



Rac1 and AMPK Account for the Majority of Muscle Glucose Uptake Stimulated by Ex Vivo Contraction but Not In Vivo Exercise

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Exercise bypasses insulin resistance to increase glucose uptake in skeletal muscle and therefore represents an important alternative to stimulate glucose uptake in insulin-resistant muscle. Both Rac1 and AMPK have been shown to partly regulate contraction-stimulated muscle glucose uptake, but whether those two signaling pathways jointly account for the entire signal to glucose transport is unknown. We therefore studied the ability of contraction and exercise to stimulate glucose transport in isolated muscles with AMPK loss of function combined with either pharmacological inhibition or genetic deletion of Rac1.

Muscle-specific knockout (mKO) of Rac1, a kinase-dead $\alpha 2$ AMPK ($\alpha 2$ KD), and double knockout (KO) of $\beta 1$ and $\beta 2$ AMPK subunits ($\beta 1\beta 2$ KO) each partially decreased contraction-stimulated glucose transport in mouse soleus and extensor digitorum longus (EDL) muscle. Interestingly, when pharmacological Rac1 inhibition was combined with either AMPK $\beta 1\beta 2$ KO or $\alpha 2$ KD, contraction-stimulated glucose transport was almost completely inhibited. Importantly, $\alpha 2$ KD+Rac1 mKO double-transgenic mice also displayed severely impaired contraction-stimulated glucose transport, whereas exercise-stimulated glucose uptake in vivo was only partially reduced by Rac1 mKO with no additive effect of $\alpha 2$ KD. It is concluded that Rac1 and AMPK together account for almost the entire ex vivo contraction response in muscle glucose transport, whereas only Rac1, but not $\alpha 2$ AMPK, regulates muscle glucose uptake during submaximal exercise in vivo.

Muscle contraction provides an important insulin-independent signal to increase glucose uptake in skeletal muscle to meet the energy demand during body movement. This increase in glucose uptake is mediated by the simultaneous stimulation of the following three key steps: glucose delivery, glucose transport across the muscle membrane, and intracellular flux through metabolic processes (1,2). The translocation of the highly conserved GLUT4 from an intracellular storage site to the plasma membrane is a central component of this contraction response (3,4). At present, no single pathway has been found to account for all contraction-stimulated glucose uptake into muscle. This is likely due to redundancy in the signaling pathways mediating glucose uptake as evolutionary pressures would have promoted the development of several pathways to ensure the maintenance of muscle energy supply during physical activity. This probably explains why no studies to date have been able to fully block the contraction-stimulated glucose uptake in muscle by genetically modifying the expression of one protein.

Like muscle contraction, passive muscle stretching (5,6) as well as AICAR-induced activation of AMPK (7) stimulate glucose uptake in skeletal muscle. Interestingly, stretching combined with AICAR stimulation is sufficient to mobilize the full glucose transport contraction response in incubated muscle (8). Although AMPK mediates AICAR-stimulated glucose uptake (8,9), a role for AMPK in exercise- and contraction-induced glucose transport regulation is controversial since some studies (8,10,11), but

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not all (9,12–14), report reduced glucose uptake in various AMPK-deficient models. This controversy might be due to varying degrees of compensation from other pathways in the genetic AMPK-deficient models or to different experimental protocols. In addition to a potential requirement for AMPK, recent studies (15,16) have shown that the Rho GTPase Rac1 is also necessary for normal regulation of contraction- and exercise-stimulated glucose uptake. Both AMPK and Rac1 are conserved from insects to mammals, and we hypothesize that evolutionary pressure has secured some redundancy of those two signaling pathways to preserve glucose uptake regulation. Indeed, AMPK and Rac1 are activated via different and independent mechanisms in muscle. AMPK is activated by increased metabolic stress that increases intracellular AMP (17). Conversely, activating AMP via stimulation with AICAR or 2,4-dinitrophenol does not activate Rac1 (15,16). Rather, Rac1 can be rapidly activated by stretching or mechanical stress during contraction (18), a stimulus that does not activate AMPK (5,6). Rac1 and AMPK thus rely on distinct signals for activation, both of which are present during muscle contraction. Another piece of evidence for distinct mechanisms by which Rac1 and AMPK mediate glucose uptake in muscle is the fact that stretch-stimulated glucose transport requires Rac1 (18) but not AMPK (6,8). In the current study, we investigated the relative necessity of Rac1 and AMPK for contraction-stimulated glucose transport. Because stretching combined with AICAR stimulation is sufficient to mobilize the full glucose transport contraction response (8), we hypothesized that Rac1 and AMPK jointly account for most, if not all, of the signaling to glucose transport during muscle contraction. To specifically study GLUT4-mediated glucose transport, we used the incubated muscle setup in which transport likely constitutes the rate-limiting step for glucose uptake into the myofibers (19–21). In addition, we investigated the relevance of those findings in the more physiological model of *in vivo* treadmill exercise.

RESEARCH DESIGN AND METHODS

Transgenic AMPK Mouse Models

Male and female C57BL/6 muscle-specific (MCK-Cre) AMPK $\beta 1\beta 2$ knockout (KO) mice (18–22 weeks of age) were used. Wild-type (WT) littermates (AMPK $\beta 1\beta 2$ flox/flox) were used as controls. The generation of the $\beta 1\beta 2$ KO mice (with double KO of the $\beta 1$ and $\beta 2$ AMPK subunits) was described previously (11). Female muscle-specific MCK-Cre dominant-negative kinase-dead $\alpha 2$ AMPK ($\alpha 2$ KD)-overexpressing mice (25–29 weeks of age) and WT controls were described previously (10).

AMPK $\alpha 2$ KD+Rac1 mKO Mice

Male and female inducible Rac1 muscle-specific KO (mKO) mice were generated as previously described (15). Male and female muscle-specific AMPK $\alpha 2$ KD+Rac1 mKO mice were obtained by crossing inducible Rac1 mKO mice homozygous for Rac1 flox and heterozygous for the Cre recombinase (under the control of doxycycline and the human skeletal

muscle actin promoter) with heterozygous AMPK $\alpha 2$ KD transgenic mice. Control WT mice, Rac1 mKO, AMPK $\alpha 2$ KD, and AMPK $\alpha 2$ KD+Rac1 mKO mice were all littermates. Control mice were carrying the Cre recombinase or the Rac1 flox on none, one, or both alleles. Rac1 mKO was induced at 10–14 weeks of age by adding doxycycline to the drinking water (1 g/L; Sigma-Aldrich) for 3 weeks followed by a washout period of 3 weeks. All mice, including controls, received doxycycline, apart from mice used for inhibitor studies.

All animals were maintained on a 12-h light/dark cycle and received standard rodent chow diet (catalog #1324; Altromin, Brogaarden, Denmark) and water *ad libitum*. All experiments were approved by the Danish Animal Experimental Inspectorate.

