Acute mTOR inhibition induces insulin resistance and alters substrate utilization in vivo

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ABSTRACT

The effect of acute inhibition of both mTORC1 and mTORC2 on metabolism is unknown. A single injection of the mTOR kinase inhibitor, AZD8055, induced a transient, yet marked increase in fat oxidation and insulin resistance in mice, whereas the mTORC1 inhibitor rapamycin had no effect. AZD8055, but not rapamycin reduced insulin-stimulated glucose uptake into incubated muscles, despite normal GLUT4 translocation in muscle cells. AZD8055 inhibited glycolysis in MEF cells. Abrogation of mTORC2 activity by SIN1 deletion impaired glycolysis and AZD8055 had no effect in SIN1 KO MEFs. Re-expression of wildtype SIN1 rescued glycolysis. Glucose intolerance following AZD8055 administration was absent in mice lacking the mTORC2 subunit Rictor in muscle, and in vivo glucose uptake into Rictor-deficient muscle was reduced despite normal Akt activity. Taken together, acute mTOR inhibition is detrimental to glucose homeostasis in part by blocking muscle mTORC2, indicating its importance in muscle metabolism in vivo.

Keywords Skeletal muscle; Metabolism; Glucose uptake; mTORC2; Rictor; Glycolysis

1. INTRODUCTION

The mammalian target of rapamycin (mTOR) is the catalytic subunit for two functionally and structurally distinct mTOR complexes (mTORC1 and mTORC2) [1]. mTORC1 is a major regulator of protein synthesis and cell growth [1]. Less is known, especially in vivo, about the more recently discovered mTORC2, but in the central nervous system, the liver and fat tissue it regulates glucose metabolism [2–5]. mTORC2 is also a major upstream kinase for the Akt Ser473 residue [6]. Understanding mTOR biology is vital in cancer, metabolism and aging research. Hyper-activated mTOR is common and possibly causal in cancer, because mutations of negative upstream regulators of the mTOR pathway lead to tumorigenesis [7]. Knockout of the mTOR target S6K1 [8] or long-term mTOR blockade by rapamycin treatment [9–13] extends lifespan in mice. However, other reports have shown detrimental health effects like insulin resistance and reduction in beta-cell mass following prolonged rapamycin treatment [14–19]. The tools to study mTOR biology on a whole-body level in mice are limited because knockout (KO) of mTOR is embryonic lethal and inducible global KO models can be “leaky” in some tissues [19]. Acute rapamycin treatment inhibits mTORC1, but it does so incompletely [20]. Chronic rapamycin treatment can abrogate mTORC2 activity [21], and insulin resistance in mice treated with rapamycin for several weeks has been attributed to impaired liver mTORC2 activity [19]. In other long-term rapamycin studies, however, in which rodents also exhibited insulin resistance, mTORC2 activity was normal judged by liver Akt S473 phosphorylation [15,17,18]. Moreover, different durations of chronic rapamycin treatment lead to different phenotypes along with varying degrees of mTORC2 activity [15]. Therefore, while the effects of rapamycin remain important to study, it may not be the best tool to study mTOR signaling per se. A new generation of mTOR kinase inhibitors (mTORKI) acutely and potently inhibits both mTOR complexes, but the in vivo effects on metabolism have not been extensively investigated. These mTOR KIs are ATP-competitive mTOR inhibitors, whereas rapamycin forms a complex with FKBP12 to allosterically inhibit mTORC1. We used the mTOR-KI AZD8055 to study the effects of acute mTOR inhibition on glucose metabolism. We contrasted our findings to acute rapamycin treatment to seek out AZD8055 effects due to mTORC2 inhibition, which we further explored by using genetic mTORC2 KO models (SIN1 and Rictor KO).

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2. MATERIALS AND METHODS

2.1. Animals
Female C57BL/6 mice (Taconic, Denmark) aged 11–12 weeks old were used, as well as female muscle specific Rictor knock-out (mKO: Rictor<sup>Cre<sup>Flxed/F<sup>loxed</sup>), HSA-<sup>Cre</sup><sup>Flxed/Flxed</sup>) and wildtype (WT: Rictor<sup>Cre<sup>Flxed/F<sup>loxed</sup>, HSA-<sup>Cre</sup><sup>Flxed/Flxed</sup>) littermates, aged 11–14 weeks. Tissues were homogenized in 5% (w/v) Tween-80 and 5% (w/v) ethanol) was diluted to 0.4 mg/ml in 5% (w/v) Tween-80 and 5% (w/v) PEG solution was used. For all treatments 10 µl per gram mouse mass were injected. Mice were acclimatized to indirect calorimetry cages (PhenoMaster, TSE, Bad Homburg, Germany) for 3 days. After an overnight fast, mice were intraperitoneal (IP)-injected with vehicle or AZD8055 (10 mg kg<sup>−1</sup>) [10] and given free access to food. For glucose tolerance test, insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was added during the last 30 min of inhibitor and DMSO presence, resulting in an insulin concentration of 60 nM. During the last 10 min of insulin stimulation 2-DG uptake was measured with <sup>3</sup>H-2-DG and <sup>14</sup>C-Mannitol radioactive tracers and 1 mM of 2-DG [25]. Muscles were washed in ice-cold KRH buffer, blotted dry and snap-frozen in liquid nitrogen, trimmed and weighed, before stored at −80 °C.

