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Measurement of insulin- and contraction-stimulated glucose uptake in isolated and incubated mature skeletal muscle from mice

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Dear Dr. Nandita Singh, Senior Science Editor of *JoVE*

Enclosed please find our manuscript entitled "Measurement of insulin- and contraction-stimulated glucose uptake in isolated and incubated mature skeletal muscle from mice" which we would like to publish as an article in *JoVE*

Since skeletal muscle takes up the majority of glucose that enters the blood circulation after a meal, it is not surprising that invention studies aimed at enhancing the ability of skeletal muscle to take up more glucose has increased substantially the last decades due to the worldwide increased prevalence of life-style related diseases including obesity and type 2 diabetes. Evidently, investigations of skeletal muscle glucose uptake in an isolated and controlled environment are becoming more and more relevant as well as widespread throughout the muscle research community. Currently, methodological studies describing a viable and proper model for the study of glucose uptake in isolated mouse skeletal muscle are not available and therefore no consensus exits on how to perform such analyses. Therefore, we now describe a method in the current manuscript that we have been using and optimizing for more than 20 years in order to provide the muscle research community with insight and knowhow on how to study and evaluate glucose uptake in isolated mouse skeletal muscle. Hopefully, this will bring about more consistent and reliable data on glucose uptake from isolated and incubated mouse skeletal muscle.

Based on the abovementioned, we believe our study is of great interest for the *JoVE* readership as it delicately describes how to measure glucose uptake in isolated mouse skeletal muscle in response to stimuli such as insulin and electrically induced contractions. Furthermore, we provide a comprehensive data set on viability of isolated and incubated mouse skeletal muscle, which has not been reported before. As such, we would be grateful if you would consider our manuscript for publication in *JoVE*.

Yours sincerely

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TITLE:

Measurement of Insulin- and Contraction-Stimulated Glucose Uptake in Isolated and Incubated Mature Skeletal Muscle from Mice

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KEYWORDS:

skeletal muscle, glucose transport, glucose uptake, insulin sensitivity, contraction, explant, ex vivo, in vitro, incubation, radioactive glucose tracers, 2-deoxy-D-glucose

SUMMARY:

Intact regulation of muscle glucose uptake is important for maintaining whole body glucose homeostasis. This protocol presents assessment of insulin- and contraction-stimulated glucose uptake in isolated and incubated mature skeletal muscle when delineating the impact of various physiological interventions on whole body glucose metabolism.

ABSTRACT:

Skeletal muscle is an insulin-responsive tissue and typically takes up most of the glucose that enters the blood after a meal. Moreover, skeletal muscle possesses the ability to increase the extraction of glucose from the blood by up to 50-fold during exercise compared to resting conditions. The increase in muscle glucose uptake during exercise and insulin stimulation is dependent on the translocation of glucose transporter 4 (GLUT4) from intracellular compartments to the muscle surface membrane, as well as phosphorylation of glucose to glucose-6-phosphate by hexokinase II. Isolation and incubation of mouse muscles such as *m. soleus* and *m. extensor digitorum longus* (EDL) is an appropriate ex vivo model to study the effects

of insulin and electrically induced contraction (a model for exercise) on glucose uptake in mature skeletal muscle. Thus, the ex vivo model permits evaluation of muscle insulin sensitivity and makes it possible to match muscle force production during contraction ensuring uniform recruitment of muscle fibers during measurements of muscle glucose uptake. Moreover, the described model is suitable for pharmacological compound testing that may have an impact on muscle insulin sensitivity or may be of help when trying to delineate the regulatory complexity of skeletal muscle glucose uptake.

Here we describe and provide a detailed protocol on how to measure insulin- and contraction-stimulated glucose uptake in isolated and incubated soleus and EDL muscle preparations from mice using radiolabeled [^{3}H]2-deoxy-D-glucose and [^{14}C]mannitol as an extracellular marker. This allows accurate assessment of glucose uptake in mature skeletal muscle in the absence of confounding factors that may interfere in the intact animal model. In addition, we provide information on metabolic viability of incubated mouse skeletal muscle suggesting that the method/model applied possess some caveats under certain conditions when studying muscle energy metabolism.

INTRODUCTION:

Skeletal muscle possesses the ability to extract large quantities of glucose from the extracellular space in response to insulin and exercise. This helps to maintain whole body glucose homeostasis and secures glucose supply during times of high energy demand. Since intact regulation of skeletal muscle glucose uptake has been shown to be important for overall health and physical performance[1,2], measurements of muscle glucose uptake during various conditions have received much attention. In humans and animals, the hyperinsulinemic-euglycemic clamp has been used as the gold standard technique to assess insulin sensitivity in vivo[3,4]. In contrast to findings obtained from an oral glucose tolerance test, the hyperinsulinemic-euglycemic clamp technique does not require intact gastrointestinal function or insulin secretion from the pancreas and thus permits insulin responses to be compared between subjects who exhibit variations in gastro-intestinal and/or pancreatic function. Measurements of muscle glucose uptake in vivo during exercise in humans have been performed frequently since the 1960s[5]. First by the use of arteriovenous balance techniques[6] and later by the use of positron emission tomography (PET) imaging in combination with a positron emitting glucose analogue e.g., ^{18}F-Fluoro-deoxy-glucose[7]. In rodents, exercise-stimulated muscle glucose uptake in vivo is typically performed by the use of radioactive or stable isotope-labeled glucose analogs[8–10].

A complementary method to measurements of muscle glucose uptake in vivo, is to isolate and incubate small muscles from rodents and subsequently measure glucose uptake using radioactive or stable isotope-labelled glucose analogues[11–13]. This method allows accurate and reliable quantification of glucose uptake rates in mature skeletal muscle and can be performed in the presence of various insulin concentrations and during contraction elicited by electrical stimulation. More importantly, measurements of glucose uptake in isolated and incubated skeletal muscle are of relevance when investigating the muscle metabolic phenotype of mice that have undergone various interventions (e.g., nutrition, physical activity, infection, therapeutics). The isolated skeletal muscle model is also a suitable tool for pharmacological compound testing

that may affect glucose uptake per se and/or modify insulin sensitivity[12,14]. In this way, the efficacy of compounds designed to regulate muscle glucose metabolism can be tested and evaluated in a highly controlled milieu before subsequent in vivo testing in pre-clinical animal models.

Under some conditions, metabolic viability may pose a challenge in the isolated and incubated skeletal muscle model system. Indeed, the lack of a circulatory system in the incubated muscles entails that delivery of substrates (e.g., oxygen and nutrients) fully depends on simple diffusion between the muscle fibers and the surrounding environment. In regards to this, it is of importance that the incubated muscles are small and thin and thus, represent less of a barrier for oxygen diffusion during incubation[15]. Especially during prolonged incubations for several hours, hypoxic states may develop due to insufficient oxygen supply resulting in muscle energy depletion[15]. Although various markers of metabolic viability in incubated rat muscle have been reported previously alongside the identification of important variables that help to maintain rat muscle viability[15], a comprehensive evaluation of metabolic viability in small incubated mouse muscles is still warranted. Hence, at present glycogen content has mainly been used by others as a marker of metabolic viability in incubated mouse skeletal muscle[16,17].

Here we describe a detailed protocol to measure basal, insulin- and contraction-stimulated glucose uptake in isolated and incubated soleus and EDL muscle from mice using radiolabeled [^{3}H]2-deoxy-D-glucose and [^{14}C]mannitol as an extracellular marker. In the present study, glucose uptake was measured during a 10-minute period and the method is presented with the use of submaximally and maximally effective insulin concentrations as well as a single contraction protocol. However, the protocols described herein can easily be modified with regards to incubation time, insulin-dosage, and electrical stimulation protocol. Furthermore, we provide a thorough characterization of various markers of metabolic viability in incubated soleus and EDL mouse skeletal muscle. The results indicate that glucose supplementation to the incubation buffer is essential to preserve metabolic viability of muscle incubated for 1 hour.

PROTOCOL:

Procedures involving research animals should be performed in accordance with relevant guidelines and local legislation. All animal experiments used for this study complied with the EU convention for the protection of vertebra used for scientific purposes and were approved by the Danish Animal Experiments Inspectorate.

1. Preparation of the experimental apparatus and suture loops

NOTE: For this study, use an integrated muscle strip myograph system with customized incubation hooks to incubate isolated mouse skeletal muscles (**Figure 1**). This system allows muscle to bathe in a physiological solution with continuous oxygenation (95% O_2 and 5% CO_2) and at constant temperature. The muscle tissue bath is coupled to a force transducer for the measurement of muscle force production during contraction. To elicit and record myo-mechanical responses during contraction, employ an electrical pulse stimulator and a data collection program, respectively. Stimulate the incubated muscles to contract by platinum

electrodes positioned centrally and on both sides of the muscle.

1.1. Switch on the myograph system and warm chambers to 30 °C. Open data collection software compatible with the myograph system and calibrate force transducers to ensure comparability between datasets.

1.2. Start by cutting ~16 cm strands of non-absorbable surgical nylon suture. Use forceps to create a loop of approximately 0.4 cm in diameter from a single strand. Repeat this until enough loops have been made. Each muscle needs two loops – one for the proximal and one for the distal tendon.

2. Preparation of solutions and incubation media

2.1. Preparation of basal incubation media

2.1.1. Prepare the following stock solutions: 2.5 M sodium chloride (NaCl, 250 mL), 0.5 M sodium bicarbonate ($NaHCO_3$, 250 mL), 0.5 M potassium chloride (KCl, 50 mL), 0.25 M calcium chloride ($CaCl_2$, 50 mL), 0.25 M potassium dihydrogen phosphate (KH_2PO_4, 50 mL), 0.25 M magnesium sulfate ($MgSO_4$, 50 mL), 110 mM sodium pyruvate (Na-Pyruvate, 100 mL), 500 mM D-mannitol (100 mL), 1 M 2-deoxy-D-glucose (4 mL), 15% solution of bovine serum albumin (BSA) dialyzed against Krebs-Ringer-Henseleit (KRH) buffer (described below in step 4) (100 mL).

NOTE: Two solutions are required to measure resting and contraction-stimulated glucose uptake. Furthermore, each single insulin concentration used for assessing insulin-stimulated glucose uptake requires two solutions. Thus, in total six different solutions are needed to measure basal, submaximal insulin-, maximal insulin-, and contraction-stimulated glucose uptake in isolated mouse skeletal muscle. In the following 'basal incubation media' refers to media without insulin or radioactive tracers. 'Incubation media' refers to media containing insulin. 'Glucose uptake incubation media' refers to media containing 2-deoxy-D-glucose and radioactive tracers in addition to insulin at a concentration identical to that used in the 'incubation media'.