Electrically Induced Muscle Contraction

Soleus and extensor digitorum longus (EDL) muscles were dissected from fed anesthetized mice (6 mg pentobarbital sodium/100 g body weight) and suspended at resting tension (4–5 mN) in incubation chambers (Multi Myograph System; Danish Myo-Technology, Aarhus, Denmark) in Krebs-Ringer-Henseleit buffer supplemented with 0.1% BSA, 2 mmol/L pyruvate, and 8 mmol/L mannitol at 30°C. For inhibitor experiments, the muscles were preincubated for 50 min in Krebs-Ringer-Henseleit buffer with Rac1 Inhibitor II (15 μ mol/L; catalog #553502, Calbiochem) or a corresponding amount of DMSO as vehicle control. Contractions were induced by electrical stimulation every 15 s with 2 s trains of 0.2 ms pulses delivered at 100 Hz (~ 35 V) for 10 min.

2-Deoxyglucose Transport

2-Deoxyglucose (2DG) transport was measured for 10 min during electrical stimulation using [3 H]2DG (0.6 μ Ci/mL), 1 mmol/L cold 2DG, and 14 C mannitol (0.180 μ Ci/mL) radioactive tracers as described previously (18).

Acclimatization to Treadmills and Maximal Exercise Capacity Test

Mice were acclimatized to the treadmill 3×5 min at 10 m/min and 2×5 min at 16 m/min at a 0° incline during the week prior to the maximal running capacity test. The test was performed at a 10° incline beginning with a 5-min warmup at 10 m/min, after which the speed was increased by ~ 1.2 m/min every minute until exhaustion. Exhaustion was defined as the speed at which the mouse was unable to keep up with the treadmill. The test was performed blinded.

Tissue-Specific 2DG Uptake Measurements During Treadmill Running

The number of mice in each group (rest/exercise) were as follows: WT group, $n = 6/10$; AMPK $\alpha 2$ KD group, $n = 8/12$; Rac1 mKO group, $n = 3/5$; AMPK $\alpha 2$ KD+Rac1 mKO group, $n = 6/9$. Due to the larger variation in data from exercised mice, more mice were allocated to exercise than rest. Each mouse was exercised at a relative work load corresponding to $\sim 65\%$ of its maximal running speed

(~14 m/min for AMPK α 2KD and AMPK α 2KD+Rac1 mKO mice, ~19 m/min for WT and Rac1 mKO mice) for 20 min at a 10° incline. To determine 2DG uptake in muscle, [3 H]2DG (PerkinElmer) was injected intraperitoneally (as a bolus in saline, 10 μ L/g body weight) containing 0.1 mmol/L 2DG and 50 μ Ci/mL [3 H]2DG corresponding to ~12 μ Ci/mouse) into fed mice immediately before the onset of exercise. Control mice were placed on a still treadmill for the same amount of time. Before and immediately after 20 min of exercise or rest, blood samples were collected from the tail vein and analyzed for glucose concentration. After exercise or rest, plasma was collected for measurement of specific [3 H]2DG tracer activity. Mice were euthanized by cervical dislocation, and gastrocnemius muscles were excised and quickly frozen in liquid nitrogen and stored at -80°C until processing. Tissue-specific 2DG-6-phosphate accumulation was measured as described previously (22) by deproteinization, using 0.1 mol/L Ba(OH)₂ and 0.1 mol/L ZnSO₄. The total tissue [3 H]2DG tracer activity found in 2DG-6-phosphate was divided by the area under the curve of the specific activity at time points 0 and 20 min and was multiplied with the average blood glucose at time points 0 and 20 min. This was related to muscle weight and the time to obtain the tissue-specific 2DG uptake as micromoles per gram per hour.

Muscle Homogenization

Muscle tissue was pulverized in liquid nitrogen and was homogenized with stainless steel beads 2 $\times$ 30 s at 30 Hz using a TissueLyser II (Qiagen, Germantown, MD) as described previously (18). After rotation end over end for 30 min, lysate supernatants were collected by centrifugation (10,000g) for 20 min at 4°C.

Glycogen and Glucose-6-Phosphate

Glycogen content was determined as glycosyl units after acid hydrolysis of muscle as previously described (23). Glucose-6-phosphate (G6P) was measured as previously described (24).

Immunoblotting

Lysate protein concentrations were measured using the bicinchoninic acid method using BSA standards and bicinchoninic acid assay reagents (Pierce). Total protein and phosphorylation levels of relevant proteins were determined by standard immunoblotting techniques loading equal amounts of protein. The primary antibodies used were phosphorylated (p)-ACC^{Ser212} (catalog #11818), p-AMPK^{Thr172} (catalog #5256), AMPK α 2 (catalog #2757), β -actin (catalog #4970), p-TBC1D4^{Thr642} (catalog #8881), p-p38MAPK^{Thr180/182} (catalog #9211), and Hexokinase II (catalog #2867; Cell Signaling Technology); p-TBC1D1^{Ser231} (catalog #07-2268; Millipore); ACC2 (a gift from Grahame Hardie, University of Dundee, Dundee, U.K.); and Rac1 (catalog #ARC03; Cytoskeleton, Inc.). Polyvinylidene difluoride membranes (Immobilon Transfer Membrane; Millipore) were blocked in TBS-Tween 20 containing 2% skim milk or 5% BSA protein for 30 min at room temperature. Membranes were incubated with primary antibodies

overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature. Bands were visualized using the ChemiDoc MP Imaging System (Bio-Rad) and enhanced chemiluminescence (ECL⁺; Amersham Biosciences).

MRI Scan

The body composition of the mice was analyzed using magnetic resonance imaging (EchoMRI 4-in-1; EchoMRI, Houston, TX).

Glucose and AICAR Tolerance Tests

Mice were fasted for 6 h from 7:00 A.M. Glucose (2 g/kg body weight) or AICAR (250 mg/kg; Toronto Research Chemicals Inc., Toronto, Canada) was administered intraperitoneally; blood was collected from the tail vein at time points 0, 20, 40, 60, and 90 min (and 120 min for the glucose tolerance test [GTT]); and blood glucose concentration was determined using a glucometer.

Statistical Analyses

Results are shown as mean $\pm$ SEM. Statistical testing was performed using *t* tests, one-way ANOVA, or multiple two-way (repeated measurements for paired data) ANOVA, as appropriate. A Tukey post hoc test was performed when ANOVA revealed significant main effects or interactions. Statistical evaluation was performed using GraphPad Prism 6 or SigmaPlot 12. The significance level was set at *P* < 0.05.

