AMPKγ3 Controls Muscle Glucose Uptake in Recovery From Exercise to Recapture Energy Stores

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Exercise increases muscle glucose uptake independently of insulin signaling and represents a cornerstone for the prevention of metabolic disorders. Pharmacological activation of the exercise-responsive AMPK in skeletal muscle has been proven successful as a therapeutic approach to treat metabolic disorders by improving glucose homeostasis through the regulation of muscle glucose uptake. However, conflicting observations cloud the proposed role of AMPK as a necessary regulator of muscle glucose uptake during exercise. We show that glucose uptake increases in human skeletal muscle in the absence of AMPK activation during exercise and that exercise-stimulated AMPKγ3 activity strongly correlates to muscle glucose uptake in the postexercise period. In AMPKγ3-deficient mice, muscle glucose uptake is normally regulated during exercise and contractions but impaired in the recovery period from these stimuli. Impaired glucose uptake in recovery from exercise and contractions is associated with a lower glucose extraction, which can be explained by a diminished permeability to glucose and abundance of GLUT4 at the muscle plasma membrane. As a result, AMPKγ3 deficiency impairs muscle glycogen resynthesis following exercise. These results identify a physiological function of the AMPKγ3 complex in human and rodent skeletal muscle that regulates glucose uptake in recovery from exercise to recapture muscle energy stores.

ARTICLE HIGHLIGHTS

- Exercise-induced activation of AMPK in skeletal muscle has been proposed to regulate muscle glucose uptake in recovery from exercise.
- This study investigated whether the muscle-specific AMPKγ3-associated heterotrimeric complex was involved in regulating muscle glucose metabolism in recovery from exercise.
- The findings support that exercise-induced activation of the AMPKγ3 complex in human and mouse skeletal muscle enhances glucose uptake in recovery from exercise via increased translocation of GLUT4 to the plasma membrane.
- This work uncovers the physiological role of the AMPKγ3 complex in regulating muscle glucose uptake that favors replenishment of the muscle cellular energy stores.

AMPK functions as a cellular energy sensor and is expressed in essentially all human and animal cells (1,2). AMPK exists as a heterotrimeric complex and is composed of a catalytic α-subunit and regulatory β- and γ-subunits (3) of which multiple isoforms exist (α1, α2, β1, β2, γ1, γ2, and γ3) (4). Isoform-specific immunoprecipitation experiments suggest that three heterotrimeric combinations are present in human skeletal muscle (α2β2γ3, α2β2γ1, and α1β2γ1), while five combinations are present in mouse skeletal muscle (α2β2γ3, α2β2γ1, α1β2γ1, α2β1γ1, and α1β1γ1) (5,6). Pharmacological activation of AMPK in skeletal muscle increases glucose uptake (7,8), and observations from rodent

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models and nonhuman primates have confirmed that targeting AMPK in skeletal muscle by selective activators is a viable therapeutic approach to reverse hyperglycemia (9,10). We and others have shown that ADaM-site–binding small-molecule activators of AMPK, including PF739, 991, and MK-8722, increase muscle glucose uptake via an AMPKα-dependent but AMPKγ3-independent mechanism (11,12). In contrast, the nonspecific AMPK activator AICAR (AMP mimetic) has repeatedly been shown to increase muscle glucose uptake via an AMPKγ3-dependent mechanism (11–13). Physiologically, AMPK is activated in skeletal muscle during exercise in a time- and intensity-dependent manner (14–17). In human skeletal muscle, exercise-induced activation of the AMPKγ3 complex is highly potent and selective, although the AMPKγ3 complex only accounts for one-fifth of all the AMPK complexes (18). Because exercise and AICAR activate the AMPKγ3 complex by promoting phosphorylation of AMPKα-T172 via changes in the intracellular AMP and ZMP pools, respectively, it has been argued that the AMPKγ3 complex regulates muscle glucose uptake during exercise and contractions. However, numerous studies involving AMPK-deficient mice have not been able to reach consensus on this matter. In a recent investigation of the literature, we found that the lack of consensus is related to methodological inconsistencies. Thus, the majority of studies, including our own, reporting impaired contraction-induced muscle glucose uptake in AMPK-deficient mice actually measure muscle glucose uptake in the period after contraction (19). Inspired by these observations, we hypothesized that AMPK, and specifically the AMPKγ3 complex, regulates muscle glucose uptake in the period after, but not during, exercise and that this physiological function of AMPKγ3 serves to recapture muscle energy stores in prior exercised muscle.

RESEARCH DESIGN AND METHODS

Study Approvals

The human study was approved by the Copenhagen Regional Ethics Committee (H-18006850), complied with the guidelines of the Declaration of Helsinki, and is registered with ClinicalTrials.gov (NCT04872426). All participants were provided with oral and written study information after which written informed consent was obtained from all participants before entering the study. Animal experiments were approved by the Danish Animal Experiments Inspectorate (license 2019-15-0201-01659) and complied with the European Union guidelines for the protection of vertebrate animals used for scientific purposes.

Animals Models

AMPKγ3 (*PRKAG3*) knockout (KO) mice were generated at The Jackson Laboratory (Bar Harbor, ME) using clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology as previously described (11). In short, Cas9 protein with a single guide RNA was used to induce a frameshift deletion in exon 6, resulting in a premature truncation of the *PRKAG3* gene and KO of the AMPKγ3 protein. AMPKγ3 R225W gain-of-function mice were generated at The Jackson Laboratory using CRISPR-Cas9 technology. In short, Cas9 protein with a single guide RNA (CATGGTGGCCAACGGTGTGA) was used together with a repair template (TCGTTCTTTCCTGCCCCTCAGATAAAGAAGGCTTTCTTTGCCATGGTGGCCAACGGTGTGTGGGCAGCTCCTCTGTGGGACAGCAAGAAGCAGAGCTTTGTGGGTGAGGAGAGGTGGCTGG) to induce the R225W mutation. Constitutive muscle-specific AMPKα1α2 double-KO mice and corresponding wild-type (WT) littermates were generated, bred, and characterized as previously described (20). Animal genotypes were determined by *1*) genomic DNA from ear snip sampling that was analyzed by standard PCR methods and *2*) analyses of AMPKγ3 and AMPKα2 protein in muscle by standard immunoblotting. Female and male mice (mean ± SD age 20.2 ± 7.7 weeks, mean ± SD weight 28.0 ± 7.7 g) were obtained from in-house breeding at a specific-pathogen-free animal facility and transported to the experimental facility at least 1 week before entering an experiment to secure acclimatization. AMPKγ3 KO, R225W, and corresponding WT mice were bred as homozygotes and represented the F1 and F2 generations. All animals were group housed unless otherwise stated, had free access to water and standard rodent chow (Altromin no. 1324), and were maintained on a 12-h light/dark cycle (lights on 6:00 A.M.) in a temperature-controlled room (22 ± 2°C).

