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IRS1^{Ser307} phosphorylation does not mediate mTORC1-induced insulin resistance

Hilde Herrema, Jaemin Lee, Yingjiang Zhou, Kyle D. Copps, Morris F. White, Umut Ozcan *

Division of Endocrinology, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA

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ABSTRACT

Increased mammalian target of rapamycin complex 1 (mTORC1) activity has been suggested to play important roles in development of insulin resistance in obesity. mTORC1 hyperactivity also increases endoplasmic reticulum (ER) stress, which in turn contributes to development of insulin resistance and glucose intolerance. Increased IRS1 phosphorylation at Ser307 *in vitro* is correlated with mTORC1- and ER stress-induced insulin resistance. This phosphorylation site correlates strongly with impaired insulin receptor signaling in diabetic mice and humans. In contrast, evidence from knock-in mice suggests that phosphorylation of IRS1 at Ser307 is actually required to maintain insulin sensitivity. To study the involvement of IRS1^{Ser307} phosphorylation in mTORC1-mediated glucose intolerance and insulin sensitivity *in vivo*, we investigated the effects of liver specific TSC1 depletion in IRS1^{Ser307Ala} mice and controls. Our results demonstrate that blockade of IRS1^{Ser307} phosphorylation *in vivo* does not prevent mTORC1-mediated glucose intolerance and insulin resistance.

1. Introduction

The rise in incidence of obesity over the last few decades has led to a robust increase in development of insulin resistance and consequently type 2 diabetes [1–3]. Despite enormous efforts, the molecular mechanisms of obesity-induced insulin resistance remain incompletely understood.

The endoplasmic reticulum (ER) plays a central role in protein homeostasis by dynamically adjusting synthesis and quality control of membrane and secreted proteins in conjunction the demands of the cell [4]. ER homeostasis is tightly regulated, as impaired ER function has detrimental consequences for the cell [5–8]. A plethora of conditions such as viral infections, increased protein synthesis, glucose deprivation, depletion of ER calcium, alterations in ER membrane composition and nutrient overload can severely affect ER homeostasis, interfere its function and create stress on the organelle (ER stress) [9,10].

ER stress leads to activation of a complex signal transduction pathway collectively called the unfolded protein response (UPR) [9,10]. UPR communicates the status of the ER to the cell through inositol-requiring enzyme-1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor-6 (ATF6) [4,11]. UPR signaling primarily aims to restore ER homeostasis by decreasing global protein translation, thereby reducing the protein load on the ER, and by activating expression of genes that assist in protein folding and increasing ER size, capacity and efficiency.

Chronic overnutrition, a major hallmark of diet-induced obesity, significantly burdens ER protein synthetic capacity, and at the same time impairs mechanisms and signaling cascades that are required to maintain or restore ER homeostasis [12]. Consequently, markers of ER stress and UPR signaling are consistently increased in metabolically active tissues in obesity [13] and these events contribute significantly to development of insulin resistance in obesity [12,14–18].

Novel, ER stress-mediated negative feedback mechanisms on insulin signaling pathways in obesity are increasingly emerging [13,19,20]. One of the identified pathways leading to development of ER stress in obesity is the mammalian target of rapamycin (mTOR) pathway [16]. mTOR is a highly conserved serine/threonine kinase that exists in two large protein complexes called mTORC1 and mTORC2 [21]. mTORC1 contains of mTOR, Deptor, PRAS40, mLST8/GβL, and raptor and is sensitive to rapamycin inhibition. mTORC2 consists of mTOR, Deptor, mSIN, Protor, mLST8/GβL, and rictor (instead of raptor) and is resistant to acute inhibition by rapamycin [22]. mTOR plays a central role in cell growth and proliferation, and communicates nutrient and energy status to the cell. mTOR-mediated control of these physiological processes are, at least in part, mediated by mTORC1-mediated regulation of translation. mTORC1 phosphorylates and activates ribosomal S6 protein kinase (S6K) that leads to increased protein translation [23]. In addition, mTORC1 phosphorylates, and thereby

* Corresponding author.
E-mail address: umut.ozcan@childrens.harvard.edu (U. Ozcan).