RESULTS

AMPK α 2KD Combined With Rac1 Inhibition Blocks Contraction-Induced Glucose Transport in Soleus and Partly in EDL Muscle

To investigate the relative contribution of Rac1 and AMPK to contraction-induced glucose transport in fully differentiated skeletal muscle, we took advantage of a classic model in exercise physiology, the electrically induced muscle contraction of incubated muscles. In this system, glucose transport by GLUT4 is thought to be the limiting factor for glucose uptake (20,21,25). We first examined resting and contraction-stimulated glucose transport in oxidative soleus and glycolytic EDL muscles from WT and muscle-specific AMPK α 2KD mice treated with either vehicle or a Rac1 inhibitor. Force production in soleus muscle was unaffected by AMPK α 2KD or Rac1 inhibition, although it was modestly impaired after 2.5 min in EDL muscles in all intervention groups compared with WT (Fig. 1A and B). We observed no difference in muscle mass between genotypes (data not shown). In soleus muscle, Rac1 inhibition reduced contraction-stimulated glucose transport by ~20%, whereas combined AMPK α 2KD+Rac1 inhibition blocked the contraction-stimulated increase in glucose transport (Fig. 1C). In EDL muscle, AMPK α 2KD and Rac1 inhibition alone each reduced contraction-stimulated glucose transport by ~30–40%, with no additive effect of combining AMPK α 2KD with Rac1 inhibition (Fig. 1D). As expected, phosphorylation of ACC^{Ser212}, the downstream target of AMPK, was reduced by 70% (soleus) and 50% (EDL) by AMPK α 2KD inhibition but

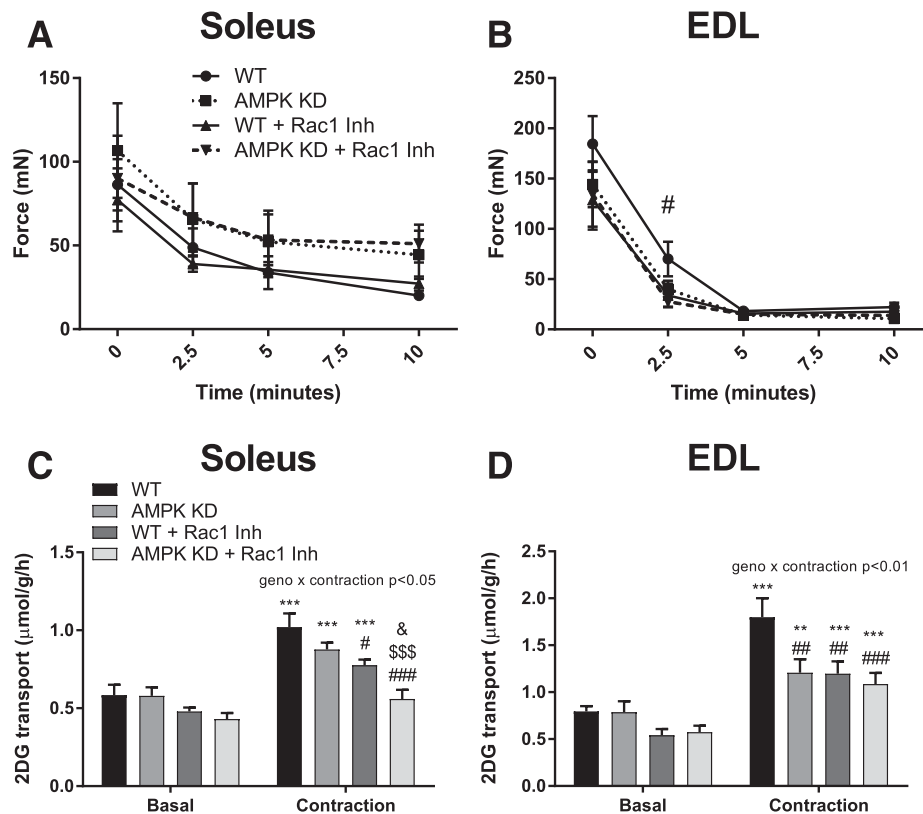


Figure 1—Force development recordings during 10-min contraction in soleus (A) and EDL (B) muscles from $\alpha 2$ KD and WT littermate mice $\pm$ 15 μ mol/L Rac1 Inhibitor II (50 min). $n = 9$. Bar graphs showing contraction-stimulated 2DG transport/signaling are indicated as follows: ** $P < 0.01$ and *** $P < 0.001$. Significantly different from WT is indicated as follows: # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$. Significantly different from AMPK $\alpha 2$ KD is indicated as follows: \$\$\$ $P < 0.001$. Significantly different from WT+Rac1 Inhibitor II is indicated by & $P < 0.05$. Values are mean $\pm$ SEM. geno, genotype; Inh, inhibition.

was unaffected by Rac1 inhibition (Fig. 2A and D, representative blots). TBC1D4 and TBC1D1 are phosphorylated by muscle contraction and exercise, with some phosphorylation sites likely downstream of AMPK, and have been proposed to be involved in contraction-stimulated glucose transport in muscle (26–29). We found that p-TBC1D4^{Thr642} increased with contraction in EDL muscle, but not soleus muscle, and equally in all groups (Fig. 2B and D). We also analyzed the phosphorylation status of p38 mitogen-activated protein kinase (MAPK), which is activated by stretch and muscle contraction and has also been implicated in glucose transport induced by those stimuli (6,30). Electrically induced contraction increased p-p38MAPK^{Thr180/182} in both muscles apart from soleus muscles overexpressing AMPK $\alpha 2$ KD (Fig. 2C and D). Our findings suggest that Rac1 and AMPK each contribute to contraction-stimulated glucose transport and that this occurs via parallel and additive pathways in soleus muscle. Furthermore, contraction-stimulated p38 MAPK phosphorylation seems to be AMPK-dependent in soleus muscle.

Rac1 Inhibition in AMPK $\beta 1\beta 2$ KO Mice Blocks Contraction-Stimulated Glucose Transport

We sought to further validate our findings in another model of genetic ablation of AMPK signaling: the muscle-

specific AMPK $\beta 1\beta 2$ KO mouse. This model displays partially reduced exercise- and contraction-stimulated glucose uptake in skeletal muscle (11). In agreement, AMPK $\beta 1\beta 2$ KO reduced contraction-stimulated glucose transport in soleus muscles by 20% with a similar trend ($P < 0.1$) in EDL muscle (Fig. 3A). When Rac1 inhibition was combined with AMPK $\beta 1\beta 2$ KO, contraction-stimulated glucose transport in both soleus and EDL muscles was further reduced (Fig. 3A), suggesting again that the signaling pathways defined by Rac1 and AMPK are distinct and can account for the major part of the contraction-induced glucose transport response ex vivo. AMPK $\beta 1\beta 2$ KO combined with Rac1 inhibition did not affect force development (Fig. 3B). As expected, AMPK $\beta 1\beta 2$ KO reduced contraction-stimulated AMPK^{Thr172} and ACC^{Ser212} phosphorylation (Fig. 3C–E). As in the AMPK $\alpha 2$ KD mice (Fig. 2), contraction increased p-p38MAPK^{Thr180/Tyr182} levels, but this response was almost abolished in soleus muscle lacking AMPK $\beta 1\beta 2$ (Fig. 3C and F). TBC1D4^{Thr642} phosphorylation increased 10–20% with muscle contraction in both muscles (main effect) and was unaffected by AMPK $\beta 1\beta 2$ KO or Rac1 inhibition (Fig. 3C and G). Collectively, these data support a model in which AMPK and Rac1 pathways independently and additively regulate contraction-induced muscle glucose transport ex vivo.