2.1.2. Prepare a KRH buffer by supplementing ultrapure water (ddH_2O) with NaCl (117 mM), $NaHCO_3$ (24.6 mM), KCl (4.7 mM), $CaCl_2$ (2.5 mM), KH_2PO_4 (1.2 mM), and $MgSO_4$ (1.2 mM). Subsequently gas the KRH buffer with 95% O_2 and 5% CO_2 for 10 min. The desired pH of the KRH buffer should be between 7.35-7.45 at 30 °C. If pH adjustment is performed at room temperature the pH of the KRH buffer should be between 7.25-7.35.

2.1.3. Add BSA (0.1%), Na-Pyruvate (2 mM), and D-mannitol (8 mM) to the gassed and pH-adjusted KRH buffer to complete the basal incubation media. Store basal incubation media in a sealed container to minimize degasification of O_2 and CO_2 and place media at 30 °C.

NOTE: Typically, the osmolarity of the KRH supplements (i.e., Na-Pyruvate, D-Mannitol, and D-glucose) is kept constant across an entire experiment to avoid shrinkage or expansion of the muscle cells. The protocol described herein uses an osmolarity of 10 mM for the KRH

supplements. If a glucose-containing buffer is needed, replace KRH supplements to accommodate the needs, e.g. 5 mM D-glucose and 5 mM D-mannitol.

2.1.4. To avoid possible BSA-associated contaminants in the incubation buffer, dialyze BSA against KRH.

2.1.4.1. To make a 15% BSA stock solution dialyzed against KRH buffer, start by dissolving 300 g of analytical grade fat-free BSA in 900 mL of KRH buffer. Next, boil the dialysis tube in redistilled water until the tubing is soft.

2.1.4.2. Fill the tubing with the BSA-KRH solution and secure the tubing ends. Place the tubing with BSA-KRH in 5 L of KRH buffer and leave it overnight at 4 °C. The following day replace the KRH buffer and leave the tubing with BSA-KRH in KRH buffer overnight at 4 °C.

2.1.4.3. Lastly, collect the BSA-KRH solution from the tubing and add KRH buffer to a final volume of 2 L (i.e., 15% BSA-KRH stock solution).

2.2. Preparation of incubation media containing insulin

2.2.1. For the incubation media containing a submaximally effective insulin concentration, add 1 µL of a 100 mU/mL insulin stock solution per mL of basal incubation media (100 µU/mL insulin).

2.2.2. For the incubation media containing a maximally effective insulin concentration, add 1 µL of a 10 U/mL insulin stock solution per mL of basal incubation media (10 mU/mL insulin).

2.3. Preparation of glucose uptake incubation media

CAUTION: Handling of radioactive material is only allowed in a restricted and controlled area by authorized personnel and some universities, research institutions and companies may require the acquisition of a "Radioactivity Use Permit". Material and waste must be handled according to appropriate local procedures, guidelines, and legislation.

2.3.1. Follow the same procedure as described in section 2.1.2.

2.3.2. Add BSA (0.1%), Na-Pyruvate (2 mM), D-mannitol (7 mM), and 2-deoxy-D-glucose (1 mM) to the gassed and pH-adjusted KRH buffer.

2.3.3. Add [^{3}H]2-deoxy-D-glucose (0.028 MBq/mL) and [^{14}C]mannitol (0.0083 MBq/mL) to the supplemented KRH buffer to complete the glucose uptake incubation media. Store at 30 °C. If [^{3}H]2-deoxy-D-glucose and [^{14}C]mannitol are dissolved in ethanol remove the ethanol by N_2 mediated evaporation before use.

2.3.4. For the glucose uptake incubation media containing a submaximally effective insulin concentration, add 1 µL of a 100 mU/mL insulin stock solution per mL of glucose uptake

incubation media (100 µU/mL insulin).

2.3.5. For the glucose uptake incubation media containing a maximally effective insulin concentration, add 1 µL of a 10 U/mL insulin stock solution per mL of glucose uptake incubation media (10 mU/mL insulin).

3. Animals and dissection of the mouse soleus and EDL muscle for incubation

NOTE: Procedures involving research animals should be performed in accordance with relevant guidelines and local legislation. The described procedure can be used with in-house bred or commercially available male and female mice of various strains and genetic backgrounds. The following procedure is provided for fed female C57Bl/6J mice. On an average, mice were 19 weeks old and weighed 25 g. The mice were maintained on a 12:12 h light-dark cycle with free access to standard rodent chow and water. Animal experiments were initiated at ~ 9:00 AM local time and all animals were sacrificed within a period of 2 h.

3.1. Add 4 mL of pre-warmed (30°C) basal incubation media to each incubation chamber and make sure the basal incubation media is continuously oxygenated with 95% O_2 and 5% CO_2.

3.2. Anaesthetize mice with an intraperitoneal injection of pentobarbital (10 mg/100 g body weight) or other available anaesthesia (e.g., tribromoethanol).

NOTE: Be aware that in some countries a licence to handle pentobarbital and other anaesthetic drugs may be a required. Before muscle dissection can be initiated, anaesthesia of each animal must be properly induced. To ensure this, tail and leg reflexes are controlled for. For optimal results dissection should be well practiced avoiding damaging the muscles during removal.

3.3. Place anaesthetized mice prone on a dissection tray (e.g., styrofoam lid) and pin down a single front paw using a needle.

3.4. Remove the skin from the lower leg and make sure that both the Achilles tendon and knee joint are visible.

3.4.1. For the dissection of soleus muscle, start by attaching a single suture loop to the Achilles tendon. Secure a pean forceps to the Achilles tendon distally of the suture loop and cut to release the soleus and gastrocnemius muscles from the paw. Carefully slide the pean forceps across the mouse thereby exposing the soleus muscle.

3.4.2. Pin down the pean forceps and place a second suture loop around the proximal tendon of the soleus muscle. Next, cut the proximal tendon and dissect soleus (including the two attached suture loops) free of gastrocnemius muscle. Quickly place the soleus muscle in the incubation chamber by attaching each suture loop to the respective hooks.

3.5. Remove the fascia covering the tibialis anterior (TA) muscle using forceps. If done correctly

distal tendons of the TA and EDL muscles should be clear white and visible; and separated from each other.

3.6. Cut the distal tendon of the TA muscle and dissect out the muscle for later analyses (e.g., genotyping). Using forceps, gently liberate the EDL muscle from the surrounding tissues but leave the muscle intact and do not cut the tendons. Place one suture loop around the distal tendon and a second suture loop around the proximal tendon of EDL.

3.7. Next, cut the tendons releasing the EDL muscle with two attached suture loops and quickly place the muscle in the incubation chamber by attaching each suture loop to the respective hooks. In order to not lose tension during incubation and especially during electrically induced contraction of the soleus and EDL muscles, it is of great importance to fix the suture loops around the tendons with tight knots.

3.9. Lastly, euthanize the animal by e.g. cervical dislocation.

3.10. When the muscles have been dissected and placed in incubation chambers, adjust the resting tension of each muscle to ~5 mN and pre-incubate the muscles for at least 10 min before initiating the experimental protocol.

4. Insulin-stimulated glucose uptake in isolated mouse skeletal muscle

4.1. Following step 3.10 replace the basal incubation media with incubation media containing no insulin (basal incubation media), a submaximally effective insulin concentration or a maximally effective insulin concentration and leave in the incubation chambers for 20 min. Space each incubation chamber by 1 min, thereby making time for the subsequent harvest of muscles.

4.2. At the end of the 20 min stimulation period, replace the incubation media with the glucose uptake incubation media containing an identical concentration of insulin and leave in the incubation chambers for 10 min, again with 1 min spacing between each incubation chamber.

4.3. After 10 min of incubation in the glucose uptake incubation media gently remove muscles from the incubation chambers and wash them in ice-cold basal incubation media. Subsequently, quickly dry the muscles on filter paper before the suture loops are removed and muscles are frozen in liquid nitrogen. It is imperative that the incubated muscles are harvested quickly if one also wishes to investigate various intracellular metabolites and protein signaling in addition to glucose uptake.

4.4. Collect 100 µL of the glucose uptake incubation media from each incubation chamber and store it at -20 °C. The amount of radioactivity in these samples will be included in the calculation of muscle glucose uptake.

5. Contraction-stimulated glucose uptake in isolated mouse skeletal muscle

NOTE: To induce contraction of isolated mouse skeletal muscle use the following protocol: 1 train/15 s, each train 1 s long consisting of 0.2 ms pulses delivered at 100 Hz. However, other similar protocols eliciting contraction of isolated mouse skeletal muscle will likely work as well. Importantly, the voltage should be adjusted to generate maximal force development of the incubated muscle, which is dependent on the experimental setup. If this is not ensured, you may risk that not all fibers of the muscle are contracting. In turn, this may induce bias in the dataset.

5.1. Following step 3.10 place the platinum electrodes centrally and on both sides of the muscles. Initiate contraction of the muscles immediately after replacing the basal incubation media with the glucose uptake incubation media. If possible, space each incubation chamber by 1 min, thereby making time for the subsequent harvest of muscles. Remember to record force production from each incubated muscle.

5.2. After 10 min of contraction in the glucose uptake incubation media, remove the platinum electrodes, gently collect the muscles from the incubation chambers and wash them in ice-cold basal incubation media. Subsequently, quickly dry the muscles on filter paper before the suture loops are removed and muscles frozen in liquid nitrogen. The entire muscle harvest procedure should be performed as fast as possible.

5.3. Collect 100 µL of the glucose uptake incubation media from each incubation chamber and store it at -20 °C. The amount of radioactivity in these samples will be included in the calculation of muscle glucose uptake.

6. Skeletal muscle homogenization and processing

NOTE: The given procedure below for muscle homogenization makes it possible to determine both glucose uptake and myocellular signaling by western blotting techniques in the same set of muscle samples.

6.1. Homogenize each muscle in 400 µL of ice-cold buffer with pH 7.5 containing 10% glycerol, 20 mM sodium-pyrophosphate, 1% IGEPAL CA-630 (NP-40), 2 mM phenylmethylsulfonylfluoride (dissolved in isopropanol), 150 mM NaCl, 50 mM HEPES, 20 mM β-glycerophosphate, 10 mM sodium fluoride (NaF), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM glycoletherdiaminetetraacetic acid (EGTA), 10 µg/ml aprotinin, 10 µg/mL leupeptin, 3 mM benzamidine, and 2mM sodium-orthovanadate using steel beads and a tissuelyser (2 x 30 s at 30 Hz). Rotate all homogenates end-over-end for 1 h at 4 °C after which they are centrifuged at 16,000 x *g* for 20 min at 4 °C. Collect the lysate (supernatant) which is used to determine muscle glucose uptake.

7. Determination of radiolabeled 2-deoxyglucose and mannitol

7.1. Add 150 µL of each muscle lysate and 25 µL of the glucose uptake incubation media from each incubation chamber to separate liquid scintillation counting vials containing 3 mL of liquid scintillation fluid. Moreover, prepare two blind control vials only containing 3 mL of liquid

scintillation fluid. Close all the vials and mix thoroughly by vortexing each vial for ~5 s.

