Skeletal muscle glucose uptake during contraction is regulated by nitric oxide and ROS independently of AMPK

Troy L. Merry,1 Gregory R. Steinberg,3 Gordon S. Lynch,2 and Glenn K. McConell1

1Exercise Physiology and Metabolism Laboratory, 2Basic and Clinical Myology Laboratory, Department of Physiology, University of Melbourne, Victoria, Australia; and 3Department of Medicine, McMaster University, Hamilton, Ontario, Canada

Submitted 9 April 2009; accepted in final form 13 December 2009

Merry TL, Steinberg GR, Lynch GS, McConell GK. Skeletal muscle glucose uptake during contraction is regulated by nitric oxide and ROS independently of AMPK. Am J Physiol Endocrinol Metab 298: E577–E585, 2010. First published December 15, 2009; doi:10.1152/ajpendo.00239.2009.—Reactive oxygen species (ROS) and nitric oxide (NO) have been implicated in the regulation of skeletal muscle glucose uptake during contraction, and there is evidence that they do so via interaction with AMP-activated protein kinase (AMPK). In this study, we tested the hypothesis that ROS and NO regulate skeletal muscle glucose uptake during contraction via an AMPK-independent mechanism. Isolated extensor digitorum longus (EDL) and soleus muscles from mice that expressed a muscle-specific kinase dead (AMPK-KD) and wild-type littermates (WT) were stimulated to contract, and glucose uptake was measured in the presence or absence of the antioxidant N-acetyl-l-cysteine (NAC) or the nitric oxide synthase (NOS) inhibitor L-G-monomethyl-l-arginine (L-NMMA). Contracted AMPK-KD activity in WT but not AMPK-KD EDL muscles. However, contraction increased glucose uptake in the EDL and soleus muscles of AMPK-KD and WT mice to a similar extent. In EDL muscles, NAC and L-NMMA prevented contraction-stimulated increases in oxidant levels (dichlorofluorescein fluorescence) and NOS activity, respectively, and attenuated contraction-stimulated glucose uptake in both genotypes to a similar extent. In soleus muscles of AMPK-KD and WT mice, NAC prevented contraction-stimulated glucose uptake and L-NMMA had no effect. This is likely attributed to the relative lack of neuronal NOS in the soleus muscles compared with EDL muscles. Contraction increased AMPKα Thr172 phosphorylation in EDL and soleus muscles of WT but not AMPK-KD mice, and this was not affected by NAC or L-NMMA treatment. In conclusion, ROS and NO are involved in regulating skeletal muscle glucose uptake during contraction via an AMPK-independent mechanism.

nitric oxide; reactive oxygen species; exercise; metabolism; contraction; glucose uptake

EXERCISE AND CONTRACTION INCREASE translocation of GLUT4 to the cell surface, facilitating glucose transport into skeletal muscle (55). Although the mechanism(s) by which contraction increases GLUT4 translocation and glucose uptake into skeletal muscle are not yet fully defined, there is consensus that the signals regulating this pathway are independent of the insulin-signaling pathway (31, 39). Numerous mechanisms have been implicated in regulating contraction-stimulated glucose uptake (43), and one of the most attractive and well-studied candidates is AMP-activated protein kinase (AMPK). While initial studies demonstrated that the AMPK activator 5-aminomimidazole 4-carboxamide ribonucleoside (AICAR) increased glucose uptake (17) and that increased AMPK activity during contraction correlates with glucose uptake (20, 38), disassociations between glucose uptake and contraction have also been reported (9, 34). Importantly, mice that overexpress a kinase dead (KD) AMPKα2 isoform, the major isoform activated with contraction (13, 52), or have a genetic deletion of AMPKα1 or α2 isoforms, have normal increases in glucose uptake during contraction (15, 25). However, this is not a universal finding since attenuation of glucose uptake during contraction in mice with deficient AMPK signaling has also been reported (14, 23, 29, 35), and these discrepancies have been attributed to differences in the contraction protocols employed (29).