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inhibits, eukaryotic initiation factor eIF4E binding protein 1 (4EBP1) [24], which releases its inhibitory action on the translation initiation complex, thereby increasing mRNA translation and protein synthesis. mTOR is regulated by a small G protein, Rheb (Ras homolog enriched in brain). The GTP-bound form of Rheb activates mTORC1 kinase activity [25]. Rheb, in turn, is inhibited by a GTPase-activating protein (GAP) called tuberous sclerosis complex 2 (TSC2) [26,27]. TSC2 exists as a complex with TSC1 and loss of function of either leads to increased mTORC1 activity.

Increased mTORC1 activity has been associated with insulin resistant conditions [28,29]. Activation of mTORC1 modulates insulin receptor signaling through inhibitory phosphorylation of IRS1 by the mTORC1 effector protein S6 kinase (S6K) [30–32]. TSC1–2 deficiency has been reported to decrease both IRS1 mRNA and protein expression levels although no detailed mechanisms have been established to date [30,32]. Importantly, hyperactivation of mTORC1 due to loss of TSC1 leads to ER stress *in vitro* [16]. TSC1-deficient cells are highly insulin resistant but regain insulin sensitivity significantly when ER stress is relieved [16]. These findings suggest that one of the factors that contribute to development of mTORC1 hyperactivity-induced insulin resistance is ER stress [16].

The observations that phosphorylation of IRS1 at Ser307 is increased in TSC1 deficient conditions *in vitro* and that this phosphorylation site has been indicated in development of insulin resistance *in vivo*, led us to assess the pathophysiological consequences of TSC1 depletion in mice that lack the ability to phosphorylate IRS1 at Ser307.

2. Materials and methods

2.1. Mice

$TSC1^{f/f}$ mice were obtained from Dr. D. Kwiatkowski [33]. $IRS1^{s/s}$ and $IRS1^{a/a}$ mice have been described in detail [34]. In short, $IRS1^{a/a}$ mice were generated using a knock-in approach: the IRS1 gene was replaced by a point-mutated Ser307Ala copy of IRS1. $IRS1^{s/s}$ control mice were generated using the same knock-in approach. $IRS1^{s/s}$-$TSC1^{f/f}$ and $IRS1^{a/a}TSC1^{f/f}$ double mutant mice were generated by crossing $IRS1^{s/s}$ and $IRS1^{a/a}$ mice with $TSC1^{f/f}$ to generate $IRS1^{s/+}$ $TSC1^{f/f}$ and $IRS1^{a/+}$ $TSC1^{f/f}$ mice. The respective IRS1 heterozygotes were used to generate $TSC1^{f/f}$ double mutants homozygous for the $IRS1^{a/a}$ or $IRS1^{s/s}$ knock-in. All the mice were on a C57BL6 background. The Animal Care and Use Committee at Boston Children's Hospital approved all mouse procedures.

2.2. Adenovirus injection through tail vein

Adenovirus was thawed at room temperature prior to injection. Adenovirus was diluted with saline to a dose of 2×10^{11} PFU (plaque forming units)/kg. The final administration volume was 100 μl per mouse. Mice were put in a restrainer and adenovirus was injected through the tail vein using a 28-gauge needle. Mild pressure was applied at the site of injection to prevent bleeding.

2.3. Biochemical reagents

Anti-phosphotyrosine (PY99), anti-β-actin, anti-insulin receptor (IR), and HRP-conjugated goat anti-mouse or goat anti-rabbit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Akt at Thr308, anti-Akt, anti-phospho-S6K at Thr389, anti-S6 K, and anti-α-tubulin were purchased from Cell Signaling Technology (Beverley, MA). cDNA Synthesis Kit, SYBR Green Supermix and detergent-compatible protein assay kit were from BIO-RAD (Hercules, CA). BM Chemiluminescence Blotting Substrate was from Roche (Indianapolis, IN). Adenoviruses expressing LacZ and Cre were generated as described previously [14].