2.2. In vivo AZD8055 and rapamycin injection experiments
AZD8055 and rapamycin (Selleckchem, Houston, USA) were formulated according to Chresta et al. [23] and Chen et al. [10], respectively. In brief, AZD8055 was prepared at 1 mg/ml in a 30% (w/v) Captisol<sup>®</sup> (Ligand Pharmaceuticals, Inc, La Jolla, CA, USA) solution and the pH was adjusted to 3. For vehicle injections, 30% (w/v) Captisol<sup>®</sup>, pH3, was used. For rapamycin, a rapamycin stock solution (12.5 mg/ml in ethanol) was diluted to 0.4 mg/ml in 5% (v/v) Tween-80 and 5% (w/v) polyethylene glycol (PEG) (Sigma–Aldrich). For vehicle injections a 5% (w/v) Tween-80 and 5% (w/v) PEG solution was used. For all treatments 10 µl per gram mouse mass were injected. Mice were acclimatized to indirect calorimetry cages (PhenoMaster, TSE, Bad Homburg, Germany) for 3 days. After an overnight fast, mice were intraperitoneal (IP)-injected with vehicle or AZD8055 (10 mg kg<sup>−1</sup>) [23]; or vehicle or rapamycin (4 mg kg<sup>−1</sup>) [10] and given free access to food for 6 h. Expired carbon dioxide (VCO<sub>2</sub>) and inhaled oxygen (VO<sub>2</sub>) were measured to calculate respiratory exchange ratio (RER). Calculations of carbohydrate and fat utilization were adopted from Couture et al. [24] and normalized to mouse body mass. For plasma glucose determination, mixed tail blood was obtained at baseline, after an overnight fast and at 1, 3, 6, and 24 h following AZD8055 injection, during which mice had free access to food, and analyzed in duplicates with a glucometer just before drug injection (Contour XT, Bayer Consumer Care AG, Basel, Switzerland). For plasma insulin determination at the 3 h time-point mixed tail blood was collected into heparinized capillary tubes and plasma insulin levels were assayed with an ELISA kit (Mouse Ultra-sensitive Insulin ELISA, #80-INSMSU-E01, Alpco, Salem, USA). Plasma fatty acids (FAs) were determined with a colorimetric commercial kit (NEFA-HR2) kit, Wako Chemicals, Germany) using a Hitachi 912 Automatic Analyzer, Germany. For glucose tolerance test Rictor WT and mKO mice were fasted for three hours, injected with vehicle or AZD8055, and two hours later IP-injected with 2 g/kg bodyweight o-glucose (0.2 g in 1 ml saline). Blood glucose concentration in mixed tail blood was obtained in duplicates with a glucometer just before drug and glucose injection (−120 and 0 min, respectively) and 20, 40, 60, 90 and 120 min after glucose injection. For the signaling experiment mice were overnight fasted, injected with vehicle or AZD8055 and granted free access to food. Two hours after injection mice were euthanized by cervical dislocation and gastrocnemius, quadriceps, liver, heart and gonadal adipose tissue were quickly dissected out and stored at −80 °C.

2.3. In vitro mouse muscle incubations and 2-deoxyglucose (2-DG) uptake
Soleus and EDL muscles from both legs were dissected out from 1 h fasted and anesthetized mice (6 mg pentobarbital and 0.24 mg lidocaine/100 g body wt). Mice were euthanized by cervical dislocation after muscles had been removed. Muscles were gently lengthened to resting tension (4–5 mN) in incubation chambers (Multi Myograph system; Danish Myo-Technology, Denmark). As described previously [25], these compartments contained 4 ml heated (30 °C) Krebs–Ringer–Henseleit (KRH) buffer supplemented with 2 mM pyruvate, 8 mM mannitol and 0.1% BSA. Muscles were incubated for 60 min with either 0.1% DMSO, 640 nM AZD8055 or 1 µM Rapamycin. When appropriate, insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was added during the last 30 min of inhibitor and DMSO presence, resulting in an insulin concentration of 60 nM. During the last 10 min of insulin stimulation 2-DG uptake was measured with <sup>3</sup>H-2-DG and <sup>14</sup>C-Mannitol radioactive tracers and 1 mM of 2-DG [25]. Muscles were washed in ice-cold KRH buffer, blotted dry and snap-frozen in liquid nitrogen, trimmed and weighed, before stored at −80 °C.