Recent studies have focused on free radical signaling of glucose uptake in skeletal muscle, with donors of nitric oxide (NO) and reactive oxygen species (ROS) shown to increase glucose uptake in resting skeletal muscle (18, 19, 24). Indeed, acute physiological increases in ROS, nitric oxide synthase (NOS) activity, and NO production occur during contraction (22, 40, 44, 46) and antioxidant treatment (46) and NOS inhibition, in some (1, 2, 26, 42, 44) but not all studies (11, 18, 45), attenuates skeletal muscle glucose uptake during contraction. Because ROS increase AMPK activity (19, 24) and antioxidant treatment during contraction attenuates increases in AMPK activity (46), it has been proposed that ROS, particularly hydrogen peroxide (H2O2), may regulate skeletal muscle glucose uptake via an AMPK-dependent mechanism (46). However, more recent studies (19) have shown that at low concentrations (600 μM) H2O2 treatment of rat EDL muscles can increase glucose uptake without affecting AMPK activity. Thus it appears that although ROS can increase AMPK activity, AMPK activation is not required for ROS-stimulated glucose uptake in noncontracted muscles. Similarly, NO has been proposed to act both upstream and downstream of AMPK (30) in regulating contraction and NO-mediated glucose uptake (12, 18, 50). However, it has been shown that NOS inhibition during contraction attenuates glucose uptake without affecting AMPK signaling (44).

Based on these equivocal findings, the aim of the present study was to determine whether NO and ROS regulate glucose uptake during contraction via an AMPK-dependent pathway by contracting isolated skeletal muscle from AMPK-KD and wild-type mice in the presence of a NOS inhibitor [L-G-monomethyl-l-arginine (L-NMMA)] or an antioxidant [N-acetyl-l-cysteine (NAC)]. We tested the hypothesis that ROS and NO regulate glucose uptake during contraction via an AMPK-independent mechanism.

Address for reprint requests and other correspondence: T. Merry, Dept. of Physiology, Univ. of Melbourne, Victoria 3010, Australia (e-mail: tmerry@pgrad.unimelb.edu.au).
RESEARCH DESIGN AND METHODS

Animals. This study used male C57Bl/6 mice aged 12–14 wk, and male and female mice with muscle-specific overexpression of a kinase dead form of the AMPKα2 isofrom (AMPK-KD), which have been described in detail previously (10, 35). AMPK-KD and littermate controls were used for experiments at 22–24 wk of age (Table 1), and since no differences were found between male and female mice for muscle glucose uptake during contraction, the results were pooled. Mice were maintained in an environmentally controlled room at 21°C with a 12-h light-dark cycle (light 0630–1830). Mice were given ad libitum access to standard rodent chow and water. The experiments were approved by the Animal Experimentation Ethics Committee of The University of Melbourne and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, as described by the National Health and Medical Research Council (Australia).

Materials and antibodies. NAC and l-NMMA were purchased from Sigma-Aldrich Chemicals (St. Louis, MO), 2-Deoxy-d-[1,2-3H]glucose and d-[1-14C]mannitol were purchased from Amersham Biosciences (Piscataway, NJ), and 5-aminomimidazole 4-carboxamide ribonucleoside (AICAR) was purchased from Toronto Chemicals (Toronto, Canada). Primary antibodies for AMPKα and α-tubulin were purchased from Cell Signaling Technology (Hartfordshire, England), and anti-phospho-ACCβ Ser217 and anti-phospho-AMPKα Thr172 were from Upstate Biotechnology (New York, NY), neuronal NOS (nNOS) was from BD Transduction Laboratories (Sydney, New South Wales, Australia), and glutathione was from Abcam (Cambridge, England). AMPKα1 and α2 antibodies used for immunoprecipitation were as previously described (5). IRDye 800-conjugated streptavidin and secondary antibodies IRDye 800-conjugated anti-mouse and anti-rabbit IgG were purchased from Rockland (Gilbertsville, PA).