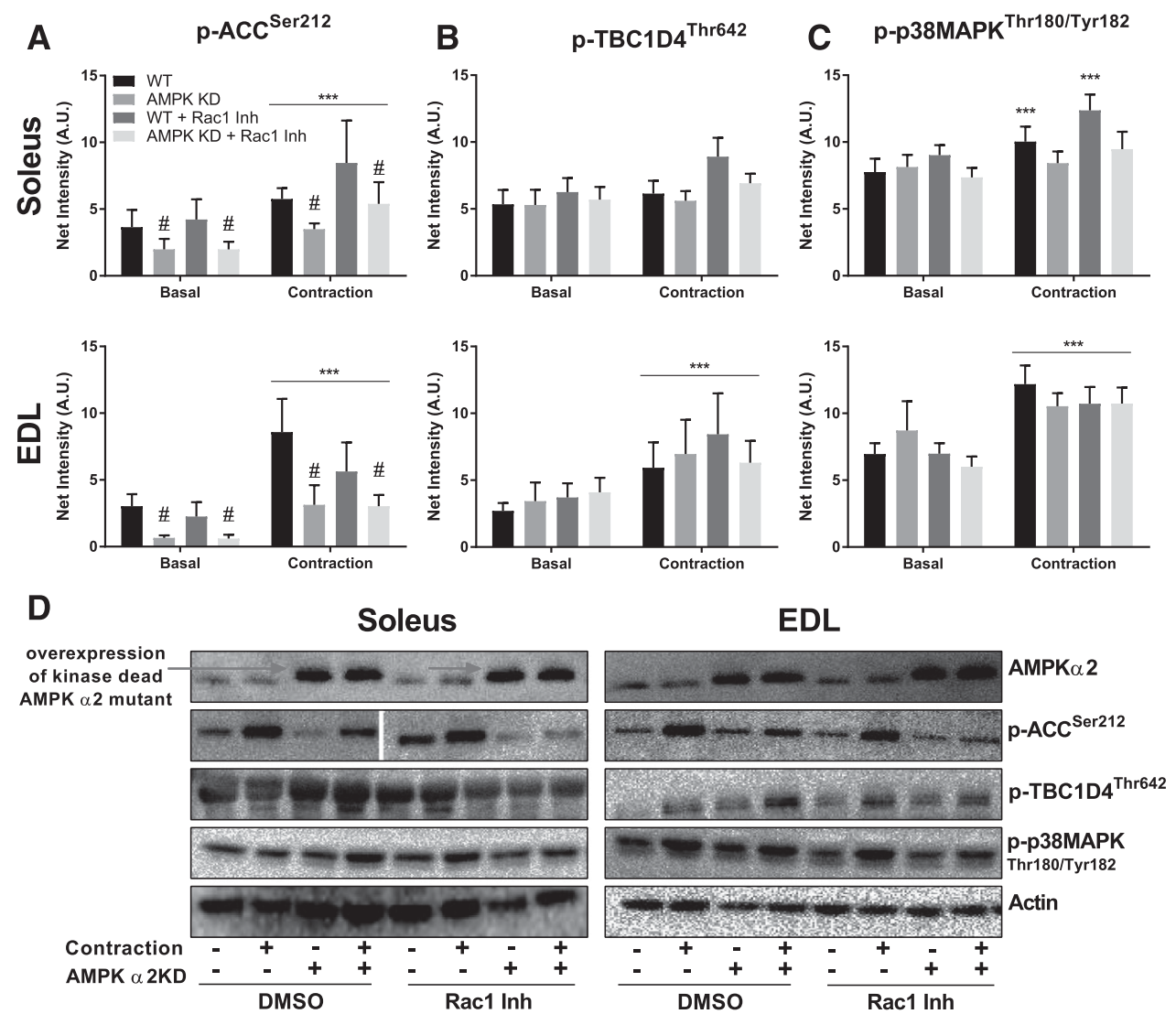


Figure 2—Bar graphs showing quantifications of p-ACC^{Ser212} (A), p-TBC1D4^{Thr642} (B), and p-p38MAPK^{Thr180/Tyr182} (C). *n* = 9. D: Representative blots of basal and contraction-stimulated p-ACC^{Ser212}, p-TBC1D4^{Thr642}, and p-p38MAPK^{Thr180/Tyr182} and protein expression of AMPK α 2 and β -actin (control). A significant difference between basal and contraction-stimulated signaling is indicated as follows: ****P* < 0.001. Significantly different from WT indicated as follows: #*P* < 0.05. Main effects are underlined. Values are mean $\pm$ SEM. A.U., arbitrary units; Inh, inhibition.

AMPK α 2KD+Rac1 mKO Mice Display Almost Abolished Contraction-Stimulated Glucose Transport

To examine to what extent the combined signals from AMPK and Rac1 are required, we crossed Rac1 mKO mice with AMPK α 2KD mice to obtain double-genetic loss-of-function mice. These mice display similar body weight (Fig. 4A), fat mass (Fig. 4B), and lean body mass (Fig. 4C) compared with WT and single Rac1 mKO or α 2KD mice at 23 weeks of age. Contraction-stimulated 2DG transport in soleus and EDL muscles was $\sim$ 20–30% reduced in AMPK α 2KD mice compared with control littermates (Fig. 4D and E). In soleus muscle, dual α 2KD+Rac1 mKO completely prevented an increase in glucose transport during contraction. In EDL muscle, combined α 2KD+Rac1 mKO displayed an additive effect on glucose transport. Despite this, glucose

transport still increased by $\sim$ 50% in response to muscle contraction compared with a 150% increase in control EDL muscles (Fig. 4E). The weights of the soleus and EDL muscles were similar between genotypes (Fig. 4F). However, force production in soleus at time points 5 and 10 min was reduced by combined α 2KD+Rac1 mKO (Fig. 4G). In EDL muscle, initial force production and force production at time point 2.5 min were reduced in both AMPK α 2KD and combined α 2KD+Rac1 mKO (Fig. 4G). We observed on average threefold overexpression of the α 2KD AMPK transgene (Fig. 4H), which severely impairs AMPK activity (10,12). Rac1 protein was reduced by 80% in soleus muscle and 90% in EDL muscle of Rac1 mKO mice. We also analyzed p-TBC1D1^{Ser231}, an AMPK substrate mainly expressed