2.4. 2-DG uptake and GLUT4 translocation and signaling in L6 myoblasts
L6 cells stably over-expressing GLUT4 with a c-myc epitope tag (L6-GLUT4myc) [26], a kind gift from Amira Klip, were grown in z-mEM media (GIBCO #1257-063) with 10% fetal bovine serum (GIBCO #26140-038), 100 units/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL Fungizone<sup>®</sup> (GIBCO #15240-062) (5% CO<sub>2</sub>, 37 °C). For uptake, translocation and signaling experiments cells were grown until confluent and the following experimental scheme was applied: Cells were serum starved for four hours. During the last 50 min of starvation, inhibitors (0.1%DMSO, 500 nM AZD8055 or 100 nM Rapamycin) were added. Twenty minutes before the end of starvation, insulin (10 or 100 nM) was added when appropriate. 2-DG uptake over five minutes was then determined as previously described by Somwar et al. [27] and related to protein concentration in each well. Background determined with cytochalasin B was subtracted and experiments were assayed in duplicates. For the GLUT4 translocation assay, the media was removed at the end of basal or insulin stimulation and cells were immediately chilled on ice, fixed with 3% paraformaldehyde, blocked in 5% goat serum, and incubated with primary anti-myc antibody (Cell Signaling Technology). The signal was detected with a secondary HRP-antibody and o-Phenylenediamine reagent (Sigma–Aldrich) was added to each well to initiate a color reaction with the secondary antibody. This was terminated with 5 M HCl, before absorbance at 492 nm was measured. Background absorbance (no primary antibody) was subtracted. Each experiment was assayed in at least triplicates.

2.5. Glucose transport into giant sarcocemmal vesicles from rats
To investigate whether AZD8055 has direct effect on glucose transporters, giant sarcolemms vesicles (GSV) were prepared from rat gastrocnemius muscle from four pooled rats as previously described [28]. For the glucose transport experiment, aliquots of freshly prepared GSV suspension were pre-incubated in KC1–MOPS buffer (140 mM KCl and 10 mM MOPS) with or without 640 or 1280 nM AZD8055 for 15 min at RT. Uptake medium was added for 45 s to yield a final concentration of 0.5 µCi ml<sup>−1</sup> <sup>3</sup>H-2-deoxyglucose (2DG), 0.1 µCi ml<sup>−1</sup> <sup>14</sup>C-Mannitol, 5 mM 2DG and 35 mM Mannitoll (all in KC1–MOPS buffer). Glucose transport was terminated by adding ice-cold stop solution (KC1–MOPS containing 50 µM cytochalasin B). Specific glucose transport was calculated as the difference between total <sup>3</sup>H-2DG and extravascular <sup>14</sup>C-2DG.

2.6. Western blot analysis
Tissues were homogenized 3 × 30 s at 30 Hz using a Tissuelyser II (Qiagen, USA) in ice-cold homogenization buffer (10% Glycerol, 20 mM
The accumulation of phosphorylated 3H-2-DG (3H-2-DG-6-P) with the 0.1 mM 2-deoxyglucose (2-DG) and 60 min fasted (Baseline mixed tail blood was collected from conscious and overnight-fasted (12 h) mice, before mice were IP injected (10 mg/kg body weight) with either saline or glucose (2 g/kg body mass) containing 0.1 mM 2-deoxyglucose (2-DG) and 60 μCi/ml 3H-labeled-2-DG [30]. Additional blood was drawn at 15 and 30 min post-injection. Immediately after the last blood draw mice were sacrificed by cervical dislocation and quadriceps muscles were rapidly excised and snap frozen in liquid nitrogen. The blood draws were used for blood glucose and plasma insulin determinations, which were carried out as described above. In addition, 10 μl of plasma was used to measure the plasma 3H activity by scintillation counting. Subsequently the area under the curve, AUC 3H-2-DG (3H-2-DG-6-P) with the precipitation method [30]. Quadriceps muscle glycogen content was measured as glycosyl units after acid hydrolysis and muscle glucose was determined fluorometrically from neutralized perchloric acid extracts [31].

2.9. Statistical analysis
The error bars represent SEM. Statistical testing was performed using two-tailed Student t-tests when comparing two means. Analysis of variance (ANOVA) or, when experiments involved multiple testing, repeated measures ANOVA with Holm Sidak post hoc tests were performed when comparing more than two means. Statistical evaluation was performed using SigmaPlot 11.0. The significance level was set at p < 0.05.

3. RESULTS

3.1. AZD8055 but not rapamycin alters substrate utilization in vivo
We injected AZD8055 or rapamycin at doses used in preclinical cancer mouse studies [10,23] into fasted C57BL/6 mice and measured metabolic flexibility with indirect calorimetry during re-feeding. AZD8055, but not rapamycin, markedly decreased the respiratory exchange ratio (RER) (Figure 1A,B) without alterations in food consumption (Figure 1C,D). AZD8055 treated mice had reduced carbohydrate and increased fat utilization (Supplemental Figure 1a,b), while rapamycin treated mice showed no changes (Supplemental Figure 1c,d).