Muscle dissection and incubation. Mice were anesthetized with pentobarbital sodium (60 mg/kg ip Nembulat; Rhone Merieux, Pinkenba, Queensland, Australia) and the proximal and distal tendons of the extensor digitorum longus (EDL) and soleus muscles of both hindlimbs were tied with 5/0-silk suture. Muscles were carefully excised tendon-to-tendon, with the proximal tendon tied to a force transducer (PanLab), and the muscles were suspended in incubation chambers (Radnoti, Monrovia, CA) filled with Ringer solution (in mM: 118.5 NaCl, 24.7 NaHCO3, 4.74 KCl, 1.18 MgSO4, 1.18 KH2PO4, and 2.5 CaCl2 pH 7.4) containing 0.01% BSA, 8 mM mannitol, and 2 mM sodium pyruvate and in the presence or absence of 10 mM 2-deoxy-D-glucose, 8 mM L-NMMA, and 2 mM sodium pyruvate. Chambers were oxygenated with 95% O2-5% CO2 gas (Carbogen; BOC Gases, Preston, Victoria, Australia) and maintained at 30°C. After all the muscles had been excised, the mice were killed by cervical dislocation while still anesthetized deeply.

Muscle contraction protocol. In contraction experiments, muscles were stimulated by square wave electrical pulses generated by a Grass S48 stimulator (model DC-300A Series II; Crown International, MA), amplified (EP500B power amplifier; Audio Assemblies, Cambellfield, Victoria, Australia), and delivered via two platinum plate electrodes that flanked but did not touch the muscle preparation. The stimulation protocol was designed to maximize glucose uptake yet maintain muscle viability and minimize fatigue. Noncontracted muscles were treated the same as contracted muscles except that they were not stimulated during the contraction period.

Glucose uptake measurements. 2-Deoxy-d-glucose uptake was measured during the final 5 min of contraction and for 5 min of recovery in contracted muscles (51) and during the final 10 min of incubation in noncontracted muscles. This involved exchanging the incubation buffer for a buffer containing 1 mM 2-deoxy-d-ribose, 100 nM 2-Deoxy-D-glucose, 8 mM L-NMMA, and 2 mM sodium pyruvate. Following incubation in this buffer, muscles were washed in ice-cold Ringer solution, blotted on filter paper, and then snap frozen in liquid nitrogen. Whole (intact) muscles were digested in 125 μl of 1 M NaOH for 10 min at 80°C and then neutralized by the addition of 125 μl of 1 M HCl, vortexed, and spun at 13,000 rpm for 2 min. The supernatant (175 μl) was recovered and added to 4.5 ml of inorganic liquid scintillation cocktail (PerkinElmer, Boston, MA). Radioactivity of both tracers was measured by a β-scintillation counter (Packard TriCarb 2900TR; PerkinElmer), and glucose uptake was calculated (50). Separate muscles were used for immunoblotting and glucose uptake. Muscles used for immunoblotting were frozen immediately after contraction.

Immunoblotting and AMPK activity. Frozen muscles were homogenized in ice-cold lysis buffer (20 μl/mg tissue; 50 mM Tris·HCl at pH 7.5 containing 1 mM EDTA, 10% vol/vol glycerol, 1% vol/vol Triton X-100, 50 mM NaF, 5 mM Na3P2O7, 1 mM DTT, 1 mM PMSF, and 5 μl/ml protease inhibitor cocktail), incubated for 20 min on ice and then centrifuged at 13,000 g for 20 min at 4°C. Protein concentration was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL) with BSA as the standard. Total protein was diluted 1:3 in sample buffer (1.5 M Tris·HCl pH 6.8, 30% glycerol, 10% SDS, 0.6 M DTT, and 0.0012% bromphenol blue), heated for 10 min at 100°C, and stored at −20°C before 80 μg of total protein was separated by SDS-PAGE, transferred to a PVDF membrane, and blocked in PBS containing 5% nonfat milk for 1 h at room temperature. For analysis of protein S-glutathionylation (the addition of glutathione to the cysteine sulffhydryls on proteins; a commonly used marker of oxidative stress; Ref. 8), muscle was extracted under nonreducing conditions with lysis and sample buffer containing 5 and 10 mM of N-ethylmaleimide, respectively, in the absence of DTT. After blocked membranes were incubated overnight at 4°C with phosphorylation-specific antibodies for ACCβ Ser217, or with primary antibodies for nNOS or glutathione, binding was detected with anti-mouse or rabbit IgG secondary antibody. Direct fluorescence was detected and quantified using the Odyssey infrared imaging system (LICOR Biosciences, Lincoln, NB). Membranes were then stripped (2% SDS (wt/vol) in 25 mM glycine pH 2.0) and reprobed with primary antibodies for ACCβ (streptavidin), nNOS, and tubulin to determine total protein levels. However, for AMPKα Thr172 phosphorylation, membranes were first probed with a AMPKα primary antibody before being stripped and reprobed with a AMPKα Thr172 phosphorylation-specific antibody, as we find that AMPKα Thr172 phosphorylation cannot be effectively stripped. Protein phosphorylation was expressed relative to the total protein abundance of the protein of interest or tubulin as indicated.