7.2. Place the vials in a liquid scintillation counter and measure radioactivity of [^{3}H]2-deoxy-D-glucose and [^{14}C]mannitol according to the manufacturer's guidelines. Record DPM (disintegrations per minute) for each liquid scintillation vial.

8. Calculation of muscle glucose uptake rates

8.1. Use the lysate from step 6.1 to measure the total protein concentration in each muscle sample using standard protein quantification methods (e.g., Bicinchoninic acid or Bradford assays). Calculate the amount of protein (mg) added to each scintillation vial.

NOTE: The rate of glucose uptake for each muscle sample is calculated by subtracting the amount of [^{3}H]2-deoxy-D-glucose located in the extracellular space from the total amount of [^{3}H]2-deoxy-D-glucose in the muscle sample using [^{14}C]mannitol as an extracellular marker. It is assumed that [^{3}H]2-deoxy-D-glucose and [^{14}C]mannitol exhibit similar diffusion properties within the muscle tissue during incubation. Perform the following calculations:

8.2. Start by subtracting the [^{3}H] and [^{14}C] DPM of the blind control samples from all muscle and media samples.

8.3. Determine the muscle extracellular space in µL (µL-ECS):

$[^{14}C]DPM_{muscle} / ([^{14}C]DPM_{media} / M_{vol})$

8.4. Calculate the amount of [^{3}H]DPM in the muscle extracellular space ($[^{3}H]DPM_{ECS}$):

$\mu L\text{-}ECS \times ([^{3}H]DPM_{media} / M_{vol})$

8.5. Calculate the amount of [^{3}H]DPM in the muscle intracellular space ($[^{3}H]DPM_{ICS}$):

$[^{3}H]DPM_{muscle} - [^{3}H]DPM_{ECS}$

8.6. Calculate the muscle glucose uptake rate (µmol / g protein / hour):

$([^{3}H]DPM_{ICS} / ([^{3}H]DPM_{media} / M_{vol}) / [2\text{-deoxy-D-glucose}])) / mg\ protein) / T_{h}$

NOTE: For all the equations above,
$[^{14}C]DPM_{muscle}$ is the amount of [^{14}C]mannitol radioactivity in a muscle sample;
$[^{14}C]DPM_{media}$ is the amount of [^{14}C]mannitol radioactivity in a media sample;
$[^{3}H]DPM_{muscle}$ is the amount of [^{3}H]2-deoxy-D-glucose radioactivity in a muscle sample;
$[^{3}H]DPM_{media}$is the amount of [^{3}H]2-deoxy-D-glucose radioactivity in a media sample;
$[^{3}H]DPM_{ECS}$ is the amount of [^{3}H]2-deoxy-D-glucose radioactivity in the muscle extracellular space;

$[^3H]DPM_{ICS}$ is the amount of [^{3}H]2-deoxy-D-glucose radioactivity in the muscle intracellular space;
µL-ECS is the muscle extracellular space in µL;
M_{vol} is the volume (µL) of incubation media used for scintillation counting (e.g. '25' as mentioned above);
T_h is the time factor used to calculate uptake rates per hour (i.e., '1/6' when incubating muscles with glucose uptake media for 10 min)

8.7. Take into consideration this example calculation. The [^{3}H] and [^{14}C] DPM of the blind control samples (17 and 6, respectively) have been subtracted from the DPM values mentioned below.

$[^{14}C]DPM_{muscle}$: 343
$[^{14}C]DPM_{media}$: 11846
$[^3H]DPM_{muscle}$: 4467
$[^3H]DPM_{media}$: 39814
M_{vol}: 25
mg protein: 0.396 (in 150 µL muscle protein lysate)
[2-deoxy-D-glucose]: 1 (mM)
T_h: 1/6 (h)

µL-ECS = 343 DPM / (11846 DPM / 25 µL) = 0.724 µL

$[^3H]DPM_{ECS}$: 0.724 µL × (39814 DPM / 25 µL) = 1153 DPM

$[^3H]DPM_{ICS}$: 4467 DPM – 1153 DPM = 3314 DPM

Glucose uptake: ((3314 DPM / (39814 DPM / 25 µL) / 1 mmol/L) / 0,396 mg protein) / (1/6 hour) = 31.53 µmol / g protein / hour

9. SDS-PAGE and western blot analyses

9.1. Prepare soleus and EDL muscle lysates in Laemmli buffer and heat for 5 min at 96 °C.

9.2. Separate equal amounts of muscle protein by SDS-SAGE on self-cast gels and transfer the proteins to polyvinylidene fluoride membranes by semidry blotting.

9.3. Subsequently, incubate membranes in Tris-buffered saline containing 0.1% Tween 20 and 2% skim milk and probe membranes with relevant primary and secondary antibodies.

9.4. Detect proteins with chemiluminescence and visualize them by a digital imaging system.

10. Muscle glycogen, nucleotides, lactate, creatine, and phosphocreatine

10.1. Use perchloric acid to extract EDL and soleus muscle samples.

10.2. Subsequently, neutralize samples and analyze them for lactate, creatine, and

phosphocreatine as previously described[18].

10.3. Analyze nucleotide content in EDL and soleus muscle by reverse-phase HPLC following extraction in perchloric acid.

10.4. Determine muscle glycogen content in whole muscle homogenate as glycosyl units after acid hydrolysis by a fluorometric method as previously described[18].

11. Statistics

11.1. Perform statistical analyses with statistical analyses software.

11.2. Use a two-way analysis of variance (ANOVA) test to assess statistical differences between values presented in **Table 1**.

11.2. Use unpaired Student *t* tests to assess statistical differences in glucose uptake between EDL and soleus within each group presented in **Figure 2**. Present data as means ± standard error of the mean (SEM). $P < 0.05$ is considered statistical significant.

REPRESENTATIVE RESULTS:

As shown in **Figure 2** the basal glucose uptake rates were similar between isolated soleus and EDL muscle from female mice. This has also been reported several times before[12,13,19,20]. Glucose uptake increased by ~0.8 and ~0.6 fold reaching 12 and 9 µmol/g protein/h in soleus and EDL muscle, respectively, in response to a submaximally effective insulin concentration (100 µU/mL). This increase was even higher (~4 and ~2 fold reaching 33 and 19 µmol/g protein/h in soleus and EDL muscle, respectively) when muscles were stimulated with a maximally effective insulin concentration (10 mU/mL). Moreover, both submaximal and maximal insulin-stimulated glucose uptake were significantly higher in soleus muscle indicating that soleus muscle exhibits enhanced insulin sensitivity and responsiveness compared to EDL muscle. This may be related to the higher expression of the glucose transporter 4 (GLUT4) as well as the insulin signaling transducer protein kinase B (Akt) in soleus muscle compared to EDL muscle[10,21–24].

Contraction-induced glucose uptake was significantly higher in EDL muscle compared to soleus muscle (**Figure 2**) as also previously reported[13,19]. Thus, glucose uptake increased by ~2 and ~2.5 fold reaching 14 and 22 µmol/g protein/h in soleus and EDL muscle, respectively, in response to electrically induced contractions. **Figure 3** shows maximal muscle force production in soleus and EDL muscle during the 10 min stimulation period. As seen and previously reported[19], the EDL muscle generates more force (225 mN in EDL vs. 150 mN in soleus) during the initial part of the stimulation period. In contrast, the EDL muscle exhibits a faster decline in force production compared to the soleus muscle later on in the stimulation period. These findings are likely due to the difference in fiber type distribution between the soleus (type 1 > type 2) and EDL (type 2 > type 1) muscle[25] as type 2 fibers generate more force but fatigue faster compared to type 1 fibers[26,27].

To evaluate the effect of insulin and contraction on intracellular signaling in isolated soleus and EDL muscle, phosphorylation of Akt Thr308, TBC1 domain family member 4 (TBC1D4) Ser588, AMPKα Thr172 and acetyl-CoA carboxylase (ACC) Ser212 was performed by western blotting techniques (**Figure 4**). As expected, the submaximally and maximally effective insulin concentration induced an increase in the phosphorylation of Akt Thr308 and TBC1D4 Ser588 while contraction induced an increase in the phosphorylation of AMPKα Thr172 and ACC Ser212. Neither insulin nor contraction led to a change in the total protein content of Akt2, TBC1D4, AMPKα2 and ACC in soleus and EDL muscle (**Figure 4**).

Examining various markers of metabolic viability of incubated soleus and EDL muscle, we observed an overall reduction in the levels of adenosine nucleotides (ATP, ADP, AMP) (~15-25%) as well as creatine (~10-35%) regardless of whether the muscles were incubated in the presence of pyruvate or glucose compared to non-incubated muscles (**Table 1**). On the other hand, the drop in glycogen levels observed in soleus and EDL muscles incubated with pyruvate was prevented if the muscles were incubated with glucose. Interestingly though, we observed that the inosine monophosphate (IMP) levels increased several folds but only in incubated soleus muscle. IMP levels typically increase in muscle during severe metabolic stress as the muscle attempts to prevent AMP accumulation by converting AMP into IMP in order to maintain the ATP/ADP ratio[28]. This indicates that the soleus muscle is somewhat more metabolically stressed compared to the EDL muscle during incubation. This notion is also supported by findings of elevated AMPKα Thr172 and ACC Ser212 phosphorylation in the soleus muscle incubated with pyruvate (**Figure 5**). Importantly, the observed increase in IMP levels as well as AMPKα Thr172 and ACC Ser212 phosphorylation decrease when the soleus muscle is incubated with glucose. Hence, it seems advantageous to incubate isolated skeletal muscle in a glucose-containing buffer to minimize fluctuations in adenosine nucleotides and prevent a drop in muscle glycogen when muscles are incubated for a prolonged period of time. With regards to 2-deoxyglucose uptake, we have evidence to suggest that incubating muscles for 6 to 8 h in the presence of pyruvate will increase basal/resting glucose uptake rates. Incubating muscles in a media containing glucose seems to prevent such increase in glucose uptake (unpublished data).

FIGURE AND TABLE LEGENDS:

Figure 1: Incubation system. (**A**) Myograph system with four single incubation chambers. (**B**) Customized incubation hooks.

Figure 2: Glucose uptake in isolated mature skeletal muscle from mice. 2-deoxyglucose uptake was determined in isolated soleus (black bars) and EDL (gray bars) muscles in response to a submaximally effective insulin concentration (100 μU/mL), a maximally effective insulin concentration (10 mU/mL), and electrically induced contractions (0.2 ms pulse, 100 Hz, 1 s/15s, 30 V, 10 min). Data were analyzed by Students *t* test within each group. ###p<0.001, ##p<0.01, and #p<0.05 vs. soleus muscle. Values are means ± SEM. n = 4-6 per group. h, hour.