3.2. AZD8055 induces insulin resistance in vivo
Glucose levels in AZD8055 injected mice were elevated 3 and 6 h after drug injection but were similar to control mice at 24 h after drug injection (Figure 2A). Along with elevated glucose levels at 3 h, AZD8055 treated mice had 3-fold higher plasma insulin levels (Figure 2B) and lower plasma FAs (Figure 2C). These data indicate whole-body insulin resistance and suggest an increased reliance on FAs as an energy substrate in agreement with the indirect calorimetry data (Figure 1A).

3.3. AZD8055 reduces mTOR and Akt signaling in vivo
AZD8055 injection blocked phosphorylation of the mTORC1 readout p70S6K1 Thr389, the mTORC2 substrate, Akt Ser473, and the mTORC2 biomarker, NDRG1 Thr346 in skeletal muscle, adipose tissue, liver, and cardiac muscle 2 h after injection (Figure 2D–F). Phosphorylation of Akt Thr308, which is essential for full Akt kinase activation and may regulate p-AktSer473 [32], was unaffected by AZD8055 in skeletal muscle and liver tissue and increased in cardiac muscle and adipose tissue (Figure 3A). Phosphorylation of Akt substrate of 160 kDa (AS160), an Akt target and a key mediator of insulin-induced glucose uptake [33,34] was unchanged in skeletal and cardiac muscle following mTOR inhibition (Figure 3B). p-AS160 was decreased following AZD8055 treatment in adipose tissue whereas it was undetectable in liver (Figure 3B). AZD8055 injection had largely no effect on the amount of total AS160, p70S6K1, Akt2 and NDRG1 expression detected by immunoblotting (Supplemental Figure 2), except for a 9% decrease in gastrocnemius p70S6K1 levels (Supplemental Figure 2c) and a 30% decrease in adipose tissue AS160 levels (Supplemental Figure 2b).

3.4. AZD8055 but not rapamycin impairs insulin-stimulated glucose uptake in vitro
Acute AZD8055 treatment (1 h) impaired insulin-stimulated glucose uptake in soleus and EDL muscle by ~30%, while rapamycin (1 h) had no effect (Figure 4A,B). Basal glucose uptake was unaffected.

2.7. SIN1 cell work
SIN1 KO MEFs were a kind gift from Bing Su. WT HA-SIN1 was re-expressed in SIN1−/− MEFs using the pMIG retrovirus vector which co-expresses GFP with HA-SIN1. 48 h after infection, GFP-positive cells were isolated by fluorescence-activated cell sorting. WT MEFs, MEFs with SIN1−/− and SIN1−/− MEFs reexpressing HA-SIN1 were washed and treated for 16 h in 0.1% FCS, or in 10% FCS supplemented with 0.1% DMSO, 10 μM Akt inhibitor MK2006 or 500 nM AZD8055 as indicated. Media lactate levels were measured using the hydrasine sink method, as described previously [29], and normalized to cellular protein levels, measured using the BCA assay (Pierce).

2.8. In vivo glucose-induced glucose uptake and glycogen and muscle glycogen determinations
Baseline mixed tail blood was collected from conscious and overnight-fasted (~12 h) mice, before mice were IP injected (10 mg/kg body mass) with either saline or glucose (2 g/kg body mass) containing 0.1 mM 2-deoxyglucose (2-DG) and 60 μCi/ml 3H-labeled-2-DG [30]. Additional blood was drawn at 30 min post-injection. Immediately after the last blood draw mice were sacrificed by cervical dislocation and quadriceps muscles were rapidly excised and snap frozen in liquid nitrogen. The blood draws were used for blood glucose and plasma insulin determinations, which were carried out as described above. In addition, 10 μl of plasma was used to measure the plasma 3H activity by scintillation counting. Subsequently the area under the curve, AUC 3H-2-DG (3H-2-DG-6-P) was calculated by the trapezoid method to estimate the 3H-2-DG levels the muscles had been exposed to [30]. Fifty milligrams of quadriceps muscle was used to determine the accumulation of phosphorylated 3H-2-DG (3H-2-DG-6-P) with the 3H activity by scintillation counting. Subsequently the area under the curve, AUC 3H-2-DG (3H-2-DG-6-P) was calculated by the trapezoid method to estimate the 3H-2-DG levels the muscles had been exposed to [30]. Fifty milligrams of quadriceps muscle was used to determine the accumulation of phosphorylated 3H-2-DG (3H-2-DG-6-P) with the
suggesting GLUT1 function was unperturbed by the mTOR blockers (Figure 4A,B). Similar to the *in vivo* effects, AZD8055 treatment inhibited readouts of both mTOR complexes (p-T70S6K1Thr389, AktThr473, NDRG1Thr346) (Figure 5B-D, G-I). Acute rapamycin only inhibited the mTORC1 readout p-p70S6K1Thr389 (Figure 5D,I), but to a lesser extent than AZD8055. In contrast to *in vivo*, AZD8055 decreased p-AktThr308 in both soleus and EDL muscles (Figure 5E,J). Rapamycin enhanced insulin-stimulated p-AktThr308 in soleus, but had no effect in EDL muscle (Figure 5E,J). p-AS160 was insulin responsive, but was not affected by the mTOR inhibitors in either soleus or EDL muscle (Figure 5F,K). AZD8055 or Rapamycin treatment had no effect on total protein expression of Akt2, NDRG1, AS160, and p70S6K1 (Supplemental Figure 3). However, insulin treatment increased the amount of total AS160 detected by immunoblotting by ~23% and ~21% in soleus and EDL muscle, respectively (Supplemental Figure 3c,h).

