Role of reactive oxygen species in contraction-mediated glucose transport in mouse skeletal muscle

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Exercise increases glucose transport into skeletal muscle via a pathway that is poorly understood. We investigated the role of endogenously produced reactive oxygen species (ROS) in contraction-mediated glucose transport. Repeated contractions increased 2-deoxyglucose (2-DG) uptake roughly threefold in isolated, mouse extensor digitorum longus (fast-twitch) muscle. *N*-Acetylcysteine (NAC), a non-specific antioxidant, inhibited contraction-mediated 2-DG uptake by ∼50% ($P < 0.05$ *versus* control values), but did not significantly affect basal 2-DG uptake or the uptake induced by insulin, hypoxia or 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, which mimics AMP-mediated activation of AMP-activated protein kinase, AMPK). Ebselen, a glutathione peroxidase mimetic, also inhibited contraction-mediated 2-DG uptake (by almost 60%, $P < 0.001$ *versus* control values). Muscles from mice overexpressing Mn^{2+}-dependent superoxide dismutase, which catalyses H_2O_2 production from superoxide anions, exhibited a ∼25% higher rate of contraction-mediated 2-DG uptake *versus* muscles from wild-type control mice ($P < 0.05$). Exogenous H_2O_2 induced oxidative stress, as judged by an increase in the [GSSG]/[GSH + GSSG] (reduced glutathione + oxidized glutathione) ratio to 2.5 times control values, and this increase was substantially blocked by NAC. Similarly, NAC significantly attenuated contraction-mediated oxidative stress as judged by measurements of glutathione status and the intracellular ROS level with the fluorescent indicator 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein ($P < 0.05$). Finally, contraction increased AMPK activity and phosphorylation ∼10-fold, and NAC blocked ∼50% of these changes. These data indicate that endogenously produced ROS, possibly H_2O_2 or its derivatives, play an important role in contraction-mediated activation of glucose transport in fast-twitch muscle.

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Exercise/contraction increases glucose transport into muscle by stimulating the translocation of Glut-4 transport proteins to the cell surface by a pathway that differs from that activated by insulin (Coderre *et al.* 1995; Lund *et al.* 1995). There is considerable evidence that AMP-activated protein kinase (AMPK) plays an important role in contraction-mediated glucose transport in skeletal muscle (Hayashi *et al.* 1998; Winder & Hardie, 1999; Kurth-Kraczek *et al.* 1999; Holloszy, 2003; Jessen & Goodyear, 2005; Sakamoto *et al.* 2005), although some studies indicate that there may be parallel signalling pathways that do not involve AMPK (Derave *et al.* 2000; Mu *et al.* 2001; Jorgensen *et al.* 2004; Jessen & Goodyear, 2005). Signalling intermediates in the latter pathways that are considered of importance are Ca^{2+}, nitric oxide (NO), the Akt substrate AS160, and possibly protein kinase C (Holloszy, 2003; Jessen & Goodyear, 2005; Rose & Richter, 2005). Interestingly, there is little mention of the possibility that reactive oxygen species (ROS) may play a role in contraction-mediated glucose transport. Recent studies have demonstrated that addition of H_2O_2 (a member of the ROS family) to isolated cells or perfused rat hearts results in activation of AMPK (Choi *et al.* 2001; Leon *et al.* 2004; Nagata *et al.* 2004), and from earlier studies it is known that addition of H_2O_2 to cells, including skeletal muscle, also accelerates glucose transport (Livingston *et al.* 1977; Cartee & Holloszy, 1990; Fischer *et al.* 1993). Reactive oxygen species are produced at a low rate in resting muscle and at a high rate in contracting muscle (Murrant & Reid, 2001). In view of these observations, we

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have: (1) assessed whether endogenously produced ROS are involved in contraction-mediated glucose transport; and (2) investigated the mechanism whereby ROS activates glucose transport during contraction.

Methods

Materials

2-Deoxy-D-[1,2-^{3}H]glucose (2-DG), carboxy-[^{14}C]inulin and [γ-^{32}P]ATP were from Amersham Biosciences. Ebselen (E3520) and *N*-acetylcysteine (NAC, A8199) were from Sigma. Human insulin (Actrapid) was from Novo Nordisk. 5-(and-6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) was from Invitrogen. Antibodies against pan-α-AMPK, phosphorylated α-AMPK (T172), acetyl CoA carboxylase (ACC) and phosphorylated ACC (S79) were from Cell Signalling Technology. All other reagents were from either Sigma or Boehringer Mannheim.