2.4. Total protein extraction from the liver and Western blot analysis

The liver tissues were lysed in ice-cold tissue lysis buffer (25 mM Tris–HCl, pH 7.4; 100 mM NaF; 50 mM $Na_4P_2O_7$; 10 mM Na_3VO_4; 10 mM EGTA; 10 mM EDTA; 1% NP-40; 10 μg/ml Leupeptin; 10 μg/ml Aprotinin; 2 mM PMSF and 20 nM Okadaic acid) and total protein was extracted as previously described [18]. Samples from tissue lysates were resolved by SDS–PAGE and then transferred to polyvinylidene fluoride (PVDF) membrane. After 1 h blocking at room temperature using 10% blocking reagent (Roche), the membrane was incubated overnight (O/N) with primary antibody in Tris-buffered saline solution/Tween (TBST) containing 10% blocking reagent at 4 °C. After O/N incubation, membrane was washed three times in TBST and incubated with secondary antibody for 1 h at room temperature. After three-time washing in TBST, the membrane was developed using a chemiluminescence assay system (Roche) and exposed to films. Relative protein levels were quantified using Image J.

2.5. Analysis of in vivo insulin signaling

For *in vivo* insulin signaling analysis, mice were anaesthetized with ketamine/xylazine after 6 h of fasting. Insulin (0.75 IU/kg) or saline was infused into the liver via the portal vein. Three minutes after infusion, liver was quickly excised, snap-frozen in liquid nitrogen and stored at −80 for later analysis.

2.6. Glucose tolerance test (GTT)

For GTT analysis, mice were intraperitoneally (i.p.) injected with D-glucose (1.5 g/kg body weight) after an overnight fast. Tail vein blood was collected at 0, 15, 30, 60, 90 and 120 min after glucose injection. Blood glucose was measured with a glucose meter (Bayer Contour).

2.7. Real-time quantitative PCR

Total RNA was extracted from the liver using Trizol reagent (Invitrogen) and transcribed into cDNA using cDNA synthesis kit (Bio-Rad). The gene expression analysis was performed by real-time PCR using SYBR Green (Bio-Rad). mRNA levels were normalized to 18S as a house keeping gene. The primer sequences used were:

18S rRNA forward: 5′-AGT CCC TGC CCT TTG TAC ACA-3′;
18S rRNA reverse: 5′-CGT TCC GAG GGC CTC ACT-3′;
G6pc forward: 5′-CCG GTG TTT GAA CGT CAT CT-3′;
G6pc reverse: 5′-CAA TGC CTG ACA AGA CTC CA-3′;
Ppargc1a forward: 5′-TGA TGT GAA TGA CTT GGA TAC AGA CA-3′;
Ppargc1a reverse: 5′-CAA TGC CTG ACA AGA CTC CA-3′;
Tsc1 forward: 5′- AGG AGG CCT CTT CTG CTA CC-3′
Tsc1 reverse: 5′-CAG CTC CGA CCA TGA AGT G-3′

2.8. Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Statistical significance was calculated by Student's *t*-test or by multifactor ANOVA. When ANOVA indicated a significant difference among the groups, a Bonferroni post hoc test was performed. Significance was accepted at the level of $p < 0.05$ (*).