3.5. AZD8055 reduces glucose uptake but not GLUT4 translocation and activity

AZD8055 reduced glucose uptake in L6 muscle cells during insulin stimulation (100 nM), while rapamycin had no effect (Figure 6A,B). This inhibitory effect on glucose uptake occurred despite normal insulin-stimulated (10 and 100 nM) GLUT4 translocation following AZD8055 and rapamycin pre-treatment (Figure 6C). To test whether AZD8055 had off-target effects on the glucose transporters we used giant sarcolemmal vesicles (GSV). In these vesicles glucose transporter translocation is absent and glucose transport relies solely on the glucose transporter present in the vesicle plasma membrane. AZD8055 had no effect on glucose uptake into these vesicles (Figure 6D), even at a higher concentration than was used in any of the experiments, while Cytochalasin B had the expected inhibitory effect (Figure 6D). Together these data indicate that AZD8055 inhibits glucose uptake in muscle cells independently of GLUT4 translocation and activity.

3.6. AZD8055 reduces glycolysis by blocking mTORC2 activity

In light of the observed dissociation between normal GLUT4 translocation, AS160 phosphorylation, and reduced glucose uptake with AZD8055 treatment, we hypothesized an intracellular glucose handling defect. Recent reports have suggested that mTORC2 is a positive regulator of glycolysis [2,35]. To this end we measured lactate release into media of mouse embryonic fibroblast cells that were missing the critical mTORC2 subunit Sin1 (Sin1 KO MEFs) and two control cell conditions: wildtype (WT) MEFs and Sin1 KO MEFs in...
which WT SIN1 was re-introduced (SIN1 KO + WT). Lactate release was increased by serum stimulation in WT MEF cells and in SIN1 KO + WT cells, but no response was observed in SIN1 KO cells (Figure 6E). AZD8055 treatment diminished lactate release in SIN1 WT and SIN1 KO + WT cells, but the Akt inhibitor, MK2206, did not. Neither drug affected SIN1 KO cells. Thus, impairing mTORC2 activity either genetically or pharmacologically reduced glycolytic flux in these cells.

3.7. AZD8055 reduces glucose tolerance by blocking mTORC2 activity in skeletal muscle

In WT mice, AZD8055 injection led to glucose intolerance (Figure 6F,G). In mice lacking the mTORC2 subunit, Rictor, in skeletal muscle (Ric mKO), AZD8055 had no effect (Figure 6F,G). Furthermore, vehicle treated Ric mKO mice exhibited glucose intolerance compared to vehicle treated Rictor WT mice (Figure 6F,G). This indicates that acute mTOR inhibition by AZD8055 perturbs glucose tolerance in part by blocking mTORC2 activity in skeletal muscle.

3.8. In vivo glucose uptake into muscle is impaired in muscle lacking mTORC2 activity despite normal AS160 phosphorylation

Given the glucose intolerance of Ric mKO mice, which has been reported by others [36], we investigated whether in vivo glucose uptake into muscle of Ric mKO mice was impaired. To this end we aimed to mimic a glucose tolerance test. Conscious mice were injected with saline or a glucose solution spiked with ^3H-2-DG to assess glucose uptake in skeletal muscle. Ric mKO exhibited glucose intolerance (Figure 7A), with no impairment in glucose-induced insulin release (Figure 7B). With saline injection, plasma insulin levels were lower at 15 min and returned to baseline levels by 30 min (Figure 7B) in both Ric WT and mKO mice. Overall glucose clearance (Kg) was 30% lower in Ric mKO muscle (Figure 7C). Glucose-induced glucose uptake (Rg), which accounts for the mass action of the plasma glucose concentrations, was 30% lower in Ric mKO muscle (Figure 7D). Muscle glycogen content and muscle free glucose concentrations were elevated by ~24% and ~16%, respectively, in Ric mKO muscle (Figure 7E,F). Phosphorylation of AktSer473 was markedly reduced and...
Figure 3: AZD8055 differentially affects p-Akt<sup>Thr308</sup> and p-AS160 in vivo (A,B) Representative western blots and quantitative analysis of p-Akt<sup>Thr308</sup> and pan-AS160 phosphorylation in mouse quadriceps, gastrocnemius, liver, heart, and adipose tissue two hours after vehicle or AZD8055 (10 mg kg<sup>-1</sup>) injection (n = 8, for adipose tissue n = 4). *p < 0.05 different from vehicle within each tissue. Data are means ± SEM.