Human Study

This study is part of a larger clinical trial designed to assess the effect of ischemia and two exercise modalities on insulin sensitivity and protein signaling in skeletal muscle of healthy male subjects. Only results on selected parameters obtained before, during, and 3 h into recovery from the two exercise modalities are included in this study.

Study Participants

Eight healthy, young (age: 27.3 ± 1.2 years), lean (BMI: 24.1 ± 1.0 kg/m^2), and moderately physically active male participants were recruited and enrolled in the study. Prior to the experimental day, VO_{2peak} (46.9 ± 1.6 mL/min/kg) was determined on a bike ergometer (Monark, Vansbro, Sweden) by an incremental test to exhaustion using measurements of VO_2 (MasterScreen CPX; Intramedic A/S, Gentofte, Denmark). Body composition (lean mass 61.6 ± 2.4 kg, fat mass 16.2 ± 2.3 kg) was determined by DXA (DPX-IQ Lunar; Lunar Corporation, Madison, WI). Furthermore, the study participants were familiarized to the one-legged knee extensor ergometer on several occasions, and at a minimum of 1 week prior to the first experimental day, peak workload (PWL) of the knee extensors was determined in both legs (56.3 ± 2.5 W). The study participants were instructed to record food intake for 3 days and to abstain from alcohol, caffeine, and strenuous physical activity 2 days before the first experimental day. Three days before the second experimental day, the study participants were instructed to adhere to their prior food recordings and again

abstain from alcohol, caffeine, and strenuous physical activity 2 days before the experimental day.

Human Experimental Protocol

On both experimental days, the study participants arrived at the laboratory in the morning (6:00 A.M.) after an overnight fast. The participants then ingested a light breakfast (oatmeal, skim milk, and sugar; 5% of daily energy intake) and rested in the supine position for 2–3 h during which catheters (Pediatric Jugular Catheterization Set, Arrow International, Reading, PA) were inserted into the femoral artery of one leg and femoral veins of both legs under local anesthesia (Xylocaine; AstraZeneca, Ballerup, Denmark). After the rest period, the study participants were randomized to perform one of two knee extensor exercise modalities with a single leg for 1 h. The exercise leg was randomized, but the same on the two experimental days. The two exercise modalities consisted of *1*) 70% of PWL (continuous moderate intensity [CON]) and *2*) 70% of PWL evenly interspersed with six 5-min intervals at 95% of PWL (intermittent high intensity [INT]) and were performed by each participant on two different days separated by at least 2 weeks. Before, during, and 3 h into exercise recovery, blood samples were obtained from the femoral arterial and venous catheters, and femoral artery blood flow was determined in both legs using the ultrasound Doppler technique (Phillips iU22; ViCare Medical A/S, Birkerød, Denmark) to determine leg glucose uptake. Biopsies of vastus lateralis muscle were obtained in the rested leg before exercise and in the exercised leg immediately after exercise. Muscle biopsy specimens were rinsed in ice-cold physiological saline (0.9%), dried on filter paper, and frozen in liquid nitrogen before being stored at −80°C until further use. One study participant withdrew from the study after having completed the CON trial. Thus, the human analyses are based on 8 in the CON exercise trial and 7 in the INT exercise trial.

In Vivo Exercise-Stimulated Muscle Glucose Clearance

All mice were single housed and fasted for 2 h with free access to water. For muscle glucose clearance measurements during exercise, mice received a single intraperitoneal injection of physiological saline (8 mL/kg body weight) containing [^{3}H]2-deoxyglucose (2.22 MBq/mL) and were left in their individual cages for 20 min before the running exercise protocol was initiated (30 min, 75% of individual maximal running speed, and 10° incline). Blood glucose and lactate measurements were obtained from the tail before and after the exercise bout (t = 0 and 30 min). At similar time points, 10-μL blood samples were collected and transferred to separate tubes containing 30 μL of BaOH. Blood-BaOH mixture samples were immediately vortexed, after which 30 μL of $ZnSO_4$ was added followed by a second vortex. Mice were euthanized by cervical dislocation after the last blood sample, and muscle tissues were dissected and frozen in liquid nitrogen for analyses of muscle glucose clearance.

For muscle glucose clearance during rest and in recovery from exercise, mice were anesthetized by a single intraperitoneal injection of pentobarbital/Xylocaine mixture (10/0.5 mg per 100 g of body weight) dissolved in physiological saline (0.9%) immediately after having rested in individual cages for 30 min without access to food or having performed the abovementioned running exercise protocol. Mice were left to recovery on a heating plate (30°C) for 30 min, after which a bolus of [^{3}H]2-deoxyglucose (2.48 MBq/mL) dissolved in physiological saline (0.9%) was administered by a single retro-orbital injection. Before and 5, 10, and 15 min after the retro-orbital injection, blood glucose measurements were obtained from the tail. At similar time points, 25-μL blood samples were collected and transferred to separate tubes containing 60 μL of BaOH. Blood-BaOH mixture samples were immediately vortexed after which 60 μL of $ZnSO_4$ was added followed by a second vortex. Mice were euthanized by cervical dislocation after the last blood sample, and muscle tissues were dissected and frozen in liquid nitrogen for analyses of muscle glucose clearance.

In Vivo Contraction-Stimulated Muscle Glucose Clearance

Fed mice were anesthetized by a single intraperitoneal injection of pentobarbital/Xylocaine mixture (10/0.5 mg per 100 g of body weight) dissolved in physiological saline (0.9%), after which the common peroneal nerve was exposed on both legs. Hereafter, an electrode was placed on one common peroneal nerve followed by in situ contraction of the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles. The contralateral leg served as a sham-operated resting control on all animals. The contraction protocol consisted of 0.5-s trains (100 Hz, 0.1 ms, 5 V) repeated every 1.5 s for 10 min. Muscle glucose clearance during in situ contraction was determined by retro-orbital injection of [^{3}H]2-deoxyglucose (as described above), during which blood glucose measurements and blood samples were obtained from the tail immediately before and at 5 and 10 min into the contraction protocol. For muscle glucose clearance measurements in recovery from in situ contraction, anesthetized and muscle-contracted animals were left to recover on a heating plate (30°C) for 30 min, after which muscle glucose clearance was determined as mentioned above. Mice were euthanized by cervical dislocation after the last blood sample, and muscle tissues were dissected and frozen in liquid nitrogen for analyses of muscle glucose clearance. Methods for blood and plasma analyses, muscle processing and homogenization, muscle glycogen, treadmill acclimatization, maximal exercise capacity test, calculation of in vivo muscle glucose clearance, ex vivo 3-*O*-methylglucose uptake in isolated skeletal muscle, AMPK activity, immunoblotting, antibodies, as well as GLUT4 immunostaining of muscle cryosections are all presented in the Supplementary Material.