3. Results

3.1. Liver-specific deletion of TSC1 increases mTORC1 activity and creates glucose intolerance

To assess the role of TSC1 depletion and subsequent mTORC1 hyperactivation on glucose homeostasis *in vivo*, we depleted TSC1 from livers of TSC1$^{f/f}$ mice by administration of adenovirus expressing Cre-recombinase (AdCre) through the tail vein. We injected 2 $\times 10^{11}$ PFU (plaque forming units)/kg AdCre into the tail vein of 8-week-old lean male TSC1$^{f/f}$ mice. Littermate controls were injected with 2 $\times 10^{11}$ PFU/kg adenovirus expressing LacZ (AdLacZ). As shown in Fig. 1A, AdCre injection through tail vein successfully depleted TSC1 in the livers and increased S6K phosphorylation at Thr389, which is an indication of increased mTORC1 activity. To investigate glucose metabolism, we first performed a glucose tolerance test (GTT) 14 days after adenovirus injection. Depletion of TSC1 led to a significantly lower disposal of glucose from the circulation during GTT (Fig. 1B). In parallel with reduced glucose tolerance, hepatic depletion of TSC1 significantly ($p < 0.05$) increased 6 h-fasted blood glucose levels (Fig. 1C). Consistent with decreased insulin sensitivity, expression of insulin-suppressed gluconeogenic genes such as glucose-6-phosphatase (*G6pc*), peroxisome proliferator-activated receptor gamma, coactivator 1a (*Ppargc1*) and phosphoenolpyruvate carboxykinase (*Pepck)* were increased in the livers of TSC1$^{f/f}$ mice injected with AdCre compared to controls (Fig. 1D).

To assess hepatic insulin sensitivity, we investigated insulin receptor signaling after infusion of insulin (0.5 U/kg) through the portal vein. Consistent with observations in TSC-deficient cells, insulin receptor tyrosine phosphorylation and Akt phosphorylation at Thr308 were reduced in livers from TSC1-deficient mice compared to their controls, suggestive of reduced insulin sensitivity (Fig. 1E). Together these results indicate that hepatic depletion of TSC1 reduced glucose tolerance, increased basal blood glucose levels and impaired insulin receptor signaling.

3.2. IRS1Ser307Ala mutation does not block development of glucose intolerance in TSC-deficiency

IRS1 proteins are extensively phosphorylated at Ser/Thr residues following insulin stimulation, which influences the outcome of IRS signaling [35]. Previous observations in the field have indicated that increased Ser phosphorylation inhibits activity of IRS proteins and plays an important role in development of insulin resistance in obesity [36,37]. Phosphorylation of IRS1 at Ser307 (Ser312 in humans) is one of the sites that is consistently hyperphosphorylated in insulin resistant humans [38] and genetically obese and diabetic *ob/ob* mice, as well as high-fat diet (HFD)-fed obese mice [36,37]. In correlation with these findings, *in vitro* studies revealed that mutation of Ser307 in IRS1 protected from tumor necrosis factor α (TNFα)-mediated reduction in IRS1 signaling [39].

Activation of JNK1 in obesity through various mechanisms has been suggested to play important roles in development of insulin resistance, in part through its ability to phosphorylate IRS1 at Ser307 [16]. In particular, it is widely held that increased phosphorylation of IRS1 at Ser307 contributes mechanistically to the development of insulin resistance. However, in a recent report