Figure 4: AZD8055, but not rapamycin, reduces muscle glucose uptake ex vivo. (A,B) Mouse soleus and EDL muscles were incubated ex vivo in the presence of 0.1% DMSO, 640 nM AZD8055 or 1 μM rapamycin for 30 min, followed by basal or insulin (60 nM) stimulation. Glucose uptake was measured with <sup>3</sup>H-2-deoxyglucose (n = 7–8). *p < 0.05 different from corresponding DMSO. Data are means ± SEM.
unresponsive to glucose stimulation (Figure 8B), while p-AktThr308 was elevated following saline and glucose stimulation in Ric mKO muscle (Figure 8C). Similarly p-AS160 Thr642 was also increased following saline and glucose stimulation in Ric mKO muscle (Figure 8E). p-GSK3ßSer9, a readout of Akt activity, was similar in Ric WT and mKO muscle and responsive to glucose stimulation (Figure 8G). GLUT4, GSK3-beta and AS160 protein expression were unchanged between the genotypes (Figure 8F,H,I), while Ric mKO muscle had 40% less Akt2 protein (Figure 8D). These data indicate that mTORC2 activity is necessary for normal muscle glucose uptake in vivo, despite unimpaired Akt activity, judged by downstream targets.

4. DISCUSSION

We provide in vivo and in vitro evidence that acute mTOR inhibition is detrimental to whole-body and skeletal muscle glucose metabolism. The effects on the whole-body include insulin resistance, increased lipid oxidation and glucose intolerance. The latter is largely due to blocking skeletal muscle mTORC2 activity. In muscle, acute mTOR inhibition leads to insulin resistance in vitro, although insulin-stimulated GLUT4 translocation is normal with AZD8055 treatment in muscle cells. Rictor deficient muscles exhibit reduced insulin-stimulated glucose uptake in vivo along with glucose accumulation. Abrogation of mTORC2 activity by SIN1 knockout or AZD8055 treatment decreased glycolysis in MEF cells. Collectively our data suggest that acute mTOR inhibition by AZD8055 or genetic abrogation of mTORC2 activity impairs glycolysis which reduces insulin sensitivity of glucose uptake in muscle.

We focused on acute mTOR inhibition with AZD8055, a highly selective and bioavailable mTOR-KI [23], to investigate experimental outcomes that stem from acute post-translational signaling changes due to inhibited mTORC1 and mTORC2 kinase activity. Others have studied the acute effect of mTORC1 inhibition by rapamycin. In contrast to the acute effect of AZD8055, acute rapamycin treatment may actually have insulin sensitizing effects. Insulin activates mTORC1 and its substrate S6K1, which phosphorylates and inhibits the insulin receptor substrate 1 (IRS-1). By blocking mTORC1, rapamycin disrupts this negative feedback loop and thereby increases IRS-1 activity, which is reflected in increased Akt activity in adipose and muscle cells [37,38]. In agreement, in humans acute rapamycin-treatment can actually improve whole-body insulin sensitivity [39]. Our data support this idea, because in soleus muscle insulin-stimulated p-AktThr308 is enhanced with rapamycin treatment. When we administered rapamycin we neither observed substrate changes, nor did we detect an inhibitory effect of rapamycin on muscle glucose uptake in vitro. Supporting our data, rapamycin injection in ob/ob mice, at a concentration similar to what we injected in the present study, had no effect on glucose or insulin tolerance, despite reducing S6K1 phosphorylation in muscle [40]. Collectively, these data suggest that the detrimental effect of AZD8055 on glucose metabolism is not mediated by mTORC1 signaling.
However, rapamycin does not fully inhibit mTORC1, whereas mTOR KIs likely do [20]. Evidence for this can also be found in the present study. AZD8055 inhibited p-S6K1 more effectively than rapamycin in incubated muscle. Therefore, AZD8055 could affect metabolism by inhibiting rapamycin-insensitive mTORC1 signaling. However, similar to acute rapamycin treatment, abrogation of mTORC1 activity by raptor deletion increases p-Akt in muscle cells and skeletal muscle [22,38]. Moreover, deletion of raptor in adipose tissue protects mice from high fat diet induced weight gain and improves their glucose tolerance [41]. This indicates that inhibited mTORC1 signaling by AZD8055 likely does not account for the metabolic effects observed by AZD8055.

Long-term rapamycin treatment has been used to inhibit mTORC1 and mTORC2 signaling. However, it takes several days for rapamycin to inhibit both mTOR complexes and this does not affect all tissues equally [1,14,15,17,18]. Long-term rapamycin treatment in mice also causes changes in gene transcription, body mass and food intake. To better understand the effect of AZD8055 on insulin action and glucose uptake in skeletal muscle we used the in vitro muscle incubation method. Insulin-stimulated glucose uptake was diminished by AZD8055.
AZD8055. Surprisingly insulin-stimulated GLUT4 translocation was normal in L6 cells, despite a similar glucose uptake defect as observed in mature striated muscle. Normal glucose transport into GSV indicates that AZD8055 has no direct inhibitory effect on the glucose transporter activity. In agreement with normal GLUT4 translocation observed in AZD8055-treated L6 myocytes, phosphorylation of AS160, a key regulator of GLUT4 translocation [33,34] and a well established Akt substrate, was normal in incubated muscle treated with AZD8055 under both basal and insulin-stimulated conditions. Overall our data suggest that glucose transport capacity was unaffected by AZD8055, because GLUT4 translocation and activity and the phosphorylation of the key regulator, AS160, were normal.