Table 1. Morphologic characteristics of AMPK-KD and WT mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>AMPK-KD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, wk</td>
<td>23 ± 0.2</td>
<td>23 ± 0.2</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>24.5 ± 0.6</td>
<td>24.1 ± 0.4</td>
</tr>
<tr>
<td>EDL muscle mass, mg</td>
<td>10.8 ± 0.5</td>
<td>10.1 ± 0.5</td>
</tr>
<tr>
<td>Soleus muscle mass, mg</td>
<td>8.8 ± 0.4</td>
<td>8.7 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 28–30 per group; AMPK-KD, kinase dead AMPKα2 isofrom; WT, wild type; EDL, extensor digitorum longus.
Immunocomplexs were washed with PBS and suspended in 50 mM Tris·HCl buffer (pH 7.4) for AMPK activity assay (7). In the presence of 200 μM of AMP, activities were calculated as picomoles of phosphate incorporated into the SAMS peptide [acetyl-CoA carboxylase (ACC)α (73-87)A77] per minute per milligrams of total protein subjected to immunoprecipitation.

Oxidant levels and NOS activity. Oxidant levels were measured using the fluorescent probe 2’,7’-dichlorodihydrofluorescein diacetate
(DCFH-DA; Molecular Probes, Eugene, OR; Ref. 37) in muscle cross sections. Following incubation muscles were mounted in OCT and frozen in isopentane cooled in liquid nitrogen. Muscles were sectioned and treated with DCFH-DA (5 μM) and allowed to dry overnight at room temperature. The oxidized derivative of DCFH-DA, dichloroflourescein (DCF), was measured using confocal microscopy (480 nm excitation, 520 nm emissions; Zeiss LSM-510 Meta confocal microscope, Carl Zeiss Microimaging, Thornwood, NY), and emission intensity was determined with Zeiss LSM-510 acquisition and analysis software. These procedures were carried out in low light conditions to avoid photo-oxidation. NOS activity was determined in separate muscles by measuring the conversion of labeled L-arginine to labeled L-citrulline (NOS activity assay; Cayman Chemicals, Ann Arbor, MI).

Statistical analysis. All data are expressed as means ± SE. Results were analyzed by SPSS statistical package using one (condition)-, two (genotype and condition)-, and three (genotype, condition and time)-factor ANOVA. If the ANOVA revealed a significant interaction, specific differences between mean values were located using the Fisher’s least significance difference test. Morphological properties were compared using an unpaired t-test. The level of significance was set at $P < 0.05$.

RESULTS

Morphological properties. Body mass, EDL, or soleus mass was not different between age and sex matched AMPK-KD and WT mice (Table 1).

AMPK signaling. EDL muscles from AMPK-KD mice showed lower ($P < 0.01$) basal AMPKα1 and AMPKα2 activity than EDL muscles from WT mice (Fig. 1, A and B). AMPKα1 activity in EDL muscles from WT and AMPK-KD mice were not affected by contraction (Fig. 1A). Contraction increased ($P = 0.001$) AMPKα2 activity in EDL muscles from WT mice by 3.5-fold but did not increase AMPKα2 activity in EDL muscles from AMPK-KD mice (Fig. 1B).