Statistical Analysis

Statistical analyses were performed using SigmaPlot version 14.0 software (Systat, Erkrath, Germany). An unpaired

two-tailed Student *t* test was used for comparisons between two groups. Two independent variables were compared using a two-way ANOVA with or without repeated measures followed by Holm-Šidák post hoc test when an interaction between variables occurred. Data were transformed to obtain equal variance when unequal variance was observed between nonpaired groups. Correlation analyses were performed by calculating the Pearson product moment correlation coefficient. $P < 0.05$ was considered to be statistically significant. Data are presented as mean ± SEM unless stated otherwise. Statistical parameters can be found in the individual figure legends. Figures were created using GraphPad Prism 9.0 software (GraphPad Software).

Data and Resource Availability

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

RESULTS

Exercise-Induced Activation of AMPKα2β2γ3 in Human Skeletal Muscle Is Associated With Enhanced Glucose Uptake in Recovery From Exercise

To provide human evidence in support of our hypothesis, we tested the effect of CON and INT one-legged knee extensor exercise to regulate AMPK activity and glucose uptake in human muscle (Fig. 1*A*). Muscle lactate release and plasma adrenaline/noradrenaline levels increased, while muscle glycogen levels decreased to a greater extent in response to INT compared with CON exercise, demonstrating that the INT exercise protocol provoked a larger metabolic stress response (Supplementary Fig. 1*A–D*). These findings were mirrored at the level of AMPK activation and its downstream targets. Thus, activation of the AMPKγ3 complex was observed in muscle after INT exercise together with enhanced phosphorylation of ACC-S221 and TBC1D1-S237, whereas the response was absent or weak after CON exercise (Fig. 1*B–G*). Muscle glucose uptake increased in response to both exercise trials but to a greater extent during INT exercise (Fig. 1*H*). This difference was driven by an enhanced blood flow rate (Fig. 1*I*). Because AMPKγ3 activity did not increase in response to CON exercise, this suggests that AMPKγ3 is not necessary to increase glucose uptake in human skeletal muscle during exercise.

In recovery from exercise, muscle glucose uptake was greater and of longer duration in response to INT compared with CON exercise (Fig. 1*H*). In contrast to findings during exercise, this difference was driven by an enhanced glucose extraction rate (Fig. 1*J*), indicating a role of the membrane permeability to glucose to maintain muscle glucose uptake elevated in recovery from exercise. In the INT exercise trial, we observed that changes in AMPKγ3 activity and muscle glucose uptake were not correlated during exercise but were positively correlated in recovery from exercise (Fig. 1*K* and *L*). Similarly, a positive correlation between changes in AMPKγ3 activity and the arteriovenous difference was observed in recovery from INT exercise (Fig. 1*M* and Supplementary Fig. 1*E*). Collectively, these data imply that the AMPKγ3 complex regulates glucose uptake in human skeletal muscle after, but not during, exercise by stimulating muscle glucose extraction. Measurements of glycogen synthase and pyruvate dehydrogenase phosphorylation, as well as protein expression of hexokinase 1, hexokinase 2 (HK2), and GLUT4 indicated that differences in muscle glucose uptake following CON and INT exercise were not related to differences in the capacity of muscle to take up, store, and/or oxidize glucose (Supplementary Fig. 1*F–L*). Furthermore, plasma insulin levels and muscle insulin signaling were not different between the two exercise modalities and, therefore, cannot explain differences in post-exercise muscle glucose uptake (Supplementary Fig. 1*M–O*).

Muscle Glucose Clearance in Recovery From Exercise Is Impaired in AMPKγ3-Deficient Mice and Associates With Increased AMPKα2β2γ3 Activity, Phosphorylation of TBC1D1-S231, and Glycogen Resynthesis in Muscle From WT Mice

To provide genetic evidence for our findings in humans, we examined glucose clearance in skeletal muscle of AMPKγ3 KO and WT mice at rest, during submaximal continuous treadmill exercise, and 30 min into exercise recovery (Fig. 2*A*). Maximal exercise capacity did not differ between genotypes (Fig. 2*B*). In response to submaximal exercise, blood glucose and lactate levels increased to a similar extent in both genotypes (Supplementary Fig. 2*A* and *B*). During exercise, glucose clearance increased ∼10-, ∼20-, and ∼50-fold in quadriceps, TA, and soleus muscle, respectively, irrespective of genotype (Fig. 2*C–E*). In contrast, we found that glucose clearance was significantly lower in quadriceps muscle from AMPKγ3 KO mice in recovery from exercise (Fig. 2*C*). We observed increased glucose clearance but reversal of AMPKγ3 activity in WT quadriceps muscle in recovery from exercise (Fig. 2*C* and *F*), suggesting that one or more AMPKγ3-phosphoregulated proteins located closer to the glucose transport event are responsible for maintaining muscle glucose clearance elevated in recovery from exercise. Phosphorylation of TBC1D1 is a likely mechanism by which AMPKγ3 stimulates muscle glucose uptake because AICAR-stimulated glucose uptake is abolished in muscle from TBC1D1 KO mice (21,22). We found that phosphorylation of TBC1D1-S231 (equivalent to human TBC1D1-S237) was still elevated in WT quadriceps muscle during recovery compared with rest (Fig. 2*G*). Together, this demonstrates that AMPKγ3 promotes muscle glucose uptake after, but not during, exercise and that reversal of glucose uptake in recovery from exercise is associated with downstream phosphorylation of TBC1D1-S231. We also observed elevated phosphorylation of TBC1D1-S231 in muscle from AMPKγ3 KO mice in response to exercise (Fig. 2*G*, *K*, and *L*). Because contraction increases phosphorylation of TBC1D1-S231 in muscle from AMPKα1α2-deficient mice (19), this suggests that kinases other than AMPK are able to phosphorylate TBC1D1-S231 in muscle cells during contractile activity. We believe that this does not contribute significantly to muscle glucose uptake in recovery from exercise.