Figure 3: Muscle force curves in response to electrically induced contractions. Peak force production during electrical stimulation was calculated for the soleus (black dots) and EDL (gray dots) muscle. Each single value corresponds to an average of the last 500 ms of each 1-s

stimulation period. Values are means ± SEM. n = 5-6 per group. s, second. mN, milli-Newton.

Figure 4: Representative western blots of Akt Thr308, TBC1D4 Ser588, AMPKα Thr172 and ACC Ser212 phosphorylation as well as Akt2, TBC1D4, AMPKα2 and ACC protein. Western blot analyses were performed on the mouse soleus and EDL muscle samples described in Figure 2B, Basal. S, submaximally effective insulin. M, maximally effective insulin. C, contraction.

Figure 5: Representative western blots of AMPKα Thr172 and ACC Ser212 phosphorylation as well as AMPKα2 and ACC protein. Western blot analyses were performed on the mouse soleus and EDL muscle samples described in Table 1.

Table 1: Comparison of metabolic viability of mouse soleus and EDL muscles incubated for 1 hour in the presence of 2 mM pyruvate or 5 mM glucose. Non-incubated muscles were dissected from anaesthetized and fed animals before being frozen in liquid nitrogen. Separate muscles were incubated for 1 h in KRH buffer supplemented with BSA (0.1%), Na-pyruvate (2 mM) and D-mannitol (8 mM) while others were incubated for 1 h in KRH buffer supplemented with BSA (0.1%), D-glucose (5 mM) and D-mannitol (5 mM) before frozen in liquid nitrogen. Data were converted into relative units to highlight observed changes in various markers of metabolic viability. Absolute values from non-incubated mouse soleus and EDL muscles are given below. Lactate (mmol/kg w.w): 137.53_{soleus}; 139.05_{EDL}. Cr (mmol/kg w.w): 9.35_{soleus}; 8.98_{EDL}. PCr (mmol/kg w.w): 1.50_{soleus}; 5.20_{EDL}. PCr/(PCr+Cr): 13.98_{soleus}; 37.30_{EDL}. ATP (mmol/kg w.w): 3.07_{soleus}; 4.24_{EDL}. ADP (mmol/kg w.w): 0.60_{soleus}; 0.51_{EDL}. AMP (mmol/kg w.w): 0.18_{soleus}; 0.08_{EDL}. IMP (mmol/kg w.w): 0.07_{soleus}; 0.14_{EDL}. AMP/ATP ratio: 0.06_{soleus}; 0.02_{EDL}. Glycogen (pmol/µg protein): 77.01_{soleus}; 67.56_{EDL}. Data were analyzed by a two-way ANOVA within each group. ###p<0.001, ##p<0.01, and #p<0.05 vs. non-incubated. ***p<0.001, **p<0.01, and *p<0.05 vs incubated 1 h with glucose. §§§p<0.001, §§p<0.01, and §p<0.05 vs EDL. Values are means ± SEM. n = 12 in non-incubated group, n = 4-6 in incubated groups. Cr, Creatine; PCr, Phosphocreatine; w.w, wet weight; h, hour.

DISCUSSION:

Intact regulation of glucose uptake in skeletal muscle is important for preserving overall health[1]. Thus, investigation of muscle glucose uptake often serves as a primary readout when evaluating various health-altering interventions. Here we describe an ex vivo method for measuring glucose uptake in isolated and incubated soleus and EDL muscle from mice in response to insulin and electrically induced contractions. The method is quick and reliable and allows a precise control of the surrounding milieu of the incubated muscle that permits accurate investigations of muscle glucose uptake rates isolated from the potentially confounding influence of hormones and substrates that can be found in the blood. The method has been used for several years in many studies and is widely adopted by the muscle research community.

The ex vivo incubation model has generally been considered a method to assess glucose transport capacity rather than glucose uptake in skeletal muscle. Glucose transport capacity in incubated muscle can be determined by measuring accumulated D-glucose over a period of time. However, this poses a problem as D-glucose is quickly metabolized in the muscle cell following

uptake. To circumvent this problem, the glucose analogue 3-*O*-Methyl-D-glucose (3-MG) has been widely used to assess glucose transport capacity, as 3-MG is not further metabolized inside the cell after being transported across the cell surface membrane. Thus, the initial rate of intracellular accumulated 3-MG serves as an index of cellular glucose transport capacity *per se* because it is not affected by other steps in the glucose metabolic pathways. However, the use of 3-MG may constitute a problem as 3-MG will accumulate thereby reducing the transmembrane gradient for 3-MG and subsequently reduce further uptake. Thus, to obtain a measure of the membrane transport capacity, the initial rate of 3-MG uptake must be estimated. In particular when transport capacity is high this may pose a problem due to a rapid efflux of 3-MG[29,30]. The potential problem with 3-MG efflux can be avoided using 2-deoxy-D-glucose (2-DG). Following transport into skeletal muscle, 2-DG is phosphorylated by hexokinase II to 2-deoxy-D-glucose-6-phosphate (2-DG-6P). As skeletal muscle lacks glucose-6-phophatase and GLUT4 cannot transport phosphorylated 2-DG, 2-DG-6P will be trapped within the muscle cell. In contrast to glucose-6-phosphate, 2-DG-6P is a very weak allosteric inhibitor of hexokinase II[30] which helps to maintain the transmembrane gradient for 2-DG. Thus, observations have shown that 2-DG uptake in incubated (rat) muscle remains linear until the intracellular 2-DG-6P concentration exceeds 30 mM, a concentration that lowers hexokinase II activity[30]. Moreover, in incubated mouse skeletal muscle 2-DG uptake remains linear for ~30 min when temperatures of the incubation buffers are 37°C or less[31]. This suggests that 2-DG can be used to measure glucose transport capacity rather than glucose uptake in incubated muscle except for situations where 2-DG-6P concentrations become very high (e.g. observed during incubations >2 hours with 1 mM 2-DG and a maximal insulin concentration[29,30]). The idea that 2-DG uptake likely reflects glucose transport capacity is also supported by findings showing that maximal insulin-stimulated 2-DG uptake is similar in incubated muscle from wild-type mice and mice that overexpress hexokinase II[32]. A potential concern when using 2-DG for muscle glucose transport measurements during contraction is an increase in the intracellular glucose-6-phosphate concentration due to an elevated rate of glycogenolysis. However, since a linear increase in (rat) muscle 2-DG uptake is observed during contraction[29], this indicates that the accumulation of glucose-6-phosphate from the breakdown of glycogen during contraction does not interfere with hexokinase II activity and thus 2-DG uptake rates. Based on this, 2-DG seems well suited for measurements of glucose transport in isolated skeletal muscle during insulin and contraction considering the limitations of 3-MG.

Although it is generally assumed that 2-DG is not further metabolized following phosphorylation by hexokinase II, it has been reported that some 2-DG is directed towards and incorporated into muscle glycogen. Thus, during a 2 h normoglycemic hyperinsulinemic clamp in rats ~30% of the 2-DG taken up by skeletal muscle is incorporated into glycogen[33]. Therefore, it could be reasoned that rates of insulin-stimulated 2-DG uptake in incubated skeletal muscle are underestimated if accumulation of 2-DG in glycogen is neglected. We have determined that the herein described protocol on how to prepare muscle lysates (supernatant) for subsequent analyses of 2-DG uptake rates in prior incubated skeletal muscle is not affected by the potential incorporation of 2-DG into glycogen. It could be argued that glycogen accumulates in the pellet when centrifuging whole muscle homogenate to generate lysate for 2-DG uptake measurements. However, when comparing the levels of radioactivity in insulin-stimulated whole muscle homogenate vs. lysate

we do not detect any significant difference (unpublished data). This suggests that incubating mouse muscle for 10 min in 1 mM of 2-DG does not cause a detectable accumulation of 2-DG in glycogen.

Determination of skeletal muscle 2-DG uptake without considering that 2-DG is distributed in both the extra- and intracellular space will lead to an overestimation of 2-DG uptake. To circumvent this, L-glucose should be used as an extracellular marker as this is not transported across the cell membrane but otherwise exhibits similar properties as D-glucose including mass, solubility, passive diffusion, binding, etc. Due to the excessive costs of manufacturing and thus purchasing L-glucose, mannitol is typically used as an extracellular marker since mannitol is not taken up by the muscle cell, is relatively inexpensive and is estimated to have a somewhat similar extracellular distribution volume as glucose and 2-DG[34].

Intrinsic cellular and molecular clocks seem to play an essential role for the regulation of whole body metabolism and energy homeostasis[35]. It has been observed that muscle-specific knockout of the core clock gene *Bmal1* impairs insulin-stimulated glucose uptake in isolated mouse skeletal muscle[36]. Furthermore, submaximal insulin-stimulated glucose uptake of isolated skeletal muscle exhibits circadian rhythm with the lowest and highest insulin response in the middle of the light and dark phase, respectively[37]. Based on these findings, it is therefore important to incorporate time of animal sacrifice into the experimental design to increase the reproducibility of data.

A major disadvantage of the ex vivo method is the lack of capillary flow in the isolated muscle. This means that delivery and removal of various substrates fully depend on simple diffusion between the muscle fibers and the surrounding environment. Consequently, validation of the metabolic viability of isolated muscle incubated ex vivo has been focused on diffusion limitations of oxygen to superficial as well as deep muscle fibers. Thus, incubated muscle, particularly highly metabolic mouse muscle, tends to develop hypoxic cores in which breakdown of glycogen occurs[16,17,38]. However, in a detailed review by Bonen and colleagues[15], it was suggested that hypoxic cores of incubated muscle likely develop when incubating muscle too thick to be properly oxygenated especially when incubating at temperatures of ≥ 37 °C. This led to the recommendation that only thin and cylindrical mouse muscles, such as soleus and EDL, should be used for incubations at 25-30 °C to avoid development of hypoxic cores. This indicates that thickness and geometry rather than mass are more important factors to consider when incubating mouse skeletal muscles. In addition, it was recommended that incubation temperature, muscle thickness as well as ATP, phosphocreatine, and glycogen content and/or release of lactate should be measured and reported routinely to evaluate the viability of the incubated muscle[15]. To our knowledge, such complete analyses of incubated muscle have mainly been reported for rat muscle[39–42] and only to a limited extent reported in muscle from mice[16,17]. To increase insight into various metabolic markers of viability in incubated mouse skeletal muscle, we assessed possible changes in intracellular content of lactate, creatine, phosphocreatine, adenosine nucleotides, glycogen, and AMPK signaling in soleus and EDL muscle following 1 hour of incubation in glucose- or pyruvate-supplemented KRH buffer. Similar to previous findings in incubated mouse soleus and EDL muscle[17], we observed that glycogen levels decreased when muscles were incubated in glucose-free media. This indicates that the absence

of glucose during incubation promotes glycogen breakdown and the subsequent entry of glucose-6-phosphate into glycolysis that may act to secure ATP production in particular if oxygen supply is inadequate. Furthermore, we found an overall reduction in the ATP and ADP nucleotide pools in incubated soleus and EDL muscle. This may imply that oxygen supply is not entirely sufficient to meet the demand of incubated mouse muscle both in the presence and absence of glucose. Since the oxidative soleus muscle relies more on oxygen for ATP production compared to the glycolytic EDL muscle, this would suggest that the soleus muscle is affected to a larger extent by the incubation procedure. In agreement, we found that the intracellular levels of IMP as well as AMPK signaling were severely increased in soleus compared to EDL muscle when incubated in the absence of glucose. This signifies that the soleus muscle has a higher degree of metabolic stress during incubation, which must be taken into consideration when evaluating data from the ex vivo mouse muscle model.