Muscles from AMPK-KD mice showed a twofold greater expression of AMPKα than muscles from WT mice (data not shown), but WT mice had greater AMPKα Thr172 phosphorylation relative to AMPKα expression than AMPK-KD mice for both EDL and soleus muscles ($P < 0.05$; Fig. 1, C and D). Contraction increased ($P < 0.05$) AMPKα Thr172 phosphorylation four- and sevenfold above basal in EDL and soleus muscles of WT mice, respectively, but did not affect AMPKα Thr172 phosphorylation in EDL or soleus muscles of AMPK-KD mice ($P > 0.05$). AMPKα Thr172 phosphorylation in EDL and soleus muscles of both genotypes was not affected by NAC or l-NMMA (Fig. 1, C and D).

ACCβ Ser221 phosphorylation in EDL and soleus muscles was greater in WT than in AMPK-KD mice ($P < 0.005$; Fig. 1, E and F). Contraction increased ACCβ Ser221 phosphorylation in EDL and soleus muscles of WT mice ($P < 0.05$) and in EDL ($P = 0.05$) but not soleus ($P = 0.17$) muscles of AMPK-KD mice (Fig. 1, E and F). NAC treatment prevented the contraction-induced increase in ACCβ Ser221 phosphorylation in soleus ($P = 0.17$) but not EDL muscles of WT mice.

Fig. 2. Representative images (A) and emission intensity (B) of dichlorofluorescein (DCF) fluorescence and S-glutathionylation of a protein band at ~270 kDa (C) and 37 kDa (D) in EDL muscles of WT and AMPK-KD mice with either basal incubation or when stimulated to contract for 10 min in the presence or absence of NAC. Data are means ± SE; $n = 4–7$ per group. A.U., arbitrary units. $^*P < 0.05$ vs. basal group of same genotype. $^#P < 0.05$ vs. contraction group of same genotype.
and did not affect ACCβ Ser221 phosphorylation in the EDL or soleus muscles of AMPK-KD mice (Fig. 1, E and F). L-NMMA treatment did not affect ACCβ Ser221 phosphorylation during contraction in either EDL or soleus muscles of either genotype (Fig. 1, E and F).

**Oxidant levels and S-glutathionylation.** Contraction increased (P < 0.05) oxidant levels by ~50% in the EDL muscles of both AMPK-KD and WT mice, and this increase was prevented by NAC (Fig. 2, A and B). S-glutathionylation is a redox signaling event and therefore was measured as a marker of oxidative stress (8). Contraction also increased (P < 0.05) S-glutathionylation of protein bands at ~270 and ~37 kDa by approximately twofold in EDL muscles of WT mice, and these increases were prevented by NAC. In EDL muscles from AMPK-KD mice contraction did not increase S-glutathionylation at ~270 kDa (Fig. 2C) but increased (P < 0.05) S-glutathionylation at ~37 kDa (Fig. 2C) by ~2.3-fold (P < 0.05), and this increase was prevented by NAC.

**NOS activity and nNOS expression.** EDL muscles from AMPK-KD mice showed a ~30% lower NOS activity than muscles from WT mice (P = 0.046; Fig. 3A). Contraction increased (P < 0.05) NOS activity approximately twofold in the EDL muscles of both WT and AMPK-KD mice, and this increase was prevented with L-NMMA (Fig. 3A).

As in previous studies (27), nNOS protein was threefold more abundant in EDL than in soleus muscles (P < 0.001), and there was no difference in nNOS protein expression in the EDL and soleus muscles of WT and AMPK-KD mice (Fig. 3B).

**Glucose uptake.** Despite inhibition of AMPK signaling in AMPK-KD mice, contraction increased (P < 0.05) glucose uptake in the EDL (2.3-fold) and soleus (1.6-fold) muscles from both WT and AMPK-KD mice to a similar extent (Fig. 4). NAC reduced resting C57Bl/6 muscle EDL muscle glucose uptake from 1.1 ± 0.1 to 0.7 ± 0.1 μmol·g⁻¹·h⁻¹ (P = 0.02). In EDL muscles from WT mice, NAC attenuated the increase in glucose uptake during contraction by ~50% (P = 0.01) and prevented increases in muscle glucose uptake in EDL muscles of AMPK-KD mice during contraction (Fig. 4A). In the soleus muscles of both WT and AMPK-KD mice, NAC prevented the increase in glucose uptake during contraction (Fig. 4B).