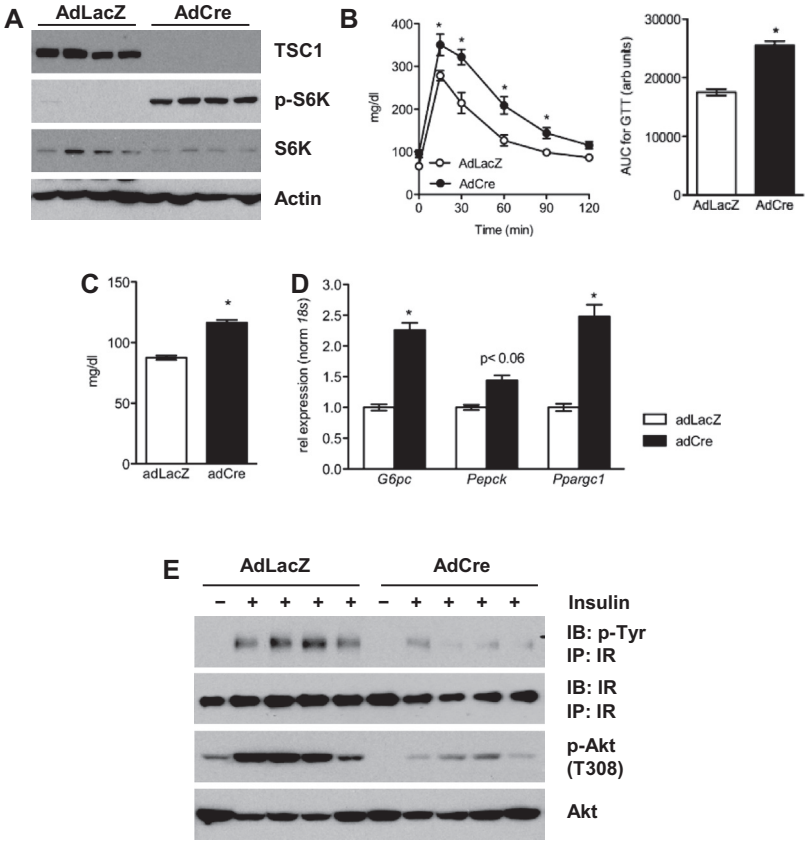

Fig. 1. Hepatic depletion of TSC1 reduces glucose intolerance and insulin sensitivity. Eight-week old male TSC1$^{f/f}$ mice were injected with adenovirus expressing LacZ (AdLacZ) or Cre (AdCre) via the tail vein. (A) Immunoblot of TSC1, phospho-S6K (Thr389), total S6K, and actin in liver lysates. (B) Glucose tolerance test (GTT) and are under the curve (AUC) for GTT. (C) Hepatic expression of *G6pc*, *Pepck* and *Ppargc1* as assessed by QPCR. (D) 6-h fasting blood glucose levels (mg/dl). (E) To assess insulin-stimulated IR signaling, livers were infused with insulin (0.5 U/kg) through the portal vein. IR tyrosine and Akt^{Thr308} phosphorylation were assessed by immunoblotting. $^{*}p < 0.05$.