Cultured muscle cells including immortalized L6 and C2C12 as well as primary human myotubes are commonly used as a surrogate for mature skeletal muscle to study the effects of various genetic and pharmacological manipulations on insulin-stimulated muscle glucose uptake. However, in several ways cultured muscle cells do not resemble mature skeletal muscle and when comparing the two model systems numerous differences become apparent. These include differences in protein expression, dimensional structure, surrounding environment, proliferation and differentiation state, fiber type composition, metabolic processes and functional properties[43] all of which may affect how the muscle cell regulates glucose uptake in response to various stimuli. Typically, greater relative effects of insulin on glucose uptake rates are observed in mature skeletal muscle compared to cultured muscle cells[44] which may indicate that cultured muscle cells to some extent lack the machinery responsible for regulating glucose uptake rates, including a high level of expression of the insulin-sensitive glucose transporter GLUT4[43]. Considering the limitations and caveats associated with the use of either cultured muscle cells or isolated skeletal muscle, it is therefore likely advantageous to take on a combined approach when studying various muscle metabolic processes such as glucose uptake.

Experimentally soleus and EDL muscles are typically regarded as representative of slow- and fast-twitch muscles, respectively. Therefore, these muscle types are ideal for mechanical studies seeking to investigate interventions that affect muscle force and fatigue development. Moreover, soleus muscle is typically reported to have enhanced insulin sensitivity and responsiveness compared to EDL muscle and thus, interventions that target muscle insulin sensitivity may affect soleus and EDL differently. In addition, the relative distribution of the different AMPK complexes differs between the soleus and EDL muscle that seems to affect the ability of AMPK activating compounds to increase muscle glucose uptake. Thus, the greatest insight into the regulation of muscle glucose uptake is likely achieved if both the soleus and EDL muscles are used during experimentation with the *ex vivo* incubation model.

The herein described method relates glucose uptake to the amount of total protein abundance determined in each muscle sample after homogenization. It is our experience that variation decreases when relating glucose uptake per amount of muscle protein instead of muscle weight (unpublished data). Furthermore, homogenization of muscle samples in a buffer used for various

biochemical assays makes it possible to determine both glucose uptake, myocellular signaling and protein activities in the same sample preparation[45]. This will often decrease the amount of mice used for a study. Nevertheless, glucose uptake can easily be determined in skeletal muscle samples that are dissolved by heating in sodium hydroxide (NaOH) followed by neutralization with hydrogen chloride[20]. Since treatment of muscle with NaOH interferes with measurements of total muscle protein concentration, glucose transport measured by this procedure must be related to muscle weight.

Based on various optimizations, our glucose uptake incubation buffer contains a specific activity of 0.028 MBq/mL of [^{3}H]2-deoxy-D-glucose and 0.0083 MBq/mL of [^{14}C]Mannitol. On one hand, this lowers the amount of lysate (150 out of 400 µL) needed in order to get sufficient and reliable radioactivity measurements. On the other hand, it increases the amount of radioactive [^{3}H]2-deoxy-D-glucose and [^{14}C]mannitol needed for each experiment, which increases experimental costs. Thus, for any experimental setup it is possible to regulate the amount of radioactivity used in the glucose uptake incubation media to fit specific requirements. However, care must be taken not to decrease amount of radioactivity in the incubation buffer to an extent that makes radioactivity measurements unreliable. This is ensured by keeping radioactivity in samples higher than the specified detection limits of the liquid scintillation counting machinery used.

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DISCLOSURES:

The authors have nothing to disclose

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Figure 1

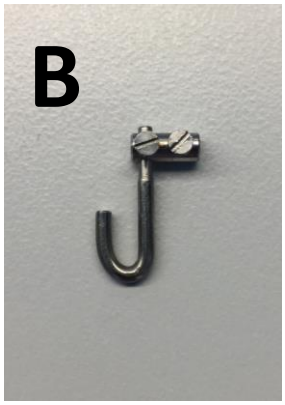

Figure 2

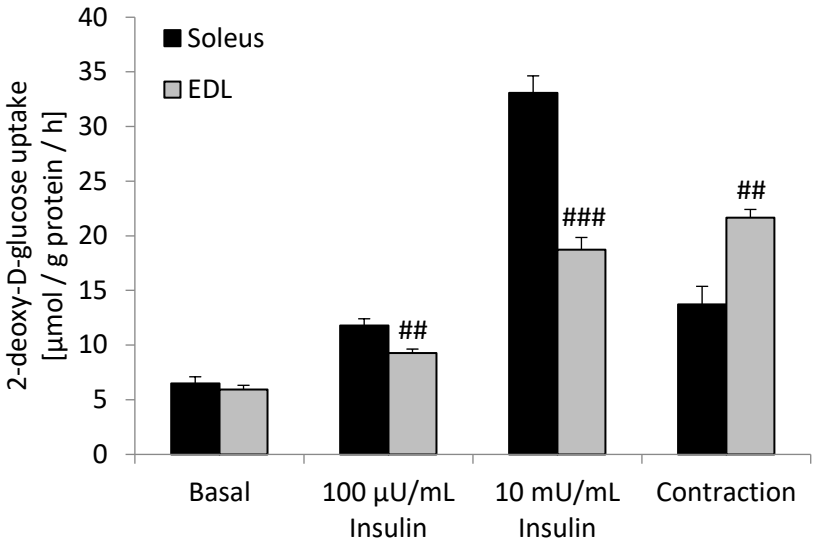

Figure 3

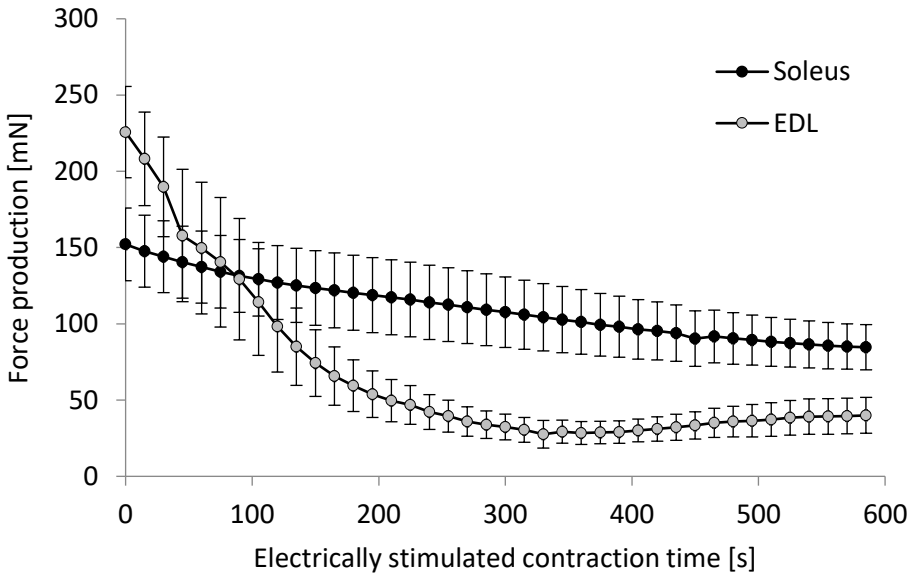

Figure 4

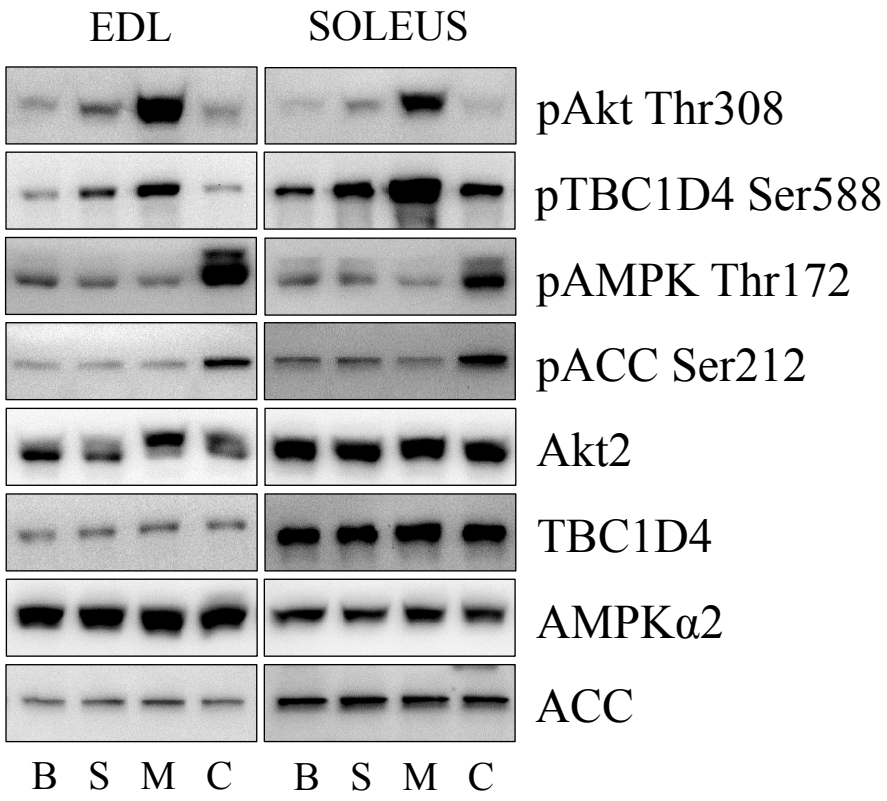

Figure 5

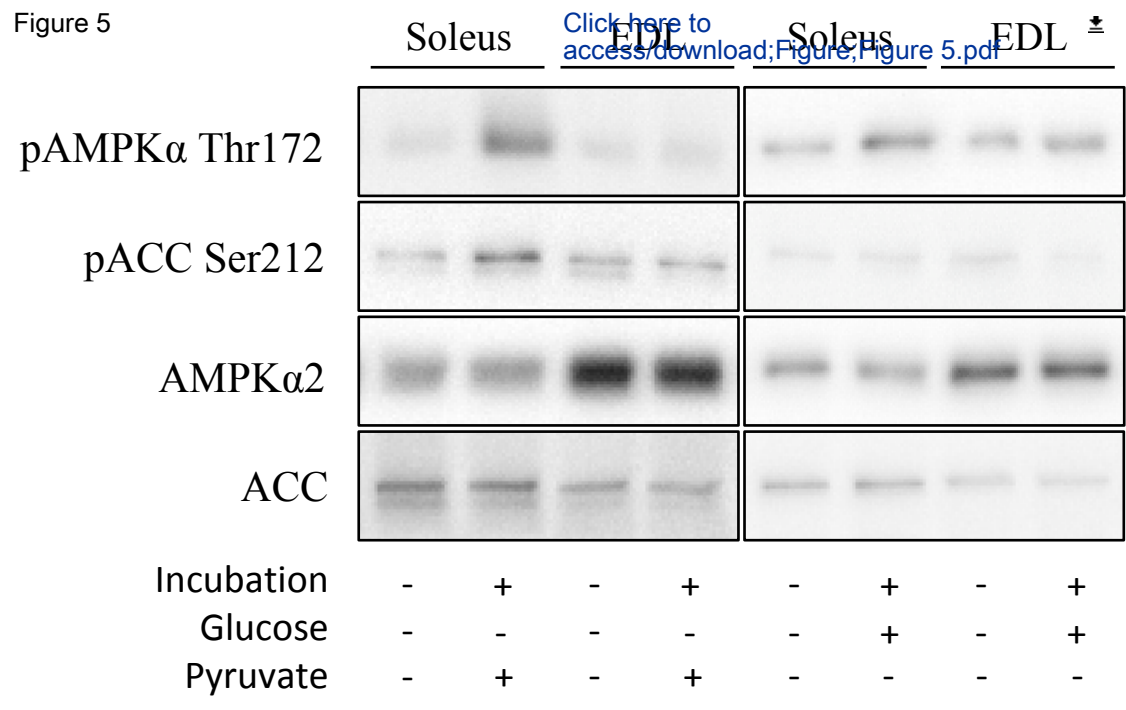

Table 1

	non-incubated		Incubated 1h with pyruvate		Incubated 1h with glucose			
	Soleus	EDL	Soleus	EDL	Soleus	EDL	Main effects	Interaction
Lactate	1.00 ± 0.01	1.00 ± 0.02	0.96 ± 0.03	0.98 ± 0.02	1.05 ± 0.01	0.99 ± 0.01	-	-
Cr	1.00 ± 0.04	1.00 ± 0.06	0.64 ± 0.07 ###	0.76 ± 0.05 ###	0.76 ± 0.07 ##	0.88 ± 0.09 ##	p < 0.001	-
PCr	1.00 ± 0.19	1.00 ± 0.06	0.80 ± 0.12	1.13 ± 0.14	0.65 ± 0.31	0.75 ± 0.08	-	-
PCr/(Cr + PCr)	1.00 ± 0.20	1.00 ± 0.07	1.17 ± 0.11	1.25 ± 0.12	0.78 ± 0.36	0.92 ± 0.11	-	-
ATP	1.00 ± 0.03	1.00 ± 0.02	0.72 ± 0.03 ###,§§§	0.99 ± 0.03 *	0.81 ± 0.06 ###	0.85 ± 0.04 ##	-	p < 0.001
ADP	1.00 ± 0.04	1.00 ± 0.04	0.75 ± 0.05 ###	0.92 ± 0.03 ###	0.84 ± 0.04 ##	0.86 ± 0.03 ##	p < 0.001	-
AMP	1.00 ± 0.11	1.00 ± 0.12	0.85 ± 0.15	0.79 ± 0.13	0.84 ± 0.18	0.75 ± 0.13	-	-
IMP	1.00 ± 0.17	1.00 ± 0.30	4.43 ± 0.67 ###,§§§	0.72 ± 0.29	3.33 ± 1.25 ##,§	1.08 ± 0.01	-	p < 0.001
AMP/ATP ratio	1.00 ± 0.12	1.00 ± 0.13	1.18 ± 0.22	0.81 ± 0.16	1.06 ± 0.23	0.90 ± 0.19	-	-
Glycogen	1.00 ± 0.08	1.00 ± 0.12	0.74 ± 0.07 (#),*	0.80 ± 0.12 (#),*	1.12 ± 0.10	1.10 ± 0.03	p = 0.035	-
pAMPK Thr172 / AMPKα2	1.00 ± 0.10	1.00 ± 0.12	3.26 ± 0.58 ###,***,§§§	1.55 ± 0.27	1.68 ± 0.19 #	1.36 ± 0.19	-	p = 0.002
pACC Ser212 / ACC	1.00 ± 0.18	1.00 ± 0.12	2.22 ± 0.58 ###,**,§§	0.96 ± 0.21	0.99 ± 0.15	0.83 ± 0.09	-	p = 0.030

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
[14C]D-mannitol	American Radiolabeled Chemicals, Inc.	ARC 0127	
[3H]2-deoxy-D-glucose	American Radiolabeled Chemicals, Inc.	ART 0103A	
2-Deoxy-D-glucose	Sigma	D8375	
4-0 USP non-sterile surgical nylon suture	Harvard Apparatus	51-7698	
ACC antibody	DAKO	P0397	
Akt2 antibody	Cell Signaling	3063	
AMPKα2 antibody	Santa Cruz	SC-19131	
aprotinin	Sigma	A1153	
benzamidine	Sigma	B6505	
Bovine serum albumin (BSA)	Sigma	A7030	
CaCl2	Merck	1020831000	
Calibration kit (force)	Danish Myo Technology A/S	300041	
Chemiluminescence	Millipore	WBLUF0500	
D-Glucose	Merck	1084180100	
D-Mannitol	Sigma	M4125	
Data collection program	National Instruments	LabVIEW software version 7.1	
Dialysis tubing	Visking	DTV.12000.09 Size No.9	
Digital imaging system	BioRad	ChemiDoc MP	
EDTA	Sigma EDS	E9884	
EGTA	Sigma	E4378	
Electrical Pulse Stimulator	Digitimer	D330 MultiStim System	
Glycerol	Sigma	G7757	
HEPES	Sigma	H7637	
IGEPAL CA-630	Sigma	I8896	
Insulin	Novo Nordisk	Actrapid, 100 IE/mL	
KCl	Merck	1049361000	
KH2PO4	Merck	104873025	
leupeptin	Sigma	L2884	
MgSO4	Merck	1058860500	
Muscle Strip Myograph System	Danish Myo Technology A/S	Model 820MS	
Na-Orthovanadate	Sigma	S6508	
Na-Pyrophosphate	Sigma	221368	
Na-Pyruvate	Sigma	P2256	
NaCl	Merck	106041000	
NaF	Sigma	S1504	
NaHCO3	VWR	27778260	
pACC Ser212 antibody	Cell Signaling	3661	
pAkt Thr308 antibody	Upstate Biotechnology	06-678	
pAMPK Thr172 antibody	Cell Signaling	2531	
phenylmethylsulfonylfluoride	Sigma	P7626	
Platinum electrodes	Danish Myo Technology A/S	300145	
pTBC1D4 Ser588 antibody	Cell Signaling	8730	
Scintillation counter	Perkin Elmer	Tri-Carb-2800TR	
Scintillation fluid	Perkin Elmer	6013329	
Statistical analyses software	Systat	SigmaPlot version 14	
TBC1D4 antibody	Abcam	ab189890	
TissueLyser II	Qiagen	85300	
Ultrapure water	Merck	Milli-Q Reference A+ System	
β-glycerophosphate	Sigma	G9422	

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
The revised manuscript has been thoroughly proofread by all authors on the manuscript.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
The revised manuscript has been formatted as recommended

3. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Danish Myo Technology (DMT Model 820M), Digitimer D330 MultiStim System, LabVIEW software, MilliQ water, Visking Dialysis Tubing, etc.
Commercial language has been removed from the revised manuscript.

4. We cannot have a separate abbreviation section. Please describe all abbreviations during the first time use.
We believe all relevant abbreviations have been described the first time of use in the revised manuscript

5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
The manuscript has been revised as recommended

6. We cannot have non-numbered step or subheadings in the protocol section. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.
The manuscript has been revised as recommended

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
The manuscript has been revised as recommended

8. The Protocol should contain only action items that direct the reader to do something.
The manuscript has been revised as recommended.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.
The manuscript protocol has been simplified as much as possible

10. Please ensure you answer the "how" question, i.e., how is the step performed?
The notion has been ensured to the best of our ability.

11. All the non-highlighted steps fors solution and reagent preparation can be moved to a separate table in .xlsx format and uploaded separately to the editorial manager account.
We have chosen to keep all the non-highlighted steps in the main text to maintain the clarity of the protocol

12. 191-198: Please convert to action steps and move some of the details to the discussion.
We disagree to the editorial comment. This paragraph referred to is not a matter of discussion, just a statement of the mice used in the particular experiment. We explicitly mention that other mouse strains are likely suitable as well for the ex vivo procedure.

13. 3.1: How do you check for the depth of anesthesia? Do you shave the animal prior to surgery? Do you place vet ointment? Do you sterilize the surgical site?
Check of proper anesthesia has been added to the revised manuscript. The animal is not shaved, vet ointment is not used and the surgical site is not sterilized.

14. 3.5: How is this done?
This procedure referred to was done using forceps. This notion has been added to the revised manuscript.

15. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
This is has been highlighted by yellow in the revised manuscript.

16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
None of the figures have been used in any previous publication.

17. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
We believe our revised manuscript to a great extent covers the abovementioned matters.

18. Please sort the materials table in alphabetical order.
The manuscript has been revised accordingly.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this manuscript, the authors describe the methodologies associated with the measurement of glucose uptake in isolated and incubated skeletal muscle preparations. The procedures are mostly well presented. The data provided are classic representations of what would be expected to happen under the experimental conditions surveyed in this manuscript. Some information of collateral procedures and on statistical analyses would improve the overall value of the manuscript.
We thank the reviewer for the helpful comments and issues raised that have improved the manuscript.

Major Concerns:
Methods and results: Some of the readers will have limited experience with this type of experimental model. The comments listed below are meant to clarify the language and the organization of some of the sections to allow all inexperienced readers to fully understand the procedures. With this in mind:
It is not clear to this reviewer whether a video will be provided to the reader. If not, then photos of the incubation systems would greatly help the unfamiliar reader comprehend the preparation. Furthermore, a photo would help the reader understand the proper positioning of the electrodes on the muscle bed.
Indeed, a video will be provided to the reader showing the incubation setup and proper positioning of electrodes centrally and on both sides of the muscles. Nevertheless, we have included photos of the incubation system to help the reader even more.

It might help an inexperienced reader to know up front how many different solutions will be needed. This could be stated at the top of the Section on Preparation of solutions and incubation media.
As suggested by the reviewer, we have included number of total solutions needed to measure basal, submaximal insulin-, maximal insulin- and contraction-stimulated glucose uptake in isolated mouse skeletal muscle.