L-NMMA did not affect resting glucose uptake in EDL muscles of C57Bl/6 mice (data not shown). However, L-NMMA attenuated the increases muscle glucose uptake during contraction in EDL muscles of WT (P = 0.03) and AMPK-KD mice (P = 0.05; ~40%), but L-NMMA did not affect glucose uptake during contraction in the soleus muscles of either WT (P = 0.34) or AMPK-KD mice (P = 0.51; Fig. 4, A and B).

**Muscle contraction.** Neither treatment nor genotype affected peak force (normalized to muscle mass) during contraction of either the EDL or soleus muscles (Fig. 5, A and B). Peak force in both muscles was obtained within the first minute of the 10-min contraction protocol and declined thereafter (P < 0.001). Similarly, force produced by EDL and soleus muscles was not affected by NAC or L-NMMA treatment in AMPK-KD or WT mice (P > 0.05).

**AICAR-stimulated glucose uptake, AMPK phosphorylation and oxidant levels.** AICAR increased glucose uptake 2.6-fold (P < 0.01) in the EDL muscles of C57Bl/6 mice, and this increase was prevented by NAC (Fig. 6A). L-NMMA had no effect on AICAR-stimulated glucose uptake in EDL muscles (Fig. 6A). NAC had no effect on basal AMPK phosphorylation in EDL muscles, but it prevented the 2.3-fold increase in AMPKα Thr172 phosphorylation in response to AICAR (P = 0.01; Fig. 6B). AICAR did not affect oxidant levels in EDL muscles, but the addition of NAC to AICAR tended to reduce oxidant levels in EDL muscles (P = 0.09; Fig. 6, C and D).

**DISCUSSION**

The most important finding of this study was that ROS and NO are involved in regulating glucose uptake during contraction via a mechanism independent of AMPK. We also provided further evidence that AMPK activity is not required for normal increases in glucose uptake during contraction of mouse skeletal muscles in vitro.

As reported previously (10, 29), AMPK-KD mice have reduced AMPKα1 and α2 activity in EDL muscles, and unlike WT mice, AMPK-KD mice show no increases in AMPKα2
activity in EDL muscles during contraction (Fig. 1). Despite this, glucose uptake increased to a similar extent with contraction in the EDL and soleus muscles of WT and AMPK-KD mice (Fig. 4). Previously, isolated muscles from AMPK-KD mice have been reported to have a slight but significant attenuation of the increase in glucose uptake during contraction (14, 23, 35). However, when the force produced by muscles from WT mice, which is greater than that in AMPK-KD mice at high stimulation frequencies (75 Hz; Ref. 29), is matched to the force of muscles from AMPK-KD mice by reducing the stimulation voltage, muscles from AMPK-KD mice have similar increases in glucose uptake during contraction as muscles from WT mice (14). Despite a similar workload, reducing stimulation voltage in muscles of WT mice may alter muscle fiber recruitment and signaling, thus potentially reducing glucose uptake (29, 47). Furthermore, recent evidence (47) suggests that force production is not necessarily synonymous with glucose uptake but rather metabolic demand. In the present study, we used a more physiologically relevant contraction protocol than used previously (18, 25, 46), specifically by using lower stimulation frequencies, train durations, and contraction rates (54). Similar to the findings of Lefort et al. (29), we found no difference in force production between muscles of AMPK-KD and WT mice (Fig. 5) using a low (50 Hz) frequency stimulation. In contrast, Lefort et al. (29) reported that contraction-stimulated glucose uptake was reduced by 50% in muscles from AMPK-KD mice. It is difficult to discern the reason for the discrepancy between our findings and those of Lefort et al. (29), but it may be attributed to differences in the contraction protocols. Lefort et al. (29) stimulated muscles at a higher intensity for a shorter duration at one train per second for 2 min, compared with our protocol of 12 contractions per minute for 10 min. This is consistent with the fact that AMPK activation is higher during more intense exercise (6, 21) and thus may be playing a greater role in regulating glucose uptake during exercise at higher intensities.