Male NMRI mice weighing 25–30 g were housed at room temperature with a 12 h–12 h light–dark cycle. Food and water were provided *ad libitum*. The mice were purchased from B&K Universal (Sollentuna, Sweden). Mice overexpressing Mn^{2+}-dependent superoxide dismutase (mitochondrial isoform; SOD2) and wild-type (WT) littermates were generated as described elsewhere (Silva *et al.* 2005). Superoxide dismutase catalyses the following reaction:

$$2 \cdot O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$$

We used mice from the PAC662D1 line exhibiting an almost sixfold increased SOD2 activity (Silva *et al.* 2005). The mice weighed 25–30 g, and the SOD2 mice did not differ from the WT littermates with respect to body weight and whole body oxygen consumption at 4 or 24°C either before or after noradrenaline injection (Silva *et al.* 2005). Furthermore, there were no significant differences in twitch kinetics, force–frequency relationship, fatigue development or recovery from fatigue between WT and SOD2 extensor digitorum longus (EDL) muscles (data not shown). This demonstrates that SOD2 overexpression did not alter contractile function, which suggests that muscle fibre composition was also not altered. Animals were killed by rapid cervical dislocation, and the EDL muscles were isolated. All procedures were approved by the Stockholm North ethics committee.

Experimental design

Contraction experiments. For contraction studies, stainless-steel hooks were tied with nylon thread to the tendons of the muscles. Muscles were then transferred to a stimulation chamber (volume ≈ 10 ml) and mounted between a force transducer and an adjustable holder (World Precision Instruments). The chamber temperature was set at 25°C with a water-jacketed circulation bath. The muscle was bathed in a Tyrode solution with the following composition (mM): NaCl, 121; KCl, 5; $CaCl_2$, 1.8; NaH_2PO_4, 0.4; $MgCl_2$, 0.5; $NaHCO_3$, 24; EDTA, 0.1; glucose, 5.5; and 0.1% fetal calf serum. The pH of the Tyrode solution was set to 7.4 by continuously and directly gassing the solution with 95% O_2–5% CO_2. Muscles were stimulated with current pulses (0.5 ms duration; ∼150% of the current required for maximum force response) via plate electrodes lying parallel to the fibres. Muscles were set to the length at which tetanic force was maximum and then bathed in a Tyrode solution that contained one of two antioxidants: either 20 mM *N*-acetylcysteine (NAC; control being addition of 10 mM NaCl) or 30 μM ebselen in DMSO (control being an equivalent volume of DMSO). As a control for the osmotic effect of NAC, 10 mM NaCl was always added to the 121 mM NaCl Tyrode solution bathing the contralateral muscle. Muscles were then allowed to rest for 60 min. Thereafter, the muscles were stimulated at 50 Hz (tetanic duration 100 ms, 2 trains s^{-1}) for 10 min. This protocol decreases muscle glycogen by ∼80% (Sandström *et al.* 2004). For measurements of AMPK and glutathione status (see below), muscles were quick-frozen in liquid N_2 within 5 s after the last tetanus. For measurement of glucose uptake, muscles were transferred at the end of the 10 min stimulation to vials containing 1.5 ml Tyrode solution lacking glucose and containing 2 mM pyruvate with or without the test drug (or vehicle) and incubated in a shaking water-bath (110 oscillations min^{-1}, 35°C, air phase in vial was continuously gassed with 95% O_2–5% CO_2) for 40 min and frozen in liquid nitrogen. Radiolabelled 2-DG (1 mM) and inulin were added 20 min before freezing as described elsewhere (Shashkin *et al.* 1995).