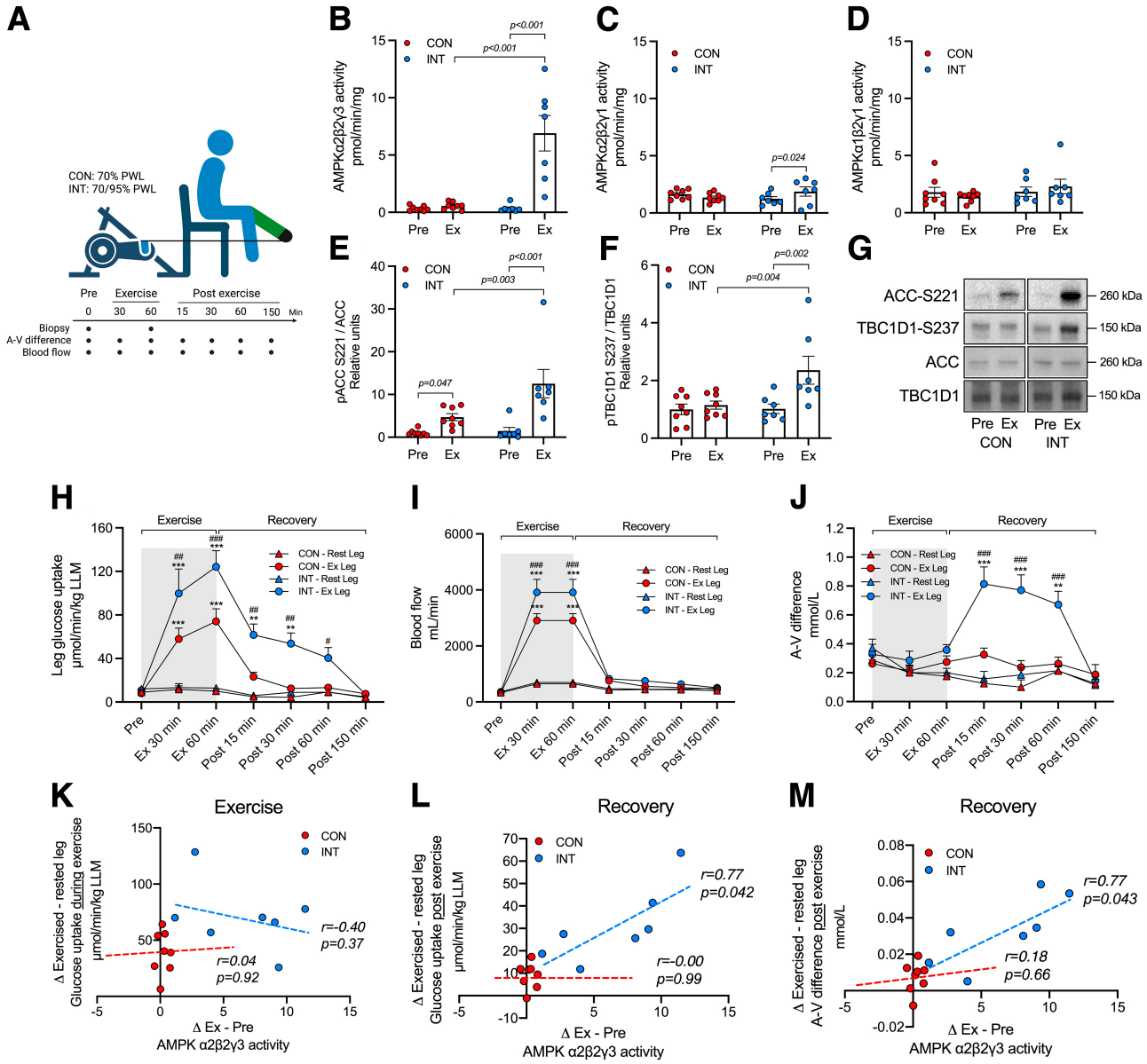

Figure 1—Exercise-induced activation of AMPKα2β2γ3 in human skeletal muscle is associated with enhanced glucose uptake in recovery from exercise. *A*: Schematic illustration of the human experimental study. Subjects were randomized to perform 1 h of one-legged knee extensor exercise either at 70% of PWL (CON) or 70% of PWL evenly interspersed with six 5-min intervals at 95% of PWL (70/95% PWL) (INT). Muscle biopsies from vastus lateralis muscle were obtained before and immediately after exercise. Before, during, and 150 min into exercise recovery, measurements of blood flow and blood glucose arteriovenous (A-V) difference (Δ) were performed to determine leg glucose uptake. *B–F*: AMPK heterotrimer–specific activity (*B–D*) and phosphorylation of AMPK downstream targets ACC-S221 (*E*) and TBC1D1-S237 (*F*) in the muscle biopsies. *G*: Representative immunoblots. *H–J*: Glucose uptake, blood flow, and A-V difference measured before (Pre), during (Ex 30 and 60 min), and in recovery from exercise (Post 15–150 min) in the rested and exercised leg. *K–M*: ΔAMPKα2β2γ3 activity (Ex value − Pre value) correlated to Δ average glucose uptake during exercise (average glucose uptake in exercised leg − average glucose uptake in rested leg), Δ average glucose uptake postexercise, and Δ average A-V difference postexercise. Data are mean ± SEM. Data in panels *B–F* were analyzed using a two-way repeated-measures ANOVA. In panels *H–J*, a single two-way repeated-measures ANOVA was used to evaluate differences between the two rested legs, and a second two-way repeated-measures ANOVA was used to evaluate differences between the two exercised legs. Differences from Pre are described for the exercised legs only. The *r* value and significance level of each correlation are indicated in the respective panels. $n = 8$ CON and 7 INT. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. PRE; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. CON. LLM, leg lean mass.

Instead, we argue that the ability of the AMPKγ3 complex to enhance and maintain phosphorylation of TBC1D1-S231 elevated after the cessation of exercise is essential to promote muscle glucose uptake in recovery from exercise.

We did not observe a genotype difference in postexercise glucose clearance for soleus and TA muscle (Fig. 2*D* and *E*). In WT soleus muscle, this can be explained by a low expression and activity of the AMPKγ3 complex (Fig. 2*H* and *I* and