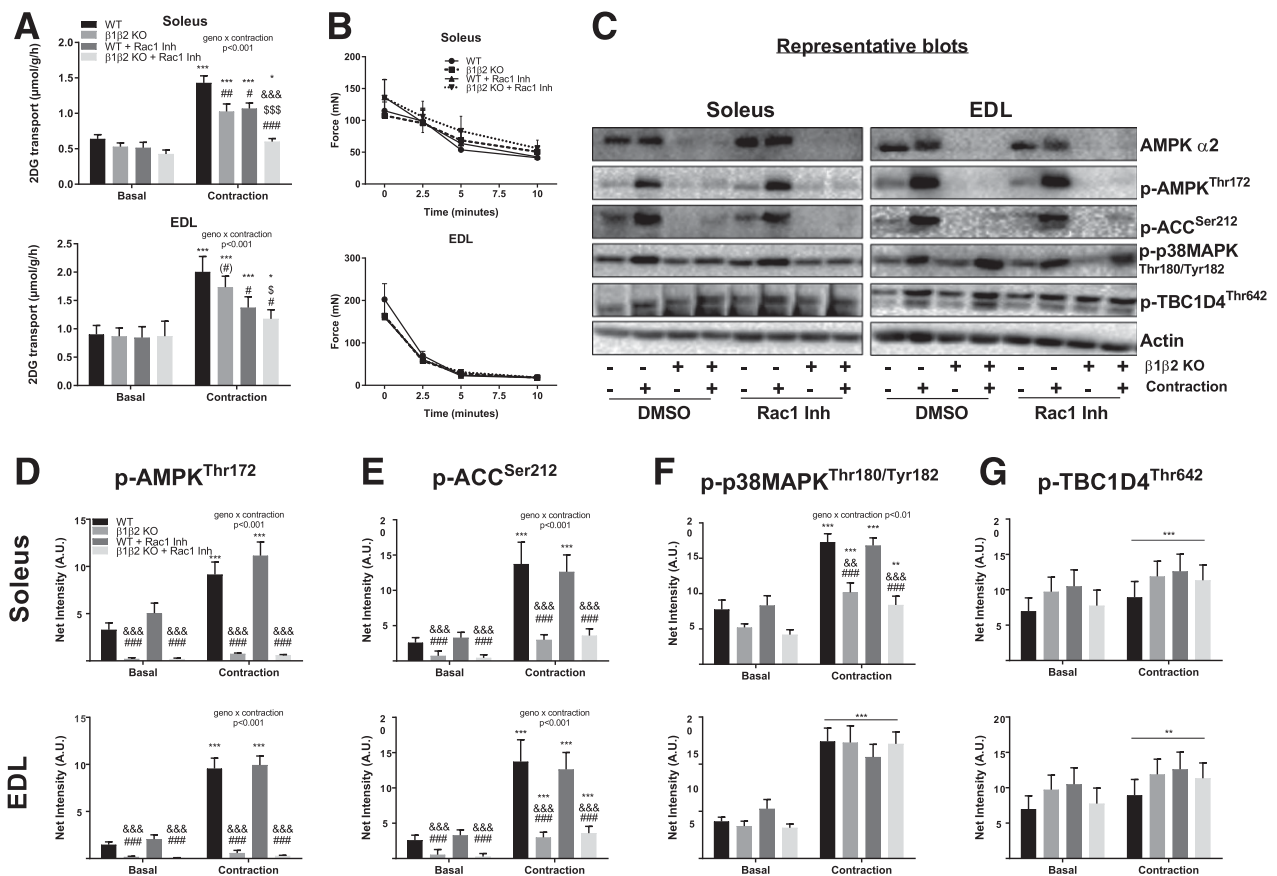


Figure 3—A: Bar graphs showing basal and contraction-stimulated 2DG transport in soleus and EDL muscles from muscle-specific AMPK β1β2 KO and WT littermate mice ± 15 μmol/L Rac1 Inhibitor II (50 min) ($n = 11-16$). **B:** Force development recordings during 10-min contraction. **C:** Representative blots of basal and contraction-stimulated p-AMPK^{Thr172}, p-ACC^{Ser212}, p-p38MAPK^{Thr180/Tyr182}, p-TBC1D4^{Thr642}, AMPK α2, Rac1, and β-actin (control) in skeletal muscle of AMPK β1β2 KO and WT mice ± 15 μmol/L Rac1 Inhibitor II. Bar graphs showing quantifications of p-AMPK^{Thr172} (**D**), p-ACC^{Ser212} (**E**), p-p38MAPK^{Thr180/Tyr182} (**F**), and p-TBC1D4^{Thr642} (**G**) ($n = 11-16$). Significant differences between basal and contraction-stimulated 2DG transport/signaling are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significantly different from WT is indicated as follows: (#) $P < 0.1$, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$. Significantly different from AMPK β1β2 KO is indicated as follows: \$ $P < 0.05$ and \$\$\$ $P < 0.001$. Significantly different from WT+Rac1 Inhibitor II is indicated as follows: && $P < 0.01$, &&& $P < 0.001$. Main effects are underlined. Values are mean ± SEM. A.U., arbitrary units; geno, genotype; Inh, inhibition.

in glycolytic muscle fibers and implicated in the regulation of exercise-stimulated glucose uptake in white, but not red, muscle types (31). P-TBC1D1^{Ser231} was significantly reduced in both the basal and contraction-stimulated state by AMPK α2KD (Fig. 4I). We observed similar results for p-ACC^{Ser212} (Fig. 4J). Rac1 mKO did not affect contraction-stimulated p-TBC1D1^{Ser231} or p-ACC^{Ser212} (Fig. 4H–J). Taken together, these findings suggest that Rac1 and AMPK can account for most of the signaling to glucose transport during ex vivo muscle contraction via independent signaling pathways.

Exercise-Stimulated Glucose Uptake Is Reduced by Rac1 mKO, With No Additive Effect of α2KD

In agreement with previous reports (12), the maximal running capacity was reduced by ~20% in AMPK α2KD mice (Fig. 5A) and was unaffected by genetic deletion of Rac1. To investigate the effect of blocking Rac1 and AMPK in vivo, we analyzed glucose uptake during submaximal exercise in gastrocnemius muscle from α2KD+Rac1 mKO

mice. As we have recently observed (16), Rac1 mKO reduced exercise-induced glucose uptake by ~40% (Fig. 5B). Glucose uptake was similarly reduced by combined α2KD+Rac1 mKO, with no additive effect upon inhibiting both pathways. The expression of α2KD AMPK isoform was unaffected by Rac1 depletion as shown in Figs. 4H and 5C. Likewise, Rac1 expression was unaffected by AMPK α2KD, and Rac1 protein was reduced by 90% in Rac1 mKO muscle (Fig. 5C and D). Important to the interpretation of our findings, GLUT4 protein content was normal in all groups (Fig. 5E). Phosphorylation of ACC^{Ser212} was reduced by AMPK α2KD but was normal in the muscles of Rac1 mKO mice (Fig. 5F).