Consequently, we explored whether AZD8055 treatment leads to an intracellular glucose handling defect. In this light it is interesting that mTORC2 has been reported as a positive regulator of glycolysis in cancer and liver cells [2,35]. We reasoned that an AZD8055-induced defect in glycolysis may adversely affect the glucose concentration gradient necessary for normal glucose uptake and that this is mediated by blocking mTORC2 activity. In agreement with our hypothesis, AZD8055 decreased glycolysis judged by lactate release in MEF cells. Similarly, disruption of mTORC2 activity in MEFs, by knockout of SIN1, also impaired glycolysis, but this defect was rescued by re-introduction of wildtype SIN1 protein. Since AZD8055 had no effect on glycolysis in SIN1 KO MEFs, but reduced glycolysis in wildtype MEFs and in SIN1 KO MEFs that received wildtype SIN1 protein, we conclude that AZD8055 impairs glycolysis by inhibiting mTORC2 activity. Furthermore, given that the Akt inhibitor, MK2206, had no effect of glycolysis, AZD8055 and mTORC2 likely impact glycolysis independently of Akt signaling.

Glucose uptake was impaired in Rictor deficient muscles in vivo. Once again this occurred despite normal Akt activity, but Rictor mKO muscles had increased muscle glucose levels. This glucose build-up is compatible with an attenuated glycolytic flux and diminishes the transmembrane glucose gradient explaining the decreased glucose uptake. The increased glycogen content in Rictor mKO muscle likely reflects the shunting of glucose into storage, which also fits with a decrease in glycolytic flux.

Of some debate is whether phosphorylation of Akt Ser473 depends on or influences Thr308 phosphorylation, and vice versa [32]. In the present study, acute mTOR inhibition by AZD8055 markedly lowered p-AktSer473 without much impact on p-AktThr308 in vivo. In incubated muscles, p-Akt (Thr308) was lower with AZD8055 treatment, but remained insulin responsive. Abrogation of mTORC2 activity by Rictor deletion in skeletal muscle also markedly lowered p-AktSer473, but this had no inhibitory effect on p-Akt (Thr308), which has been reported in muscle by others [22,36]. Deletion of the kinase responsible for Akt Thr308 phosphorylation, PDK1, has reportedly no inhibitory effect on p-AktSer473 in liver and cardiac tissue [42-44]. Together these data indicate that Ser473 or Thr308 phosphorylation do not influence one another markedly. Furthermore, the effect of decreased p-AktSer473 on overall Akt activity is elusive. Judging by in vivo substrates, such as GSK3 and TSC2 phosphorylation, Akt activity is largely normal with genetic disruption of mTORC2 (Rictor or SIN1 KO) and subsequent reduction in p-AktSer473 [3,36,45-47]. In contrast, Akt phosphorylation sites on FoxO1 and FoxO3a have been found to be impaired with blockade of mTORC2 activity by several groups [3,4,36,45,46]. It remains to be seen whether reduced p-AktSer473 affects a certain subset of Akt substrates.

The role of mTORC2 in metabolism in mature skeletal muscle in vivo has not previously been studied. Together with in vitro evidence by others [36,48], our data suggest that mTORC2 may play a role in muscle insulin resistance. Although the liver, like muscle, is an important organ in regulating whole-body glucose homeostasis [49], and liver mTORC2 activity was inhibited following AZD8055 injection, the observation that AZD8055-induced glucose intolerance is absent in Rictor mKO mice indicates that reduced muscle mTORC2 activity accounts for most of the impairment in whole-body glucose metabolism with acute global mTOR inhibition. In contrast, chronic rapamycin treatment leads to whole-body insulin resistance by disrupting liver mTORC2 signaling and increasing liver glucose output [19]. The difference between our study and the chronic rapamycin study [19] likely indicates the different responses to acute and chronic inhibition of the mTOR complexes.
In conclusion, we have shown that acute mTOR inhibition by AZD8055 leads to insulin resistance and glucose intolerance along with changes in substrate utilization. Insulin-mediated glucose transport into muscle cells is perturbed despite normal GLUT4 translocation, which points to an intracellular glucose handling defect. Fittingly, AZD8055 suppressed glycolysis through inhibition of mTORC2, and furthermore Rictor deficient muscle exhibited impaired in vivo glucose uptake along with muscle glucose accumulation. These findings warrant future investigations into the physiological role of mTORC2 in skeletal muscle, which as a large glucose sink is indispensable for normal glucose homeostasis. In addition, the severe metabolic consequences following AZD8055 treatment may limit the clinical usefulness of mTOR-KIs.