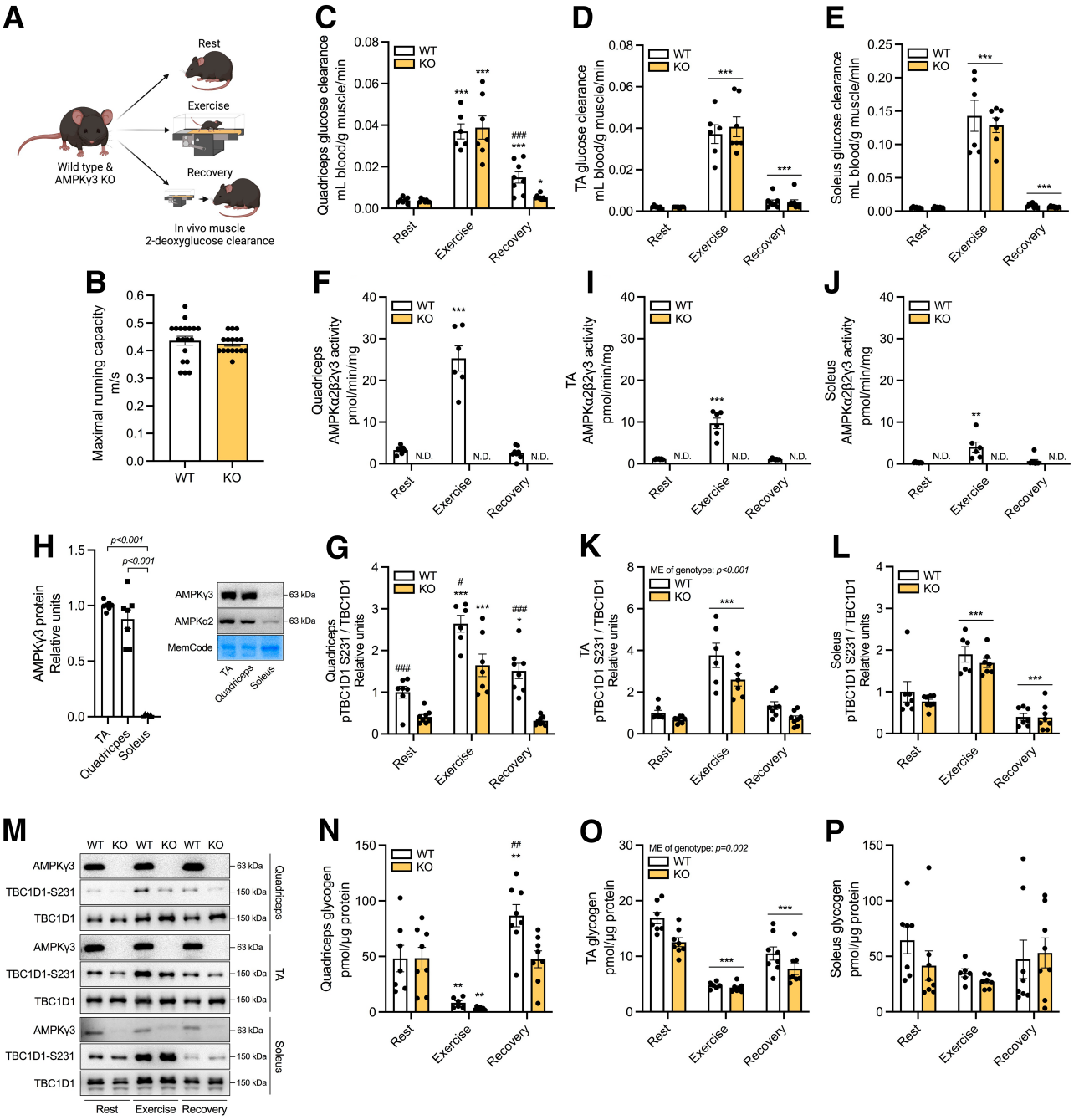

Figure 2—Muscle glucose clearance in recovery from exercise is impaired in AMPKγ3-deficient mice and associates with increased AMPKα2β2γ3 activity, phosphorylation of TBC1D1-S231, and glycogen resynthesis in muscle from WT mice. *A*: Schematic illustration of the mouse exercise study. In vivo muscle glucose clearance was assessed in male mice at rest, during exercise, and 30 min in recovery from exercise. *B*: Maximal running capacity during an incremental treadmill running test (WT $n = 19$ and KO $n = 16$). *C–E*: Glucose clearance in quadriceps, TA, and soleus muscle from WT and AMPKγ3 KO male mice. *F*, *I*, and *J*: AMPKα2β2γ3 activity in quadriceps, TA, and soleus muscle from WT and KO mice. *H*: AMPKγ3 protein content in quadriceps, TA, and soleus muscle from WT male mice. *G*, *K*, and *L*: Phosphorylation of TBC1D1 S231 in quadriceps, TA, and soleus muscle from WT and KO male mice. *M*: Representative immunoblots. *N–P*: Glycogen content in quadriceps, TA, and soleus muscle from WT and KO male mice. Data are mean ± SEM. Data in panel *B* were evaluated using Student *t* test, while data in panels *F* and *H–J* were evaluated using a one-way ANOVA. Data in panels *C–E*, *G*, *K*, *L*, and *N–P* were evaluated using a two-way ANOVA. WT $n = 7$, 6, and 8 and KO $n = 8$, 7, and 8 (rest, exercise, and recovery, respectively). Solid horizontal lines indicate main effect (ME). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. rest; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. KO. N.D., not detected.

Supplementary Fig. 2*C*). In WT TA muscle, which expresses AMPKγ3 protein to a similar extent as WT quadriceps muscle (Fig. 2*H*), AMPKγ3 activity was ∼60% lower compared with quadriceps muscle during exercise (Fig. 2*J* and Supplementary Fig. 2*C*). This low AMPKγ3 activity is likely insufficient to induce genotypic differences in postexercise glucose clearance because when conditions are applied to WT TA muscle that increases AMPKγ3 activity to levels seen in WT quadriceps

