophy observed in both MIGIRKO and streptozotocin diabetic mice.

One interesting and unexpected observation in IRMOE mice is the effect of muscle IR overexpression on other tissues. For instance, β -cells from IRMOE mice have enhanced insulin secretion in response to glucose, suggesting the existence of a myokine that targets β -cells and acts as an insulin secretagogue. Additionally, insulin action and metabolic response in liver and white fat are also affected. Thus, IRMOE mice have decreased hepatic gluconeogenesis during fasting, resulting in lower fasting blood glucose levels. This occurs with no change in expression of major liver gluconeogenic genes or reduction in the level of gluconeogenic amino acid substrates. Likewise, despite improved systemic insulin sensitivity and glucose tolerance, IRMOE mice on HFD have increased plasma FFA and TG levels. The higher levels of FFAs are likely due to increased hydrolysis of stored TG in white adipose tissue, as indicated by increased HSL and ATGL expression in eWAT of IRMOE mice, while the increase in plasma TG reflects decreased VLDL receptor and LPL expression in muscle of the IRMOE mice, which would lead to decreased TG uptake (49,50) and increased hydrolysis of TGs into FFAs (51). Indeed, like IRMOE mice, mice lacking in VLDLR are protected from diet-induced obesity and are more glucose tolerant than their wild-type counterparts, despite having increased plasma TG levels (52).

Although it does not affect activity level, IR overexpression in muscle to some extent mimics the effect of exercise. Indeed, exercise has been shown to elevate IR expression in skeletal muscle (53) and stimulate white fat lipolysis, leading to increased plasma FFA levels for 12–16 h postexercise (54), while leading to reduced liver TG content (55). Myokines such as FGF21 are known to mimic some of these effects (56), yet exactly which myokines are responsible for the improved metabolism in fat and liver still remains to be determined. Gene expression analysis reveals a fourfold increase in IGFBP5 in muscles of IRMOE mice, and this change is also observed at the protein level. Previous studies have shown that IGFBP5 knockout mice challenged with HFD exhibit increased weight gain, increased fasting blood glucose and insulin levels, and increased insulin resistance compared with controls on HFD (57)—all changes opposite those in the IRMOE mice. Given that IRMOE mice gain much less weight compared with control when fed with HFD, we cannot rule out the possibility that the lower body weight of IRMOE mice *per se* also contributes to their improved metabolic phenotype. Nonetheless, improved peripheral insulin resistance in HFD-fed IRMOE mice definitely contributes to reduced delivery of FFAs to the liver for lipid synthesis.

In summary, muscle-specific overexpression of IR protects mice from diet-induced obesity, improves glucose homeostasis, and leads to better metabolic profiles in WAT and liver. However, IRMOE mice develop postreceptor insulin resistance in muscle, as well as elevated plasma FFA and TG levels, on HFD. These secondary changes are

outweighed by the beneficial effects of IR overexpression on glucose homeostasis. Our study not only shows the importance of muscle IR signaling in regulating systemic glucose and lipid homeostasis but also uncovers some potential positive or negative regulators of insulin signaling. Understanding this molecular network will help identify new targets with therapeutic potential to benefit patients with diabetes and metabolic disease.

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