The reader would also appreciate knowing the terminology that will be used to identify the different media throughout the text. For example, this sentence in section 2.3 is not clear for an inexperienced reader: "Typically, the osmolarity of

the KRH supplements is kept at a total of 10 mM across ….". The word "KRH supplement" may need to be defined.
We have revised the sentence mentioned by the reviewer thus indicating each component of the KRH supplements "(i.e. Na-Pyruvate, D-Mannitol, and D-glucose)". In addition, we have included a note in the first paragraph of section 2 that describes the different media used throughout an experiment and revised the manuscript accordingly.

The chemical components of the solutions should also be written out.
As suggested by the reviewer, chemical components of the KRH solutions have been written out at the first mentioning in the revised manuscript.

In the caution for radioactivity, I would suggest adding: "…and in some universities, research institutions and companies may require the acquisition of a "Radioactivity Use Permit".
The suggestion has been added to the revised manuscript.

In sections 2.8 and 2.9, which incubation media should the researcher use. Here the reader could potentially be referred to the appropriate section. Alternatively a table that illuminates the components of each incubation media along with their purpose would be advantageous.
Based on our careful revisions made due to the reviewers 3rd comment, we believe that our detailed description is now adequate for readers to understand which media needs to be used at the different steps.

In section 3.2, I would suggest adding that in some countries a license to handle pentobarbital and other anesthesia drugs may be required.
The suggestion has been added to the revised manuscript.

In section 3.4, some photos of the steps would help an inexperienced researcher visualize the procedure.
A video will be provided to the reader showing all parts described in section 3.4, which will help the inexperienced reader to a greater extend than photos.

Section 3 would be strengthened by adding a description and/or discussion of the advantages of using the soleus vs. the EDL and or of using both muscles in an experiment.
The suggestion has been added to the discussion of the revised manuscript

Many of the sentences throughout the text but especially in the methods section lack the appropriate3 determiners (i.e. the use of "a" versus "the" etc…). Here are three examples that warrant a careful editing of the whole manuscript:
Line 220, Typo: Cut the distal tendon of the TA muscle and dissect out …..
Line 228, Typo: When the muscles have been dissected….
Line 234, Typo: Following step 3.6, replace the basal incubation media with….concentration and leave in the incubation chambers for 20 min. Space each incubation chamber by 1 min thereby making time for the subsequent harvesting of the muscles.

To the best of our abilities, we have added the appropriate determiners throughout the revised manuscript.

Line 307: for inexperienced readers, define what BCA stands for.
BCA has been defined in the revised manuscript

Line 316: you may want to add: Background DPM is obtained by using the DPM counts obtained from the two blind control vials.
This matter has been corrected in the revised manuscript.

Line 356: please define Akt and TBC1D4.
We have defined Akt in the revised manuscript. TBC1D4 is defined at a later point in the revised manuscript since TBC1D4 was removed in line 356 due to comments from another reviewer.

Line 402: some description should be provided for figure 3. For example, the nutritional state of the animals used to harvest the control muscle could be included.
We have added a description to the figure legend (now #5) that describes that the western blotting analyses were performed on samples used in table 1.

Furthermore, the methods used to perform the Western Blots should be briefly described (potentially in the methods). Lastly, bands for the muscles incubated with pyruvate should be included.
We have added a paragraph in the methods section briefly describing the western blot analyses. Bands for muscles incubated with pyruvate were already included in figure 5 at the initial submission. However, we realize that the figure did not show this properly. Thus, figure 5 has been revised to accommodate this ambiguity.

Table 1: Given the emphasis placed on incubation with pyruvate versus glucose, some information on the effects of these different incubation media on glucose uptake would be helpful. As above, the methods used to perform the assays associated with the data in the table should be described.
Only 2mM pyruvate and 1mM 2-deoxyglucose, and not D-glucose, are present in the glucose uptake incubation media during the actual measurement of glucose uptake. Increasing the 2-deoxyglucose concentration to 5mM will increase the glucose uptake rate by 5-fold (data not shown). This is also supported by findings in isolated rat skeletal muscle showing that glucose uptake remains linear at rest, during insulin stimulation and contraction until glucose concentrations exceed approximately 10mM (Nesher et al., Am J Physiol Cell Physiol, 1985).
Based on pilot experiments involving incubation of EDL and soleus muscle for 6-8 h, we have evidence to indicate that incubating muscles only in the presence of pyruvate will increase 2-deoxyglucose uptake rates in the basal/rested state. Incubating muscles in a glucose containing media can prevent such increase in basal glucose uptake when the incubation time exceeds 6-8 hours. This notion has been added to the revised manuscript.
We have added a paragraph (section 10) in the manuscript protocol briefly describing how data in table 1 have been generated.

A statistical analysis section should be provided.
A statistical analyses section has been added to the revised manuscript.

Discussion
Line 514: "… ATP production in particularly if oxygen supply is inadequate." This does not quite make sense.
Breakdown of glycogen and the subsequent entry of glucose-6-phosphate into glycolysis give rise to 2 ATP in the absence of oxygen. We acknowledge that this has not been described in full detail. Thus, this issue has been added/mentioned in the revised manuscript.

Line 516: according to the statistical data provided in the table this is not exactly correct; only ATP is different between incubation protocols. It would be important to provide the statistical analyses used to provide these differences.
We see the point put forward by the reviewer. We have revised the manuscript accordingly. Statistical analyses of the values provided in table 1 have been provided in the 'statistical analyses section'.

Lines 521-522: this is only true in the pyruvate incubation situation; not the glucose incubation.
We have revised the manuscript accordingly.

Reviewer #2:
Manuscript Summary:
In the manuscript entitled „Measurement of insulin- and contraction-stimulated glucose uptake in isolated and incubated mature skeletal muscle from mice " the authors provide a stepwise description of a very important technique in the field of metabolic research, the analysis of contraction- and insulin-mediated glucose transport into isolated murine skeletal muscle. The protocol has been prepared thoroughly and the descriptions give substantial support in setting up this method in a lab. Moreover, the very detailed instructions may even be helpful to optimize already established ex vivo uptake assay protocols. Especially scientists who are new in this field will profit from the elaborate introduction into the topic.
We thank the reviewer for the positive and constructive view of our work.

Major Concerns:
None.

Minor Concerns:
Only some minor issues may be addressed:
-Line 133: For beginners, it may be helpful to see a graphical outline of the loop settings. Otherwise, the mere description of the muscle mounting on the hooks could be difficult to translate into practice. In addition, it might be of importance to stress the point how crucial it is to fix the knots around the tendons tightly in order to not lose the tension during the following incubation steps.
We fully agree to the reviewer's suggestion. As currently planned, creation of suture loops for muscle incubation as well as mounting of suture loops on muscle

tendons will be filmed for the video related to the article. Thus, we believe a graphical outline is redundant in this context.
We have stressed in section 3.5 that it is crucial to fix the suture knots around the tendons tightly in order to not lose tension during incubation and especially during contraction.

-Lines 147-157: Structurally, it may make more sense to add these points (2.2 and 2.3) as 2.1.1 directly below the buffer preparation (2.1) instead of setting them up as individual paragraphs.
As suggested by the reviewer, we have changed the structure of the sections.

-Line 178: Do the radioactive stock solutions require the removal of dispensable ethanol?
At present, we use radioactive tracers (3H and 14C) dissolved in a sterile and isotonic NaCl solution. If radioactive tracers are dissolved in ethanol and high specific activities are preferred, it may be advantageous to remove dispensable ethanol. This issue has been added to the manuscript in section 2.3.3.

-Line 207: Regarding animal welfare it may be recommended to explain how to make sure that the narcosis is properly induced (check reflexes etc.) before starting the preparation.
A statement concerning induction of proper anesthesia of each animal before starting dissection has been added to section 3.2.

-Line 228: Do all muscle types (e.g. EDL and Soleus) require the same basal tension?
I an ideal world each single isolated muscle requires its own individual basal tension. However, identifying the optimal length (basal tension) typically requires repeated stimulation of the muscle until the force production does not increase anymore. Indeed, this may impact the glucose uptake measurements and therefore a 5 mN resting tension has been chosen as this generally leads to an electrically induced maximal force production in both soleus and EDL.

-Line 366: It may be recommendable to provide a calculation example with real numbers.
As suggested by the reviewer, we have added a calculation example with real numbers to the manuscript.

Reviewer #3:
Manuscript Summary:
The manuscript provides an informative description of a useful method from a group that is very experienced with this technique. The clarity and precision of the text could be improved in a number of passages. Most of these recommendations are relatively minor. In some passages, statements are made without providing supportive evidence by using citations or showing data, and the way some of the statements are written might be interpreted by a naïve reader as an established fact. Providing more supporting evidence would be an improvement in these passages. Otherwise the language might be revised to

explicitly communicate when the recommendation is not supported by empirical data. Making these revisions would improve the manuscript.
We thank the reviewer for the valuable inputs.

Major Concerns:
MAJOR
There are several aspects of the text that if not revised might lead some readers to misunderstand some of the issues that are relevant to performing the method and interpreting the results from the method. This issues should not be difficult to address.

Line 154-156 - The sentence appears to suggest that 10 mM is specifically a crucial value, but that is unlikely to be true, and no data are included to indicate that. Should probably indicate that using an equal total osmolarity across treatment groups being compared is a good idea to avoid the potential influence of differences in osmolarity.
We agree with the reviewer and have revised the manuscript accordingly.

The paragraph from 470-481 has several issues that might be confusing or misleading.
Lines 472-473 - The sentence about 2DG incorporation into glycogen needs to be revised to indicate this result was in vivo in humans.
The findings regarding 2DG incorporation into glycogen were observed in skeletal muscle from rats. This notion has been added to the revised manuscript.

Lines 475-481 - The statement about the "herein described protocol" may be unintentionally misleading. It is not supported by data that is included in this study nor by a citation. If neither data nor a citation is provided, it could at least be indicated by "(unpublished data)."
In accordance with the reviewer's suggestion, we have included '(unpublished data)' at the end of the paragraph.

The distinction between the terms "homogenate" and "lysate" is potentially confusing. If the intention is "whole homogenate" versus "supernatant" after a particular centrifugation condition (time and g-force), that could be indicated.
We have revised the sentence now stating 'whole muscle homogenate" as well as including 'supernatant' in parenthesis in front of 'lysate' to decrease confusion. The protocols on how to make whole muscle homogenate from skeletal muscle tissue as well as lysate following centrifugation of whole muscle homogenate are described in section 6. Thus, we find it redundant to also mention centrifugation time and g-force in the discussion.