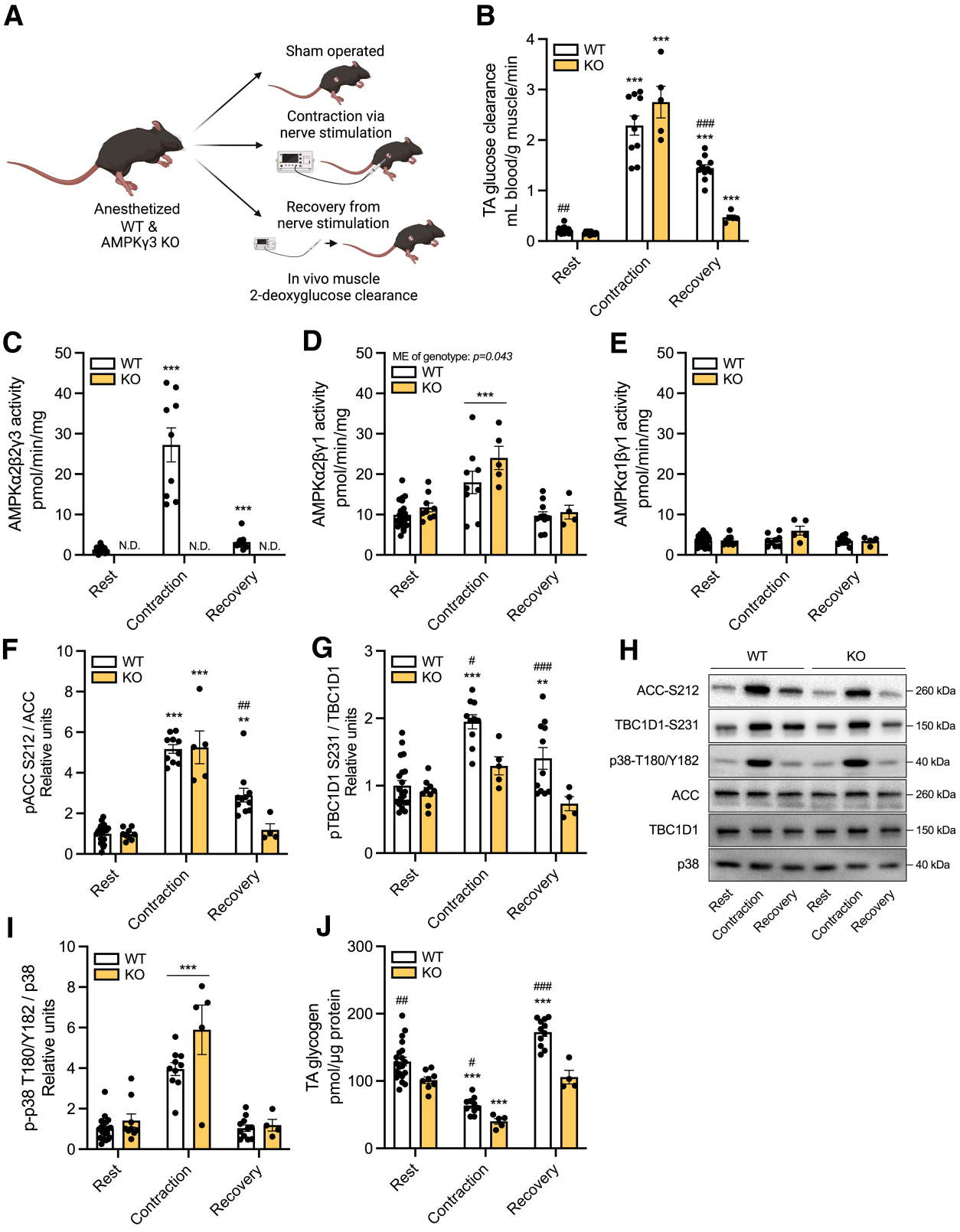

Figure 3—Muscle glucose clearance in recovery from contraction is impaired in AMPKγ3-deficient mice and associates with increased AMPKα2β2γ3 activity, phosphorylation of TBC1D1-S231, and glycogen resynthesis in muscle from WT mice. *A*: Schematic illustration of the mouse in situ contraction study. In vivo glucose clearance was assessed in rested muscle, acutely contracted muscle, and in muscle 30 min after contraction. *B–J*: TA glucose clearance, AMPKα2β2γ3 activity, AMPKα2βγ1 activity, AMPKα1βγ1 activity, phosphorylation of ACC-S212, phosphorylation of TBC1D1-S231, representative immunoblots, phosphorylation of p38-T180/Y182, and TA glycogen content measured in rested, contracted, and prior contracted (recovery) TA muscle from WT and KO female mice. Data are mean ± SEM. Data in

muscle during exercise, then such genotypic differences are present (see next section). Moreover, phosphorylation of TBC1D1-S231 was not elevated in WT soleus and TA muscle in recovery from exercise (Fig. 2*K–M*). Similar to our findings in humans, this suggests that the degree of exercise-induced AMPKγ3 activation in mouse muscle determines the magnitude of glucose uptake in recovery from exercise. The activities of the remaining AMPK complexes were not compromised in muscle from AMPKγ3 KO mice and could therefore not explain our findings (Supplementary Fig. 2*D–I*). We observed that the exercise-induced increases in muscle glycogen utilization and p38 phosphorylation were similar between genotypes (Fig. 2*N–P* and Supplementary Fig. 2*J–M*), signifying that muscles of both genotypes were subjected to similar exercise-provoked metabolic stress. Interestingly, we found that resynthesis of glycogen following exercise was suppressed in quadriceps muscle from AMPKγ3 KO mice and that glycogen was supercompensated in WT quadriceps muscle (Fig. 2*N*). We did not observe this in either WT soleus or TA muscle (Fig. 2*O* and *P*), which is consistent with the lack of genotypic differences in muscle glucose uptake. This suggests that the ability of AMPKγ3 to maintain elevated muscle glucose uptake supports resynthesis of muscle glycogen stores in recovery from exercise.

Muscle Glucose Clearance in Recovery From Contraction Is Impaired in AMPKγ3-Deficient Mice and Associates With Increased AMPKα2β2γ3 Activity, Phosphorylation of TBC1D1-S231, and Glycogen Resynthesis in Muscle From WT Mice

To substantiate our in vivo findings, we sought to demonstrate a similar phenotype using a well-controlled experimental setup that takes advantage of direct nerve stimulation to elicit muscle contraction in anesthetized mice (19) (Fig. 3*A*). We found that glucose clearance was comparable in TA muscle from AMPKγ3 KO and WT mice during in situ contractions (Fig. 3*B*). In contrast, muscle glucose clearance was significantly lower in TA muscle from AMPKγ3 KO compared with WT mice 30 min after the contraction period (recovery) (Fig. 3*B*). In an AMPKγ3 gain-of-function mouse model (R225W), muscle glucose clearance was partially rescued in recovery from contraction (Supplementary Fig. 3*A* and *B*). In muscle from WT mice, AMPKγ3 activity was increased by ∼20-fold during contraction and by ∼2.5-fold in recovery compared with rest, and activities of the remaining AMPK complexes were not compromised in muscle from the AMPKγ3 KO mice at any time (Fig. 3*C–E*). The increased AMPKγ3 activity in WT muscle during recovery was associated with increased phosphorylation of ACC-S212 and TBC1D1-S231 (Fig. 3*F–H*). Muscle protein expression of GLUT4 was similar between genotypes, but HK2 protein expression was slightly decreased (∼20%) in muscle from AMPKγ3 KO mice (Supplementary Fig. 3*C–E*). Because exercise-induced glucose clearance is not compromised in glycolytic skeletal muscle from heterozygous HK2 KO mice (23), differences in postexercise/contraction-induced muscle glucose clearance are likely not explained by a minor decrease in HK2 muscle protein expression. Similar to in vivo exercise, we found that the contraction protocol increased phosphorylation of p38 and glycogen utilization to a similar extent in muscle from both genotypes and that resynthesis of glycogen in recovery was suppressed in muscle from AMPKγ3 KO mice (Fig. 3*H–J*). We confirmed the impairment in muscle glycogen resynthesis during recovery in our muscle-specific AMPKα1α2 double-KO mouse model that exhibits intact muscle HK2 protein levels and decreased muscle glucose clearance in recovery from exercise and contractions (19) (Supplementary Fig. 3*F*). Together, these findings further support a role of the AMPKγ3 complex for the regulation of muscle glucose uptake after, but not during, exercise that promotes resynthesis of the muscle glycogen stores.