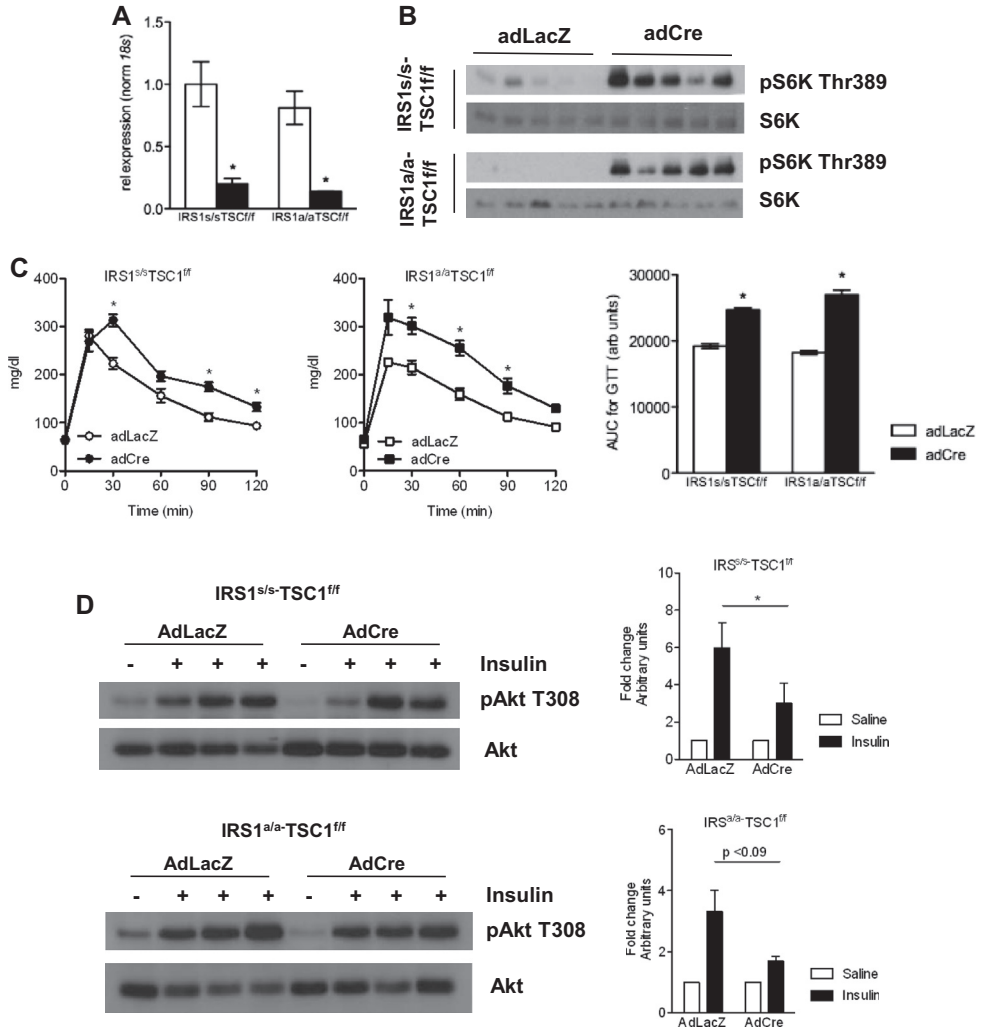

Fig. 2. Hepatic deletion of TSC1 increased mTORC1 signaling and reduced glucose tolerance to same extent in IRS1$^{s/s}$–TSC1$^{f/f}$ and IRS1$^{a/a}$–TSC1$^{f/f}$ mice. (A) mRNA levels of *Tsc1* in the livers of IRS1$^{s/s}$–TSC1$^{f/f}$ and IRS1$^{a/a}$–TSC1$^{f/f}$ mice injected with either AdCre or AdLacZ. (B) Immunoblot of phospho-S6K (Thr389) and total S6K levels in liver lysates of IRS1$^{s/s}$–TSC1$^{f/f}$ mice and IRS1$^{a/a}$–TSC1$^{f/f}$ mice. (C) Glucose tolerance test (GTT) and are under the curve (AUC) for GTT. GTT were performed 2 weeks after AdLacZ- or AdCre injection in IRS1$^{s/s}$–TSC1$^{f/f}$ mice and IRS1$^{a/a}$–TSC1$^{f/f}$ mice. (D) Insulin-stimulated Akt^{308} phosphorylation following infusion of portal vein with insulin (0.5 U/kg). Immunoblotting was used to detect Akt^{308} levels. $^{*}p < 0.05$.

White and coworkers showed that mutation of S307 in knock-in mice, contrary to the general belief, worsened glucose homeostasis and reduced insulin sensitivity [34]. Thus, these results suggest that IRS1 phosphorylation at Ser307 is required for maintenance insulin receptor signaling.

To investigate whether Ser307 phosphorylation in IRS1 plays a role in mTORC1 hyperactivity-mediated glucose intolerance *in vivo*, we crossed TSC1$^{f/f}$ mice with mice in which the endogenous IRS1 gene was replaced by a Ser307Ala mutant copy of IRS1 (IRS1$^{a/a}$ mice) [34] to generate IRS1$^{a/a}$–TSC1$^{f/f}$ mice. Control mice were generated by crossing TSC1$^{f/f}$ mice with knock-in mice expressing the endogenous form of IRS1 (IRS1$^{s/s}$ mice) [34] and will be referred to as IRS1$^{s/s}$–TSC1$^{f/f}$ mice.