AUTHORS’ CONTRIBUTIONS

MK and EAR designed and conceived the studies and wrote the manuscript. MK performed all the mouse studies and the subsequent tissue and plasma analyses, along with data analyses. LSH greatly assisted in all the animal studies and provided guidance in preparation of the manuscript. DJF, JRK, KC and GY performed the WT and SIN1 KO experiments in MEF cells. AJO with assistance from MK performed the L6 cell experiments. TEJ assisted in the in vivo studies in the Rictor mKO mice. MAR generated the Rictor mKO mice. PS performed genotyping of the Rictor mKO mice. ABJ prepared the giant sarcolemmal vesicles. DEJ, BK supervised part of the studies and provided critical guidance in the preparation of the manuscript. All authors read and provided comments on the final version of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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protein regulates GLUT4 translocation. Journal of Biological Chemistry 278: 14599—14602.

[35] Masui, K., Tanaka, K., Akhavan, D., Babic, I., Gini, B., Matsutani, T., et al., 2013. MTOR complex 2 controls glycolytic metabolism in glioblastoma through Fox0 acetylation and upregulation of C-Myc. Cell Metabolism 18:726—739.

[36] Kumar, A., Harris, T.E., Keller, S.R., Choi, K.M., Magnuson, M.A., Lawrence, J.C., 2008. Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances basal glycogen synthase activity. Molecular and Cellular Biology 28:61—70.

[37] Tremblay, F., Gagnon, A., Veilleux, A., Sorisky, A., Marette, A., 2005. Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. Endocrinology 146:1328—1337.

[38] Tzatsos, A., Kandror, K.V., 2006. Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent MTOR-mediated insulin receptor substrate 1 phosphorylation. Molecular and Cellular Biology 26:63—76.

[39] Krebs, M., Brunnair, B., Brehm, A., Artwohl, M., Szendroedi, J., Nowotny, P., et al., 2007. The mammalian target of rapamycin pathway regulates nutrient-sensitive glucose uptake in man. Diabetes 56:1600—1607.

[40] Miller, A.M., Brestoff, J.R., Phelps, C.B., Berk, E.Z., Reynolds, T.H., 2008. Rapamycin does not improve insulin sensitivity despite elevated mammalian target of rapamycin complex 1 activity in muscles of Ob/Ob mice. American Journal of Physiology — Regulatory, Integrative and Comparative Physiology 295:R1431—R1438.

[41] Polak, P., Cybulski, N., Feige, J.N., Auwerx, J., Rüegg, M.A., Hall, M.N., 2008. Adipose-specific knockout of raptor results in lean mice with enhanced mitochondrial respiration. Cell Metabolism 8:399—410.

[42] Mora, A., Davies, A.M., Bertrand, L., Sharif, I., Budas, G.R., Jovanović, S., et al., 2003. Deficiency of PDK1 in cardiac muscle results in heart failure and increased sensitivity to hypoxia. The EMBO Journal 22:4666—4676.

[43] Mora, A., Lipina, C., Tronche, F., Sutherland, C., Alessi, D.R., 2005. Deficiency of PDK1 in liver results in glucose intolerance, impairment of insulin-regulated gene expression and liver failure. Biochemical Journal 385:639—648.

[44] Zhao, X., Lu, S., Nie, J., Hu, X., Wen, L., Wu, X., et al., 2014. Phosphoinositide-dependent kinase and MTORC2 synergistically maintain postnatal heart growth and heart function in mice. Molecular and Cellular Biology 11:1966—1975.

[45] Guertin, D.A., Stevens, D.M., Thoreen, C.C., Burds, A.A., Kalaany, N.Y., Moffat, J., et al., 2006. Ablation in mice of the MTORC components raptor, rictor, or MLST8 reveals that MTORC2 is required for signaling to Akt-FOXO and PKCa, but not S6K1. Developmental Cell 11:859—871.

[46] Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S.Y., et al., 2006. SIR1/MIP1 maintains rictor-MTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell 127:125—137.

[47] Shiota, C., Woo, J.T., Lindner, J., Shelton, K.D., Magnuson, M.A., 2006. Multiallelic disruption of the rictor gene in mice reveals that MTOR complex 2 is essential for fetal growth and viability. Developmental Cell 11:583—589.

[48] Ye, L., Varamini, B., Lamming, D.W., Sabatini, D.M., Baur, J.A., 2012. Rapamycin has a biphasic effect on insulin sensitivity in C2C12 myotubes due to sequential disruption of MTORC1 and MTORC2. Frontiers in Genetics 3.

[49] Moore, M.C., Coate, K.C., Winnick, J.J., An, Z., Cherrington, A.D., 2012. Regulation of hepatic glucose uptake and storage in vivo. Advances in Nutrition: An International Review Journal 3:286—294.