AMPKγ3 Increases Glucose Permeability and GLUT4 Abundance at the Plasma Membrane to Enhance Glucose Uptake in Recovery From Contraction

AMPK has been proposed to increase muscle glucose uptake/clearance via its ability to increase GLUT4 translocation to the cell surface membrane independently of the canonical insulin signaling pathway (24–26). Therefore, we investigated potential changes in the glucose permeability and GLUT4 abundance at the muscle plasma membrane to mechanistically explain our findings on muscle glucose clearance in recovery from exercise and contraction. Initial investigations revealed that in situ contractions increased in vivo glucose clearance (13-fold) and GLUT4 translocation (1.2-fold) in EDL muscle from AMPKγ3 KO and WT mice to a similar extent (Fig. 4*A–C*). This adds to the notion that AMPKγ3 is not involved in regulating glucose uptake and GLUT4 translocation in muscle during contractions. Correlative analyses did not support an association between in vivo muscle glucose clearance and GLUT4 translocation during contractions (Supplementary Fig. 4*A* and *B*), implying that delivery, phosphorylation, and metabolism of glucose are important determinants for glucose uptake in vivo (27).

Next, we investigated glucose transport in isolated and incubated EDL muscle 1 h after in situ contractions using [^{3}H]-3-*O*-methylglucose (3-OMG) as a glucose analog (Fig. 4*D*). Cellular uptake of 3-OMG does not depend on phosphorylation by HK2 and, therefore, provides a direct measurement of the muscle membrane permeability to glucose (28). We observed

panel *C* were evaluated using one-way ANOVA, while data in panels *B*, *D–G*, *I*, and *J* were evaluated using two-way ANOVA. WT n = 21, 10, and 11 and KO n = 9, 5, and 4 (rest, contraction, and recovery, respectively) except for AMPK activity in WT mice (n = 21, 9, and 11) and TA glycogen (WT n = 20, 10, and 11 and KO n = 8, 5, and 4). Solid horizontal lines indicate main effect (ME). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. rest; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. KO. N.D., not detected.

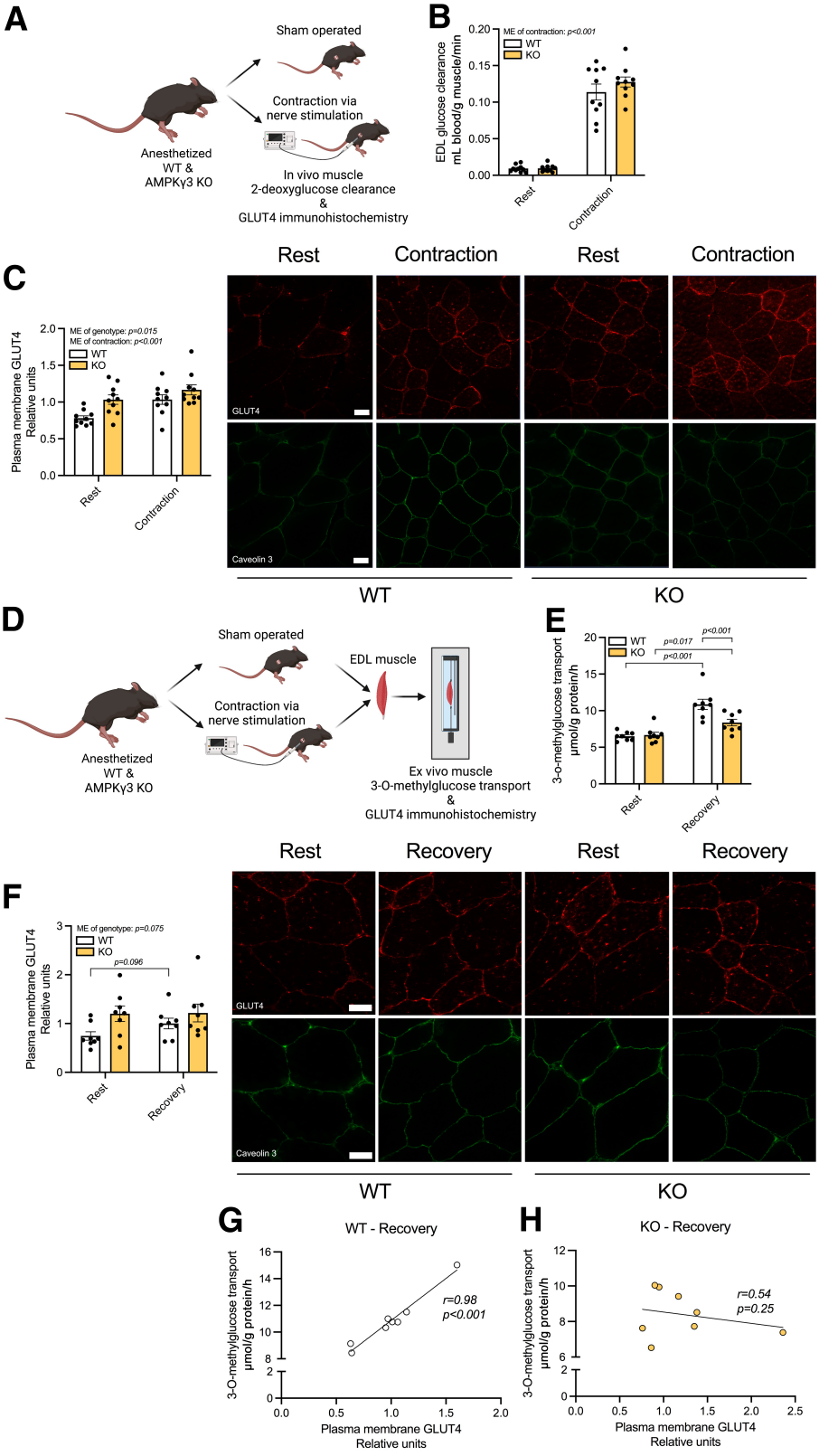

Figure 4—AMPKγ3 increases glucose permeability and GLUT4 abundance at the plasma membrane to enhance glucose uptake in recovery from contraction. *A*: Schematic illustration of the acute in situ contraction study. In vivo glucose clearance and plasma membrane GLUT4 abundance were assessed in rested and acutely contracted muscle. *B* and *C*: Glucose clearance and plasma membrane GLUT4 abundance in rested and contracted EDL muscle from WT and AMPKγ3 KO male mice (n = 10). Panel *C* also shows representative GLUT4 and caveolin-3 (plasma membrane marker) immunostainings. Scale bar = 20 μm. *D*: Schematic illustration of the acute in situ contraction and incubation study. Ex vivo glucose transport and plasma membrane GLUT4 abundance were assessed in incubated EDL muscle from

that 3-OMG transport was significantly lower in EDL muscle from AMPKγ3 KO mice compared with WT mice 1 h after contractions (Fig. 4*E*). In the same set of muscles, GLUT4 translocation tended ($P = 0.096$) to be increased in prior contracted muscle from WT mice but not in prior contracted muscle from AMPKγ3 KO mice (Fig. 4*F*). Notably, correlative analyses revealed that 3-OMG transport was strongly and positively associated with GLUT4 translocation in prior contracted muscle from WT mice only (Fig. 4*G* and *H* and Supplementary Fig. 4*C* and *D*).