To assess intracellular ROS formation in contracting muscle, small bundles of 5–20 fibres were mechanically dissected from the EDL muscle. Bundles were loaded with the ROS-sensitive indicator by incubation for 90–120 min in 10 μM CM-H_2DCFDA at room temperature. Thereafter, bundles were transferred to the muscle trough and stretched to the length at which tetanic force was maximal. Muscles were then washed for 30 min with 5.5 mM glucose Tyrode solution (± 20 mM NAC). A BIO-RAD MRC 1024 and a Nikon Diaphot 200 inverted microscope with a ×20 objective lens (NA 0.75) were used. In the cell, esterases cleave CM-H_2DCFDA to release CM-H_2DCFH, which is converted to a fluorescent product (CM-H_2DCF) when exposed to ROS (Xie *et al.* 1999). CM-H_2DCF was excited with 488 nm light, and the emitted light collected through a 515 nm long-pass filter. Confocal images were taken of the muscle fibres at rest and then after 10 min of intermittent

tetanic contractions (same stimulation protocol as for the whole EDL muscle). When NAC was added, it was after the loading period and remained in the medium until after the postcontraction scans were made. CM-H_2DCF fluorescence was measured before and after contraction and after the bundle was exposed to 1 mM H_2O_2 for 5 min. Confocal images were stored and analysed offline with ImageJ (available at http://rsb.info.nih.gov/ij/). Changes in intracellular CM-H_2DCF fluorescence intensity are expressed as a percentage of that before the series of contractions or H_2O_2 exposure.

Rested muscle experiments. A series of glucose uptake experiment was also performed in resting muscle preparations. These muscles were incubated in 1.5 ml of the pyruvate-supplemented Tyrode solution at 35°C in the shaking water-bath as described above. To examine the effects of antioxidants on basal glucose uptake, muscles were incubated in the presence of NAC or ebselen (with appropriate controls) in the shaking bath for 80 min and then frozen. To examine the effects of hypoxia with or without NAC on glucose uptake, muscles were continuously gassed with 95% N_2–5% CO_2 for a total of 80 min (Cartee *et al.* 1991). *N*-Acetylcysteine was present throughout the 80 min incubation period in half the samples. Since ∼60 min are required to observe the maximal effect of hypoxia on glucose transport in isolated muscle preparations (Cartee *et al.* 1991), no preincubation with NAC in 95% O_2–5% CO_2 was performed. To examine the effects of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; final concentration, 2 mM) on glucose uptake, muscles were incubated first for 30 min and then AICAR was added to the muscles and the incubation was continued for an additional 80 min (Kurth-Kraczek *et al.* 1999). *N*-Acetylcysteine was present throughout the 110 min incubation period in half the samples. AICAR is converted in the cell to AICAR-monophosphate, which is an AMP mimetic that activates AMPK and AMPK kinase (Young *et al.* 1996; Kurth-Kraczek *et al.* 1999). To examine the effects of insulin (final concentration, 20 mU ml^{-1}) on glucose uptake, muscles were preincubated for 30 min; insulin was added and the incubation was continued for an additional 50 min. *N*-Acetylcysteine was present throughout the 80 min incubation period in half the samples. 2-Deoxyglucose and inulin were present during the last 20 min of incubation in these experiments. During the insulin experiments, bovine serum albumin (0.1% v/v) was present in the pyruvate-supplemented Tyrode solution to inhibit insulin binding to the glass vial.

To study the effects of exogenous H_2O_2 (final concentration = 3 mM) on glutathione status, muscles were incubated in 1.5 ml of the 5.5 mM glucose Tyrode solution in the shaking bath at 35°C for 90 min and then frozen. H_2O_2 was added after the initial 60 min of incubation. NAC was present throughout the 90 min incubation period in half the samples. To study the effects of NAC on glutathione status and AMPK activity in resting muscles, muscles were incubated in 1.5 ml of the 5.5 mM glucose Tyrode solution in the shaking bath at 25°C for 60 min and then frozen. NAC was present throughout the 60 min incubation period in half the samples. The latter experiments were performed at 25°C, because the contraction-mediated changes in glutathione status and AMPK activity were also studied at this temperature.

To study the effects of exogenous H_2O_2 (final concentration, 3 mM) on glucose uptake, muscles were incubated in pyruvate-supplemented Tyrode solution in the shaking bath for 80 min at 35°C (see above). Hydrogen peroxide was added to half the muscles after 30 min. Isotopes were present during the last 20 min of incubation. To examine the effects of exogenous H_2O_2 on AMPK activity, muscles were incubated in the shaking bath for 60 min at 25°C in 5.5 mM glucose Tyrode solution (since basal and contraction-mediated AMPK activities were studied under these conditions). Hydrogen peroxide was added to half the muscles after 30 min.