Muscle glycogen was reduced after exercise in all groups, but the levels were generally lower in the AMPK α2KD and α2KD+Rac1 mKO mice (Fig. 5G) and so was G6P after exercise (Fig. 5H). Blood glucose was ~2 and ~3 mmol/L, respectively, higher at the end of exercise in AMPK α2KD and α2KD+Rac1 mKO mice (Fig. 5I). Because of the higher

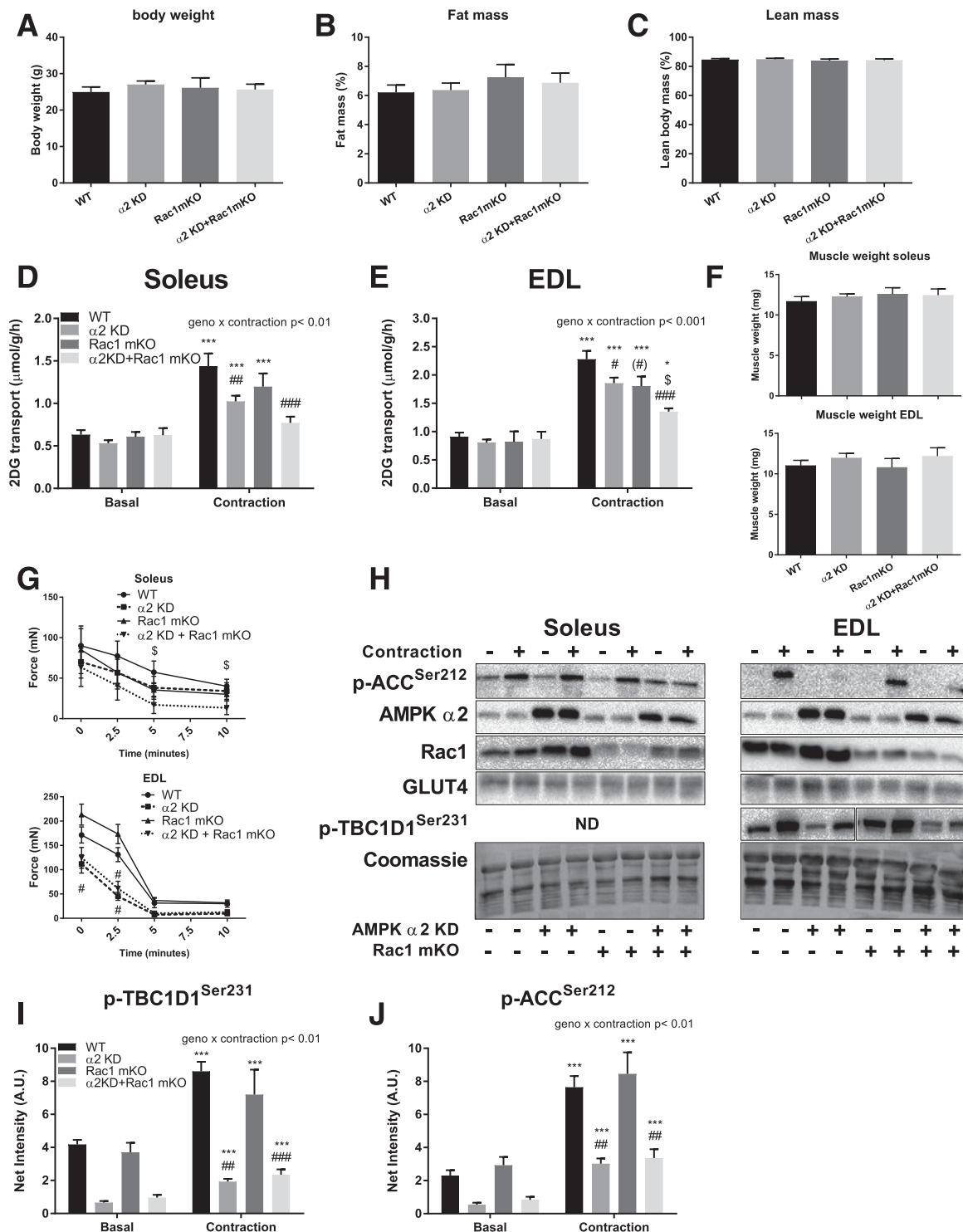


Figure 4—Body weight ($n = 5$ – 18) (A), percentage of fat mass ($n = 5$ – 18) (B), and percentage lean body mass ($n = 5$ – 18) (C) in WT, $\alpha 2$ KD, and inducible Rac1 mKO, or $\alpha 2$ KD+Rac1 mKO mice. Electrically induced 2DG transport in isolated incubated soleus (D) and EDL (E) muscles. F: Muscle weight of soleus and EDL muscle (based on the average of two same muscles from each mouse). G: Force development recordings during contraction in soleus and EDL muscle. H: Representative blots of p-ACC^{Ser212}, $\alpha 2$ AMPK, Rac1, and GLUT4 in soleus and EDL muscles and TBC1D1^{Ser231} only in EDL muscle. I: p-TBC1D1^{Ser231} in EDL muscle. J: p-ACC^{Ser212} in EDL muscle. Number of mice in each group are as follows: WT group, $n = 14$; $\alpha 2$ KD group, $n = 15$; Rac1 mKO group, $n = 6$; $\alpha 2$ KD+Rac1 mKO group, $n = 8$. Significant differences between basal and contraction-stimulated 2DG transport are indicated as follows: * $P < 0.05$ and *** $P < 0.001$. Significantly different from WT is indicated as follows: (#) $P < 0.1$, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$. Significantly different from $\alpha 2$ KD is indicated as follows: \$ $P < 0.05$. Values are mean $\pm$ SEM. A.U., arbitrary units; geno, genotype; ND, not determined.