DISCUSSION

Using AMPKγ3 KO mice, we provide genetic evidence to support that exercise-induced activation of the AMPKγ3 complex in skeletal muscle functions to maintain muscle glucose uptake elevated in the period after, but not during, exercise by preserving GLUT4 in the muscle plasma membrane to promote muscle glycogen resynthesis. These findings are fully in line with our previous work describing a role of AMPKα1α2 in regulating muscle glucose uptake in recovery from exercise and contractions (19). Our findings in mice also seem valid for human skeletal muscle because CON exercise increased muscle glucose uptake independently of changes in AMPKγ3 activity and because greater AMPKγ3 activity following INT exercise correlated with muscle glucose uptake after, but not during, exercise. On the basis of these findings, we now propose that a physiological function of the AMPKγ3 complex in skeletal muscle is to secure a faster normalization of the myocellular energy and fuel status in recovery from exercise rather than to secure energy supply during exercise.

Others have observed impairments in muscle glycogen resynthesis in AMPKγ3 KO mice 2.5 h after a swimming exercise (13), but these findings were not associated with a difference in muscle glucose uptake between WT and AMPKγ3 KO mice (29). The discrepancy between these and our findings likely relates to the temporal assessment of muscle glucose uptake rates because it has been demonstrated that the AMPK-dependent increase in muscle glucose uptake promotes glycogen synthesis due to enhanced allosteric activation of glycogen synthase by glucose-6-phosphate (30). Thus, impaired muscle glycogen resynthesis after exercise in AMPKγ3-deficient mice is likely a consequence of impaired muscle glucose uptake after exercise.

We have shown several times that the AMPKγ3 complex is potently activated in human skeletal muscle during exercise (18,31–33). Exercise-induced activation of the AMPKγ3 complex is likely mediated by the binding of AMP that does not allosterically activate the complex but rather promotes covalent activation by increasing phosphorylation of AMPKα-T172 (34). This is mediated via enhanced phosphorylation by the upstream kinase LKB1, as well as by decreased dephosphorylation by protein phosphatases (34). Depending on the intensity and duration of the exercise, activity of the AMPKγ3 complex is increased several hours into recovery (31,33,35,36). Because exercise-induced changes in the muscle adenine nucleotide pools return to preexercise levels within minutes after the cessation of exercise (37,38), we speculate that AMP binding to the AMPKγ3 complex persists for several hours following exercise, which stimulates phosphorylation of AMPKα-T172 to promote muscle glucose uptake.

Based on our previous findings in muscle from TBC1D1 KO mice demonstrating impaired glucose uptake in recovery from contraction (19), as well as observations of enhanced phosphorylation of TBC1D1 in recovery from exercise/contractions, it seems likely that an AMPKγ3-TBC1D1 signaling axis regulates muscle glucose uptake after exercise. However, the idea that AMPK increases muscle glucose uptake by delaying GLUT4 endocytosis as previously proposed (39) does not go hand in hand with downstream phosphorylation and inhibition of TBC1D1, as this would be expected to accelerate GLUT4 endocytosis since TBC1D1 is an inhibitor of GLUT4 trafficking (40). We now speculate that the AMPKγ3-TBC1D1 signaling axis drives muscle glucose uptake in the period after exercise by promoting re-exocytosis of GLUT4.

The dissociation between increased AMPK activity and lack of effect on muscle glucose uptake during exercise is puzzling but indicates redundancy of the AMPK signaling pathway; thus, we hypothesize that the AMPK-stimulated GLUT4 trafficking only becomes relevant when the exercise stimulus ceases, which maintains elevated muscle glucose uptake in recovery from exercise. Our speculation that AMPKγ3-dependent phosphorylation of TBC1D1 is important for muscle glucose uptake in recovery from exercise is supported by findings showing that muscle overexpression of TBC1D1 mutated at four phosphorylation sites targeted by AMPK (S231A, T499A, S660A, and S700A) diminishes in situ contraction-stimulated glucose uptake measured as the combined uptake of glucose during (15 min) and after (30 min) contraction (41). In contrast, measurements of muscle glucose uptake in the period after in situ contraction is not affected in TBC1D1-S231A knock-in mice (42) or by muscle overexpression of TBC1D1 mutated at single phosphorylation sites (S231A, S660A, or S700A) (41). This indicates that multisite phosphorylation of TBC1D1 by

prior rested and in situ contracted legs of anesthetized mice. *E* and *F*: 3-OMG transport and plasma membrane GLUT4 abundance in rested and prior contracted (recovery) EDL muscle from WT and AMPKγ3 KO male mice ($n = 8$). Panel *F* also shows representative GLUT4 and caveolin-3 immunostainings. Scale bar = 20 μm. *G* and *H*: Correlation between 3-OMG transport and plasma membrane GLUT4 abundance in prior contracted muscle from WT and AMPKγ3 KO male mice. Data are mean ± SEM. Data in panels *C*, *D*, *E*, and *F* were evaluated using a two-way repeated-measures ANOVA. The *r* value and significance level of each correlation are indicated in the respective panels. ME, main effect.

AMPKγ3 is necessary to maintain elevated glucose uptake in recovery from exercise/contraction.

It should be noted that correlative analyses did not support an association between in vivo muscle glucose clearance and GLUT4 translocation during contraction (Supplementary Fig. 4*A* and *B*). This implies that delivery, phosphorylation, and metabolism of glucose are important determinants for contraction-induced glucose uptake in vivo (27). However, the disassociation may also reflect methodological limitations like the relatively (to the plasma membrane thickness) low resolution of the confocal microscope (43) or the lack of the T-tubule surface area in our GLUT4 immunostaining analyses (44). Furthermore, potential changes in GLUT4 intrinsic transporter activity (45) could also explain the dissociation between in vivo muscle glucose clearance and GLUT4 translocation during contraction.

In conclusion, our work provides new insight into the physiological role of the AMPKγ3 complex in regulating postexercise muscle glucose uptake. The effect of AMPKγ3 on muscle glucose uptake is mediated by enhanced translocation of GLUT4 to the plasma membrane, and our data suggest that this is important for resynthesis of muscle glycogen stores after exercise. Further studies are required to delineate the exact phosphorylation sites on TBC1D1 and intracellular GLUT4 compartments responsible for relaying AMPKγ3 signaling to stimulate muscle glucose uptake and glycogen resynthesis in the postexercise period. Considering our previous findings on muscle insulin sensitization by exercise (46,47), it is intriguing to speculate that the AMPKγ3 complex may also be involved in regulating muscle insulin sensitivity in recovery from exercise.

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