Analytical

2-Deoxyglucose uptake. For analysis of 2-DG uptake, frozen muscles were added to preweighed Eppendorf tubes containing 0.5 ml of 1 N NaOH. The muscle was weighed and then digested at 70°C for 15 min. The tubes were cooled on ice and centrifuged at 23 000*g* for 5 min. Aliquots of the supernatant were added to scintillation cocktail and counted for ^{14}C and ^{3}H as described earlier (Shashkin *et al.* 1995).

Glutathione. For analysis of glutathione, a kit was used (Biooxytech GSH/GSSG-412, Oxis Health Products, Portland, OR, USA). Muscles were freeze-dried, dissected free of non-muscle constituents, powdered and thoroughly mixed. The powders were divided into two aliquots, which were homogenized in ground glass homogenizers containing ice-cold 5% metaphosphoric acid (80 μl (mg dry weight)$^{-1}$) with and without 1-methyl-2-vinyl-pyridinium trifluoromethane sulphonate (M2VP; 10% v/v), a scavenger of reduced glutathione (GSH). The homogenates were centrifuged at 23 000*g* for 15 min at 4°C. The pellets were digested with 1 N NaOH (60°C) and assayed for protein with the Bio-Rad assay (BIO-RAD). For measurement of reduced + oxidized glutathione (TGSH = GSH + oxidized gluthathione [GSSG]), 4 μl supernatant were mixed with 96 μl assay buffer, and for GSSG estimation, 5 μl of the supernatant with M2VP were mixed with 95 μl GSSG assay buffer. For both assays, the samples were mixed with 300 μl of chromagen,

glutathione reductase and NADPH, and absorbance (reduction of dithiobis-2-nitrobenzoic acid at 412 nm) was measured after 4.5 min in a spectrophotometer. Preliminary experiments demonstrated that the assays were linear with respect to the extract volume used and reaction time, and GSH was not detectable in the presence of M2VP (data not shown).

AMPK. AMPK activity was analysed by assessing the incorporation of radiolabelled phosphate from ATP into SAMS peptide (Winder & Hardie, 1996) with some modifications. Briefly, muscles were freeze-dried and treated as above. Muscles were homogenized in ice-cold buffer (100 μl (mg dry weight)$^{-1}$) consisting of (mM): Tris, 10; sucrose, 250; NaF, 50; EDTA, 1; β-mercaptoethanol, 10; and one tablet protease inhibitor cocktail (Roche) per 50 ml of buffer, pH 7.5. The homogenate was centrifuged at 23 000*g* for 30 min at 4°C. The supernatant was divided into aliquots. One aliquot was assayed for protein (see above). Another aliquot was diluted with seven volumes of homogenization buffer, and 10 μl of the diluted extract were mixed with 30 μl reaction buffer, resulting in the following final concentrations (mm): Hepes (pH 7.0), 40; SAMS peptide, 0.2; NaCl, 80; EDTA, 0.8; AMP, 0.2; dithiothreitol (DTT), 0.8; $MgCl_2$, 5; ATP, 0.2; [γ-^{32}P]ATP, 2 μCi; and glycerol, 8% (v/v);. The assay was performed at 37°C for 10 min. Thereafter, 30 μl of the mixture were spotted onto Whatman P81 discs, washed in 1% phosphoric acid, dried and counted. Blanks consisted of mixtures spotted without incubation. The assay was linear with respect to the used extract volume and reaction time, and no activity was detected in the absence of SAMS peptide (data not shown). In preliminary experiments, we also assayed the AMPK activity in ammonium-sulphate-precipitated extracts as described by others (Winder & Hardie, 1996). While this procedure also yielded linearity with respect to extract volume and reaction time, there was a large loss of enzyme activity when compared to results from untreated extracts (data not shown). These findings are in agreement with results recently reported by others (Derave *et al.* 2000). Because of the prevention of enzyme loss and the decrease in preparative steps, assays were performed on untreated supernatants.