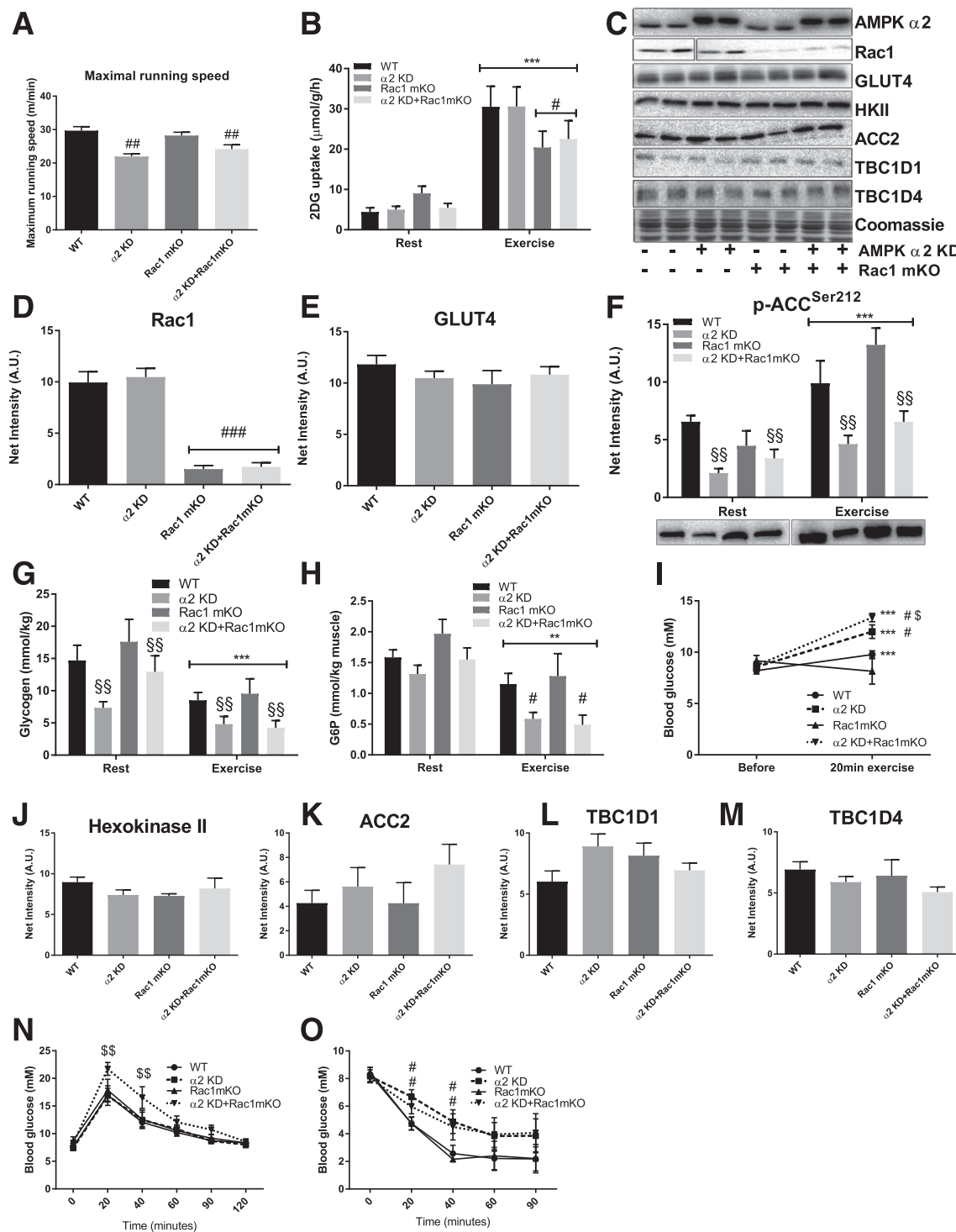


Figure 5—A: Maximal running speed of WT, α2KD, inducible Rac1 mKO, or α2KD+Rac1 mKO mice. **B:** Resting and exercise-stimulated glucose uptake (65% maximal running speed) in gastrocnemius muscle. The number of mice in each group (rest/exercise) were as follows: WT group, $n = 6/10$; AMPK α2KD group, $n = 8/12$; Rac1 mKO group, $n = 3/5$; AMPK α2KD+Rac1 mKO group, $n = 6/9$. **C:** Representative blots showing α2 AMPK, Rac1, GLUT4, HKII, ACC2, TBC1D1, and TBC1D4 expression and Coomassie staining as a control in gastrocnemius muscle. Bar graphs showing quantifications of Rac1 (**D**), GLUT4 (**E**), and p-ACC^{Ser212} (**F**). Muscle glycogen (**G**) and muscle G6P (**H**) concentrations in gastrocnemius muscle in the rested state or after exercise. **I:** Blood glucose level before and after exercise. HKII (**J**), ACC2 (**K**), TBC1D1 (**L**), and TBC1D4 (**M**) gastrocnemius muscle protein expression. Blood glucose during a GTT (**N**) and an AICAR tolerance test (**O**). Number of determinations in each group were as follows: WT group, $n = 14$; α2KD group, $n = 15$; Rac1 mKO group, $n = 6$; α2KD+Rac1 mKO group, $n = 8$. Significant differences between resting and exercise-stimulated values are indicated as follows: $**P < 0.01$ and $***P < 0.001$. Significantly different from WT is indicated as follows: $\#P < 0.05$, $##P < 0.01$, $###P < 0.001$. Significantly different from WT is indicated as follows: $\$P < 0.05$, $\$\$P < 0.01$. The main effect of α2KD is indicated as follows: $\$\$P < 0.01$. Values are mean $\pm$ SEM. A.U., arbitrary units.

plasma glucose concentration in these mice, a slightly greater driving force for glucose uptake is present in the $\alpha 2$ KD and $\alpha 2$ KD+Rac1 mKO mice compared with WT and Rac1 mKO mice. None of the groups displayed altered muscle HKII expression (Fig. 5C and J) or protein expression of ACC2, TBC1D1, and TBC1D4 (Fig. 5C and K–M).

Finally, we further characterized whole-body glucose metabolism in these mice using glucose and AICAR tolerance tests. Both AMPK $\alpha 2$ KD and Rac1 mKO mice displayed normal glucose tolerance in response to an intraperitoneal glucose challenge (Fig. 5N). Interestingly, combined $\alpha 2$ KD+Rac1 mKO resulted in significant glucose intolerance evidenced by 30% higher blood glucose levels 20 and 40 min into the test (Fig. 5N). The *in vivo* effect of AICAR administration has not previously been analyzed in Rac1 mKO, and it was unknown whether $\alpha 2$ KD+Rac1 mKO displayed additive effects. As observed previously (9), AICAR sensitivity was reduced by $\sim 40\%$ in AMPK $\alpha 2$ KD. We observed no additive effect of combined $\alpha 2$ KD+Rac1 mKO, and Rac1 mKO alone did not affect AICAR sensitivity (Fig. 5O). These data suggest that Rac1 is not involved in AICAR-stimulated glucose uptake *in vivo*, which is in agreement with our previous findings *ex vivo* (15).

DISCUSSION

In the current study, we show that when Rac1 and AMPK are simultaneously blocked either by pharmacological or genetic means, glucose transport in response to contraction in incubated muscle is severely impaired. This is the first study to explain the molecular mechanisms responsible for a large majority of contraction-stimulated glucose transport. Such findings are in consonance with previous work where activation of metabolic stress signaling, requiring AMPK, together with stretch stimulation, requiring Rac1 (18), accounted for the entire glucose transport response (8). Furthermore, we find that in contrast to Rac1, $\alpha 2$ AMPK does not seem to regulate glucose transport during treadmill exercise *in vivo*, at least at the submaximal exercise intensity studied here.

Our findings are of physiological and pathophysiological importance since exercise bypasses insulin resistance to increase glucose uptake in skeletal muscle. On the basis of our study, activation of $\alpha 2$ AMPK as well as Rac1 thus represents important alternatives to stimulate glucose uptake in insulin-resistant muscle. Indeed, the activation of both AMPK (27,32) and Rac1 (33,34) each suffices to increase GLUT4 translocation in muscle.