The nonspecific antioxidant NAC attenuated the increase in glucose uptake during contraction (Fig. 4) of glycolytic (EDL) muscles from WT mice to a similar extent as reported previously (Fig. 4; Ref. 46). However, Sandstrom et al. (46) also reported that NAC attenuated the increases in AMPK activity and AMPK phosphorylation during contraction, suggestive of a causative relationship between ROS, AMPK, and glucose uptake.

Fig. 4. 2-Deoxy-D-glucose uptake in EDL (A) and soleus (B) muscles from WT and AMPK-KD mice with either basal incubation or when stimulated to contract for 10 min in the presence or absence of L-NMMA and NAC. Data are means ± SE; n = 6–12 per group. *P < 0.05 vs. basal group of same genotype. #P < 0.05 vs. contraction of same genotype.

Fig. 5. Force production of isolated EDL and soleus muscles of WT and AMPK-KD mice at the beginning of each minute of the 10-min contraction protocol (see RESEARCH DESIGN AND METHODS for details). Data are means ± SE; n = 6–12 per group.
uptake during contraction. In the present study, we showed that in addition to NAC attenuating increases in glucose uptake during contraction in glycolytic muscles, NAC also prevented increases in glucose uptake during contraction in oxidative (soleus) muscles (Fig. 4). Furthermore, we found that NAC attenuated increases in skeletal muscle glucose uptake during contraction even in the absence of any increases in AMPKα1 or α2 activity during contraction (Fig. 1). In support of previous findings, we report that skeletal muscle oxidant levels are increased during contraction (41) and that this increase is prevented by NAC (46). In the present study, we measured DCF fluorescence as a marker of oxidant levels in cross-sections of EDL muscles. Although DCFH can be oxidized to DCF by both reactive nitrogen species (36) and ROS (37), because NAC prevented contraction-induced increases in DCF, we are confident of this method as a marker of muscle ROS levels. Furthermore, oxidative stress promotes the addition of glutathione to protein residuals (S-glutathionylation; Ref. 8). Since contraction-stimulated increases in S-glutathionylation of protein bands at ∼37 kDa in AMPK-KD and ∼270 and ∼37 kDa in EDL muscles of WT mice and since this increase was prevented by NAC (Fig. 2), this provides further evidence that contraction increased muscle ROS levels. Therefore, our findings suggest that ROS are involved in regulating glucose uptake in both glycolytic and oxidative muscles independent of AMPK.

In contrast to Sandstrom et al. (46), we found that NAC did not affect AMPK Thr172 phosphorylation during contraction in EDL muscles. The reason for this discrepancy is also difficult to discern, but it could be attributed to minor differences in methodology such as the incubation temperature (25°C in Ref. 46 vs. 30°C used in the present study), longer NAC preincubation period (60 min in Ref. 46 vs. 30 min used in the present study), and differences in contraction protocol as discussed above. Regardless, our finding that NAC did not affect muscle AMPK Thr172 phosphorylation during contraction in WT mice supports the involvement of ROS in regulating skeletal muscle contraction-stimulated glucose uptake independently of AMPK. In agreement, recent studies (19, 24) have reported that exogenous ROS can increase glucose uptake in isolated glycolytic and oxidative skeletal muscles independent of AMPK. Higaki et al. (19) showed that the phosphatidylinositol 3-kinase inhibitor wortmannin prevented exogenous H2O2-stimulated skeletal muscle glucose uptake. However, contraction increases skeletal muscle glucose uptake via a phosphatidylinositol 3-kinase-independent pathway (31, 39). Therefore, it is likely that