It is unclear if this text indicates that 2DG is not incorporated into glycogen in mouse muscle under these conditions. If glycogen is in the pellet, then this result is in conflict with the statement on lines 472-473 about 2DG incorporation into glycogen based on results in human muscle which was stated as a fact.
Based on in house analyses, we believe that a fixed fraction of the muscle (including glycogen and protein) is found in the pellet following centrifugation of whole muscle homogenate. However, based on the lack of radioactivity found in the pellet,

we believe that the described protocol for measuring glucose uptake does not entail (a measureable) accumulation of 2DG into glycogen likely for two reasons:

1) Incubating mouse muscle for 10 min in 1mM of 2DG is too short of a time for 2DG to accumulate in glycogen in contrast to findings in rats (120 min at normoglycemic/hyperinsulinemic conditions)

2) mouse muscle contains approximately 5 times less glycogen than rat muscle which decreases the likelihood of 2DG accumulation in glycogen to such an extent that we can detect it with our LSC machinery.

Issue #1 has been added to the revised manuscript.

Line 501 - The statement about the mass of muscles studied could be unintentionally misleading. Please see the following text from the review by Bonen et al. "Mouse soleus and EDL muscles were thought to present less of an impediment to oxygen diffusion in vitro because they are small (8-10 mg). However, the mouse has a higher metabolic rate than the rat, and thus the delivery of oxygen to the deeper fibers may be compromised in these muscles because the peripheral fibers may consume oxygen at a greater rate."
The issue of differences in metabolic rates for muscles form mouse vs. rats should be discussed in the context of muscle mass, and the importance of muscle geometry and thickness being more important than muscle mass should be addressed.

We do not believe it is in the scope of this manuscript to discuss differences in metabolic rates for muscles derived from rats and mice. The reasons for such differences may be plenty and not relevant to perform and assess studies of insulin and contraction-induced glucose uptake in isolated mouse skeletal muscle. However, we have revised the manuscript highlighting the importance of geometry and diameter being more important than muscle mass (even though the ratio between the muscle mass and the physiological cross sectional area seems fairly constant between various mouse muscle types (Charles et al., PlosOne, 2016, DOI:10.1371/journal.pone.0147669))

Minor Concerns:
Line 43 - Electrically induced (no hyphen) contraction is not identical to exercise. Could revise to, "insulin and electrically induced contraction (a model for exercise):

We agree to the reviewers comment. As suggested, these changes have been added to the revised manuscript.

Lines 67-69 - It is unclear exactly what is meant by "does not require intact gastrointestinal function or insulin secretion from the pancreas." I can guess, but the text should be revised to improve clarity.

We acknowledge the ambiguity. In contrast to the insulin clamp, data obtained from an oral glucose tolerance test (which is typically performed in metabolic studies of humans) are dependent on gastrointestinal function and insulin secretion. This matter of subject has been included in the revised manuscript.

Line 91 - Suggest revision to, "under some conditions, metabolic viability…"

The suggestion has been added to the revised manuscript.

Line 104 - "Here we describe a detailed…"
The suggestion has been added to the revised manuscript.

Line 107 - "during a 10 min period"
The suggestion has been added to the revised manuscript.

Line 114 - Measurements were only made at 1 h, not for greater or equal to 1 h, and 1 h isn't really a prolonged period. Suggest: "incubated for 1 h" to be precise.
The suggestion has been added to the revised manuscript.

Lines 183 and 186 - The term "maximal" is widely used, but 10 mU/mL is not maximal - you could use always increase the concentration higher. The more precise term is maximally effective. However, to demonsrate a dose is maximally effective, it is necessary to perform a dose-response and show a plateau effect for glucose uptake in spite of increasing insulin dose. The terms that could be used are submaximally *effective* and maximally effective (assuming that you have demonstrated maximal effectiveness) or physiologic and supraphysiologic.
We fully agree with the reviewer – from the data presented in the current manuscript, we cannot be sure that a maximal effect of 10 mU/mL insulin on glucose uptake was achieved. However, based on previous dose-response experiments performed in house, we have observed that 10 mU/mL insulin induces a maximal response on glucose uptake in isolated soleus and EDL muscle. In the light of minimizing the use of animals for experimental research, we reserve the right to imply that 10 mU/mL insulin is maximally effective on glucose uptake. The terms "submaximally effective" and "maximally effective" have been added to the revised manuscript.

Line 203 - Should comment on the rationale for fasting and the length of the fast.
Muscle from both fed and fasted mice can be used for measurements of glucose uptake by the ex vivo incubation model. Since other organs of the mouse may be used for investigations following dissection of EDL and soleus muscle, the use of either fed or fasted mice typically depends on the requirements of the individual researcher. Therefore, we believe that commenting on a potential rationale for fasting is not in the scope of the manuscript.

Line 316 - Need to explain what "background DPM" means and how it is measured.
We apologize for the ambiguity. "Background DPM" refers to the DPM obtained from the blind control samples described in section 7.1. We have corrected this matter by deleting "background DPM" and replacing it with "DPM of the blind control samples".

Lines 356 - Although Akt and TBC1D4 expression might be greater in soleus or EDL, it is unclear that is a causal relationship. Probably not true for TBC1D4 because TBC1D4 has an inhibitory function, and overexpressing TBC1D4 has been shown to not alter glucose uptake by Laurie Goodyear.
We acknowledge that increased expression of TBC1D4 in skeletal muscle is likely not driving enhanced muscle glucose uptake in contrast to Akt2 (Cleasby et al., Molecular Endocrinology, 2007). Thus, we have removed TBC1D4 from the

sentence describing potential mechanisms responsible for elevated insulin-stimulated glucose uptake in soleus vs. EDL muscle.

Line 410 - Revise to "before being frozen
The suggestion has been added to the revised manuscript.

Line 423 - "Intact regulation of glucose uptake in skeletal muscle is important for preserving overall health"
The suggestion has been added to the revised manuscript.

Line 424 - "Thus, investigation of muscle glucose uptake often serves"
The suggestion has been added to the revised manuscript.

Line 427 - "The method is quick"
The suggestion has been added to the revised manuscript.

Line 429 - "muscle glucose uptake rates isolated from the potentially confounding influence of hormones and substrates"
The suggestion has been added to the revised manuscript.

Line 434 - "generally been considered a method"
The suggestion has been added to the revised manuscript.

Line 455 - "30 mM, a concentration that lowers"
The suggestion has been added to the revised manuscript.

Line 459 - "The idea that 2-DG uptake likely reflects"
The suggestion has been added to the revised manuscript.

Line 496 - change "profound" to "deep"
The suggestion has been added to the revised manuscript.

Line 514 - "in particular if"
The suggestion has been added to the revised manuscript.

Line 537 - Need to clarify what is meant by "a wider range of glucose uptake rates." Is the intention to refer to the relative magnitude (% or fold) increase for insulin stimulated versus basal values?
We have clarified the meaning of "a wider range" in the revised manuscript. As suggested by the reviewer, our intention is to refer to the relative increase in insulin-stimulated glucose uptake vs. basal values between mature skeletal muscle and cultured muscle cells.

Line 539 - "including a high level of expression"
The suggestion has been added to the revised manuscript.

Lines 542-544 - Including data about the variation being less for values expressed relative to total protein abundance vs. muscle weight would be helpful.

We do not find it very relevant to include such data in the manuscript. However, we have included '(unpublished data)' at the end of the paragraph to imply that such data have been generated.

Lines 549-550 - The intended meaning of the statement "Typically glucose transport…" is unclear. Potentially it is meant to indicate that heating in NaOH interferes with measuring total protein concentration, but that should be clarified.
We acknowledge the ambiguity. We have revised accordingly to the reviewer's suggestion.

Line 555-556 - Mannitol is a sugar, but I don't believe it is typically considered a glucose analog.
We agree. The manuscript has been revised accordingly.

Lines 560-561 - The term "dpm" should be used rather than "counts." It is unclear if it is intended that 10,000 dpm are recommended for 2DG and also separately for mannitol. The statement about "as a rule of thumb" about the amount of radioactivity needed to "minimize uncertainty" seems rather vague. If there are quantitative data to support the assertion, it would be appropriate. It seems to be more of a guess or expectation as it is written.
We realize that our statement concerning the total number of counts/DPM needed to reach reliable measurements is depended on the detection limits/sensitivity of the liquid scintillation counting (LSC) machinery used. Therefore we have revised the sentence now describing that radioactivity of samples should be kept at levels higher than the specified detection limit of the LSC machinery used.

Several references have problems that seem to be related to authors with an initial of "A" - #15, 16, 28, and 38.
The matter has been corrected in the revised manuscript.

Figure 1 - Replace "Submaximal" with 100 uU/mL and "Maximal" with 10 mU/mL.
The suggestion has been added to the revised manuscript.

Figure 2 X-axis label - Replace "Time" with Electrically Stimulated Contraction Time.
The suggestion has been added to the revised manuscript.

Reviewer #4:
Manuscript Summary:
The manuscript describes a method to examine insulin and contraction stimulated glucose uptake in vitro in mouse skeletal muscle. They describe this method for the extensor digitorum longus (EDL) and the soleus in mice. This is relevant to whole body glucose maintenance as skeletal muscle is the major sink for postprandial glucose uptake.
We thank the reviewer for the raised issues, which is indeed relevant.

Major Concerns:

None

Minor Concerns:
The authors explain their procedure in nice detail. The discussion addresses some of the caveats including discussion of muscle viability (oxygenation, etc) and the use of C2C12 models. Using a combined approach to these type of studies is important because of these caveats. This combined approach concept is not clearly emphasized in the discussion.
In the discussion, we have included a sentence that argues for a combined approach of cultured cells and isolated skeletal muscle when studying metabolic processes such as glucose uptake.

In addition, proteins involved in skeletal muscle glucose metabolism and insulin sensitivity are circadian in muscle (Dyar et al, 2013; Harfmann et al, 2016) and therefore it is important to incorporate this into the experimental design (ie time of sacrifice, etc) to increase the reproducibility of data. In addition, it warrants some discussion.
In section 3, we have now included start time of experiment and time of animal sacrifice. Furthermore, we have mentioned and discussed the matter put forth by the reviewer in our discussion.

Reviewer #5:
Manuscript Summary:
The manuscript shows how a protocol of isolation and incubation of mouse muscles soleus and EDL using radiolabeled [3H]2-deoxy-D-glucose and [14C]mannitol as an extracellular marker is an appropriate ex vivo model to study the effects of insulin and electrically-induced contraction on glucose uptake in skeletal muscle. The findings are descriptive and correlative, the protocol is of interest to the field and it is a novel work.
We thank the reviewer for the appreciation and interest of our work.

Major Concerns:
I recommend that the authors evaluate and show the activation of Akt and AS160 in soleus and EDL after incubation with submaximal and maximal insulin concentrations (Figure 1).
We have now included data in the manuscript (new figure 3) showing enhanced phosphorylation of Akt and AS160 in incubated skeletal muscle following submaximal and maximal insulin stimulation. To add further to such analyses, we have also included data on phosphorylated AMPK Thr172 and ACC Ser212 to indicate enhanced intracellular signaling by contraction.