To date, no single signaling protein has been able to fully account for contraction-mediated glucose transport. This is likely due to the redundancy and complexity of an evolutionarily well-conserved system to ensure sufficient glucose delivery to the working muscle. Evidence for the massive complexity of the exercise signaling network came from a recent study (35) where $\sim 1,000$ phosphorylation sites were found to be regulated by exercise in human muscle. Our findings that dual inhibition of AMPK and Rac1 has an

additive effect on contraction-stimulated glucose transport support the argument that several partly redundant pathways regulate glucose transport in muscle. It is also in agreement with our previous work suggesting that Rac1 and AMPK each regulate glucose uptake via independent mechanisms (15,16,18). Certainly, stretch-stimulated glucose transport partially relies on Rac1 but not AMPK, whereas AICAR-induced glucose transport requires AMPK but not Rac1 (15). Furthermore, Rac1 activation is normal in muscles from AMPK $\alpha 2$ KD mice after exercise (15) and vice versa (15,16), confirming that these signaling pathways are independent. While AMPK is activated by metabolic cues, such as increased AMP/ATP ratio, Rac1 can be activated by applying mechanical stress (stretching) (15,18,36). Accordingly, if mechanical stress on the muscles is prevented, the increment in glucose transport induced by electrically induced contraction is significantly reduced (8,18,37). This suggests that mechanical stress is an important and perhaps previously underappreciated signal to glucose transport during contraction. Rac1 likely mediates a major part of the stretch-sensitive component of contraction-stimulated glucose uptake since Rac1 inhibition did not affect glucose transport during muscle contraction when force development was prevented (18). In contrast, AMPK ablation completely abolished contraction-induced glucose transport when force development was prevented (8), suggesting a role for AMPK, but not Rac1, in regulating glucose transport during metabolic but not mechanical stress.

In some experiments, we observed reduced force generation in transgenic groups. This could confound our findings since reduced force production has been associated with decreased glucose transport, as discussed above. However, our use of several different approaches shows that although force development is reduced in the double-transgenic model presented in Fig. 4, force development was not affected in several of the other experimental models (Figs. 1 and 3), showing that the effect of blocking both Rac1 and AMPK on glucose transport is unlikely to be caused by reduced force development.

We note that contraction-stimulated glucose transport was not completely blocked in all experiments by the dual inhibition of Rac1 and AMPK signaling. Similarly, Rac1 is only partially regulating stretch-stimulated glucose transport (18). This suggests that a third or more of systems independent from AMPK and Rac1 are also involved in the regulation of glucose transport in response to muscle contraction. One potential candidate for this could be p38 MAPK. p38 MAPK has been found to regulate stretch-stimulated glucose transport in muscle by one study (6) but not another (8), and p38 MAPK has been linked to contraction-stimulated glucose transport (30). Furthermore, we show in the current study that Rac1 does not affect contraction-stimulated activation of p38 MAPK, leaving this signaling pathway to stretch-stimulated glucose uptake intact during muscle contraction. It is thus possible that the residual glucose uptake seen during some conditions in the current study, despite simultaneous blockade of

Rac1 and AMPK, is due to signaling via p38 MAPK. Future studies should investigate this and other potential alternative pathways further.

In vivo, AMPK α 2KD+Rac1 mKO mice did not display additive effects on glucose uptake in response to treadmill running. These findings indicate that the rate-limiting steps for glucose uptake and/or their regulation differ between the ex vivo and in vivo setting. In particular, studies have suggested that the rate-limiting step for glucose uptake in vivo is not solely determined by the ability of muscle to transport glucose across the muscle membrane. Rather, the control of glucose uptake in vivo is distributed among the ability to supply glucose to muscle, the membrane glucose transport capacity, and the ability of the muscle to metabolize glucose (1,2,38). In both AMPK α 2KD and α 2KD+Rac1 mKO mice, higher blood glucose concentrations during exercise compared with WT and Rac1 mKO mice suggests there is a higher supply of glucose to the exercising muscles, potentially increasing muscle glucose uptake by creating a higher glucose gradient from blood to muscle. In addition, muscle G6P was lower during exercise in both AMPK α 2KD and α 2KD+Rac1 mKO than in WT and Rac1 mKO mice (Fig. 5H), suggesting less inhibition of HKII and therefore a greater ability to dispose of the glucose taken up by the blood. The lower muscle G6P concentration in AMPK α 2KD and α 2KD+Rac1 mKO mice compared with WT and Rac1 mKO mice may be due to the generally lower muscle glycogen concentration that is a common finding in AMPK-deficient muscles (39–41). The higher glucose gradient and lower G6P may compensate for possible lower glucose transport capacity in AMPK α 2KD and α 2KD+Rac1 mKO mice. Our data showing no role of AMPK during submaximal running in mice are in agreement with previous data in running AMPK α 2KD mice (12) and in α 1 α 2 double mKO mice (41) but in contrast to findings in the β 1 β 2 double mKO mice (11) and another study in α 2KD mice (42). These differences may be due to different exercise protocols and/or the extent of compensation for the loss of AMPK activity. Taken together, these divergent results do not allow firm conclusions about whether AMPK controls muscle glucose uptake during exercise in vivo.

Exercise improves insulin sensitivity in the hours after exercise (43,44). Intriguingly, we recently showed that short-term exercise could completely restore insulin sensitivity in insulin-resistant Rac1-deficient muscle (45). Thus, although Rac1 is necessary for the normal regulation of contraction-stimulated glucose transport, exercise does not require Rac1 to increase insulin sensitivity in muscle. This is important because insulin-resistant muscle displays defective Rac1 signaling (46). With regard to AMPK, AICAR-induced AMPK activation improves insulin sensitivity (32). Furthermore, exercise does not improve insulin sensitivity in muscles of AMPK α 1 α 2 muscle-specific double KO mice (47). These findings implicate AMPK but not Rac1 in the insulin-sensitizing effect of exercise.

Like the upstream mechanisms activating Rac1 and AMPK, the downstream mechanisms by which they regulate glucose transport also seem to be distinct. In various cell types, Rac1 regulates the cortical actin cytoskeleton (48,49). In L6 muscle cells, Rac1-dependent actin remodeling was necessary for GLUT4 vesicles to fuse with the plasma membrane and to allow glucose uptake in response to insulin (50). Indeed, the actin cytoskeleton seems to participate in contraction-stimulated glucose uptake downstream of Rac1, since the actin cytoskeleton depolymerizing agent Latrunculin B reduced contraction-stimulated glucose transport in soleus and EDL muscle in a manner that was not additive with pharmacological Rac1 inhibition (15). On the other hand, AMPK in part may regulate GLUT4 translocation via the Rab GTPase-activating proteins TBC1D4/TBCD1-mediated release of GLUT4 vesicles from intracellular compartments (27,51). Another possibility is that AMPK might decrease the rate of internalization of GLUT4 transporters in muscle during exercise since AMPK has been shown to function as a brake on endocytosis of GLUT4 in the heart (52). AMPK may thus participate mainly in controlling the rate of internalization of GLUT4 vesicles from the surface membrane, whereas Rac1 mediates the cytoskeletal rearrangement necessary for GLUT4 to be incorporated into the surface membrane.

In summary, we found that in incubated mouse skeletal muscle, Rac1 and AMPK both regulate glucose transport in parallel and additively. The function of these two pathways is necessary for a major part of glucose transport during ex vivo muscle contraction. In contrast, the present investigation indicates that muscle glucose uptake during submaximal treadmill-running exercise depends on Rac1 and not α 2 AMPK in mice.

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and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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