Fig. 6. 2-Deoxy-β-glucose uptake (A), AMPKα Thr172 phosphorylation relative to total AMPKα protein abundance (B), representative images (C), and emission intensity (D) of DCF fluorescence in EDL muscles of C57Bl/6 mice when incubated in the presence and absence of NAC, 5-aminoimidazole 4-carboxamide ribonucleoside (AICAR), NAC + AICAR, or AICAR + L-NMMA. Data are means ± SE; n = 4–6 per group. *P < 0.05 vs. basal.
during contraction ROS regulate skeletal muscle glucose uptake through a pathway that differs from that of exogenous H$_2$O$_2$. Interestingly, ROS appear to regulate p38 MAPK (p38) phosphorylation during exercise (16), and p38 has been implicated in the regulation of skeletal muscle contraction-stimulated glucose uptake (49). Furthermore, ROS have been proposed to increase stretch-stimulated skeletal muscle glucose uptake via p38 (4). Given the numerous pathways beginning to be recognized as being redox sensitive (22), it is important to investigate whether ROS regulate skeletal muscle glucose uptake during contraction via p38 or alternative pathways. Indeed, ROS have already been implicated in the regulation of pathways involved in glucose uptake signaling including ONOO$^-$ signaling (56) and cyclic guanosine monophosphate (cGMP)-dependent protein kinase activity (3, 53).

Interestingly, AICAR-stimulated AMPK phosphorylation and glucose uptake in EDL muscle were prevented by NAC. Although AICAR stimulation did not increase basal oxidant levels in EDL muscles, NAC tended to reduce AICAR oxidant levels. This suggests that basal ROS levels may be required for AICAR-stimulated AMPK phosphorylation and glucose uptake. However, it is important to acknowledge that the mechanisms through which AICAR activates AMPK and stimulates glucose uptake are considerably different from those of contraction (25) and the effect of NAC on AICAR-stimulated glucose uptake appears to depend on treatment order (46).

The role of NO in the regulation of skeletal muscle glucose uptake during contraction is controversial, with some studies reporting that inhibition of NO$^-$ during contraction attenuates the increases in glucose uptake (1, 2, 26, 42, 44), while others have reported no effect (11, 18, 45). These differences are also likely attributed to inconsistencies in methodology (33). In the present study, L-NAME treatment attenuated the increase in glucose uptake in EDL but not soleus muscles of AMPK-KD and WT mice during contraction (Fig. 4). Since NO$^-$ activity and glucose uptake in EDL muscles of WT and AMPK-KD mice were increased with contraction and L-NAME prevented these increases (Fig. 3), this suggests that NO is involved in the regulation of glucose uptake during contraction independent of AMPK in muscles with a higher proportion of glycolytic fibers. This was supported by the finding that L-NAME also did not affect contraction-stimulated AMPK Thr$^{172}$ or ACC$\beta$ Ser$^{271}$ phosphorylation in EDL muscles (Fig. 1). However, as reported previously (28), EDL muscles from AMPK-KD mice showed ~30% lower NOS activity than in WT mice. This supports evidence that AMPK phosphorylates NOS (7) and that this phosphorylation increases NOS activity. Interestingly, the lower absolute NOS activity in EDL muscles of AMPK-KD mice did not affect glucose uptake during contraction. This suggests that because muscles from AMPK-KD and WT mice showed similar increases in NOS activity and glucose uptake during contraction (Fig. 4), it may be that the contraction-stimulated increase in NO production from basal, rather than the absolute NO concentration, is more important in regulating glucose uptake during contraction. As discussed above, it has been reported previously that during intense stimulation protocols ex vivo that the muscles of AMPK-KD mice have attenuated increases in glucose uptake during contraction compared with muscles from WT mice. Therefore, whether this lower glucose uptake is a result of lower AMPK or lower NOS activity requires further examination. Interestingly, L-NAME did not affect glucose uptake during contraction in the soleus muscles of WT or AMPK-KD mice, suggesting that NO plays a greater role in regulating glucose uptake during contraction in glycolytic vs. oxidative muscles. Indeed, nNOS protein abundance was similar between genotypes, and lower in the soleus than in EDL muscles (Fig. 3) consistent with reports that soleus muscles produce less NO than EDL muscles (27).

In conclusion, this study provides evidence that ROS and NO are involved in regulating skeletal muscle glucose uptake during contraction independently of AMPK.

ACKNOWLEDGMENTS

We thank Morrie Birnbaum for the AMPK-KD mice, and Dr. Glenn Wadley, Dr. Chris van der Poel, and Dr. Kelly Linden for expert technical assistance.

DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