As shown in Fig. 2A, administration of AdCre via tail vein into 8 week-old male IRS1$^{s/s}$–TSC1$^{f/f}$ and IRS1$^{a/a}$–TSC1$^{f/f}$ mice depleted *Tsc1* and increased phosphorylation of S6K at Thr389 indicating that mTORC1 activity was induced (Fig. 2B). We assessed the consequences of TSC1-depletion on glucose tolerance 2 weeks after AdLacZ or AdCre administration in IRS1$^{s/s}$–TSC1$^{f/f}$ and IRS1$^{a/a}$–TSC1$^{f/f}$ mice by subjecting the mice to a GTT and observed that TSC1-depletion significantly reduced glucose tolerance in both genotypes (Fig. 2C). The relative area under the curve was significantly increased in both genotypes following depletion of TSC1. Nevertheless, the reduction in glucose tolerance following TSC1-depletion was similar in IRS1$^{s/s}$–TSC1$^{f/f}$ and IRS1$^{a/a}$–TSC1$^{f/f}$ mice, suggesting that lack of Ser307 phosphorylation in IRS1 does not protect against mTORC1-induced glucose intolerance.

Next, we assessed hepatic insulin sensitivity. Insulin (0.5 U/kg) was infused into the livers of AdLacZ or AdCre-injected IRS1$^{s/s}$–TSC1$^{f/f}$ and IRS1$^{a/a}$–TSC1$^{f/f}$ mice via the portal vein. Akt (Thr308) phosphorylation was analyzed with immunoblotting (Fig. 2D). Depletion of TSC1 reduced insulin-stimulated phosphorylation of Akt (Thr308) in a similar fashion in IRS1$^{s/s}$–TSC1$^{f/f}$ mice and in IRS1$^{a/a}$–TSC1$^{f/f}$ mice. Taken together, these results indicate that Ser307 phosphorylation of IRS1 does not play a role in mTORC-1 mediated insulin resistance.

4. Discussion

Maintenance of ER homeostasis is crucial to adjust protein synthetic capacity to cellular demand, which is largely determined by energy and nutrient status. Conditions that impair ER integrity or increase the demand on ER to an extent that outpaces its capacity,

can lead to ER stress and increased UPR signaling [9,40]. It is at this crossroad that mTORC1 signaling and ER homeostasis meet: overt activation of mTORC1 leads to extensive induction of protein synthesis, which increases the demand on ER capacity and can eventually lead to ER stress. Indeed, chronic activation of mTORC1 due to loss of TSC1 leads to ER stress *in vitro* [16]. In addition, increased mTORC1 activity has been shown to negatively regulate insulin signaling by decreasing IRS1 protein and mRNA levels [32]. Increased mTORC1 signaling, via activation of its downstream effector S6K, leads to phosphorylation of IRS1 on numerous Ser/Thr residues, which eventually results in reduced insulin signaling and Akt activation [29].

TSC1-deficient MEFs are highly insulin resistant, a phenotype that is also contributed by ER stress. Since ER stress increases $IRS1^{Ser307}$ phosphorylation, we investigated the role of this phosphorylation site in mTORC1-induced insulin resistance. Our results indicate that Ser307 phosphorylation in IRS1 does not play a role in development of glucose intolerance and insulin resistance in livers of mice in which mTORC1 signaling is hyperactive. Our observations on TSC1 deficiency-induced glucose intolerance *in vivo* indicate that TSC1-deficiency, and consequently mTORC1 hyperactivity, causes variable glucose intolerance, dependent upon unknown factors; crossing C57BL6 $TSC1^{f/f}$ mice into a different C57BL6 background strain (produced and maintained in another institute) yielded wild-type control mice that failed to exhibit significant glucose intolerance (not shown). Regardless, in mice wherein mTORC1 hyperactivity clearly did cause glucose intolerance, this was not ameliorated by $IRS1^{Ser307}$ mutation.

Observations in this study further suggest that usage of $IRS1^{Ser307}$ phosphorylation as a general insulin resistance marker might not reflect mechanistic importance. This phosphorylation site has been used as a marker for insulin resistance in the field for some time; current evidence, with the results obtained in this study, indicates that this belief needs to be re-evaluated.

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