Western blots were performed for phosphorylated and total AMPK and ACC. Briefly, 20 (total and phosphorylated ACC) or 25 μg (total and phosphorylated AMPK) of supernatant (see above) protein were separated by SDS-PAGE (4–12% Bis-Tris Gels; Invitrogen) and transferred onto polyvinylidine fluoride (PVDF) membranes (BIO-RAD). Membranes were blocked in 5% (w/v) non-fat milk in Tris-buffered saline containing 0.05% Tween 20 followed by incubation with primary antibodies (all at 1:1000 dilution) overnight. Membranes were then washed and incubated with secondary antibody (donkey antirabbit at 1:2000 dilution for all). Immunoreactive bands were visualized using enhanced chemiluminescence (Super Signal, Pierce, Rockford, IL, USA.). Band densities were analysed with ImageJ (NIH, USA; http://rsb.info.nih.gov/j/).

Values for glutathione and AMPK are expressed relative to dry weight. Differences between groups were maintained also if the results were adjusted for protein. Force was sampled on-line and stored on a desktop computer for subsequent analysis. Tetanic force was measured as the peak force during the 100 ms of stimulation.

Statistics

Signficant differences between means were determined with Student's *t* test for paired samples. $P < 0.05$ was regarded as significant. Values are presented as means ± S.E.M.

Results

N-Acetylcysteine and 2-DG uptake

Contraction increased 2-DG uptake roughly threefold (Fig. 1*A*). In the presence of NAC, the rate of 2-DG uptake after contraction was significantly lower than during control conditions. Thus, NAC abolished ~50% of contraction-mediated 2-DG uptake. *N*-Acetylcysteine did not affect basal 2-DG uptake, which is primarily ascribed to the availability of Glut-1 transport proteins in the cell membrane (Mueckler, 1994). However, contraction accelerates glucose transport via translocation of Glut-4 transport proteins to the cell surface. Therefore, we determined whether other interventions that increase glucose transport via translocation of Glut-4 proteins (Holloszy, 2003; Jessen & Goodyear, 2005; Rose & Richter, 2005) are affected by NAC. We found that insulin, hypoxia and AICAR increased 2-DG uptake to values similar to that seen with contraction, but NAC did not significantly affect these increases. It could be argued that NAC interfered with contraction-mediated 2-DG uptake owing to an inhibition of force production. However, force generation during the repeated contractions in the presence of NAC was virtually identical to control values (Fig. 1*B*).

It should be noted that since contractile function in isolated mouse EDL deteriorates after ~20 min at 35°C (Zhang *et al.* 2006), and a 60 min preincubation period was required, the contraction-mediated 2-DG uptake was induced at 25°C. However, insulin-, hypoxia- and AICAR-mediated 2-DG uptake were induced at 35°C. All 2-DG uptake measurements were performed at 35°C. Therefore, caution should be exerted in directly comparing 2-DG uptake between different modes of glucose uptake induced at different temperatures.

N-Acetylcysteine and ROS formation

The above results with NAC implicated endogenously produced ROS in contraction-mediated glucose uptake. Glutathione status is commonly used as a measure of intracellular oxidative stress and was therefore studied in the next series of experiments. Glutathione peroxidase catalyses the following reaction: $H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$; thus changes in GSH and GSSG will reflect changes in H_2O_2. First, we incubated muscles with 3 mM H_2O_2 in the absence and presence of NAC. Hydrogen peroxide resulted in a marked degree of oxidative stress as evidenced by a decrease in TGSH (Fig. 2). The decrease in TGSH was probably associated with an increase in mixed disulphides, since protein glutathionylation occurs with excessive oxidative stress (Ghezzi, 2005). Hydrogen peroxide also increased the GSSG/TGSH ratio to ∼250% of control values, and NAC counteracted the H_2O_2-dependent effect. We then investigated the effect of contraction in the absence and presence of NAC on glutathione status. *N*-Acetylcysteine did not affect glutathione status in the basal state, but resulted in significantly smaller changes in GSSG and the GSSG/TGSH ratio following contraction (Fig. 3). In summary, NAC decreased the oxidative stress associated with addition of exogenous H_2O_2 and repeated contractions.

We then used CM-H_2DCF to measure intracellular ROS formation in bundles of EDL fibres. Repeated contractions resulted in a significant increase in fluorescence, reflecting an increase in ROS levels, whereas no increase was observed

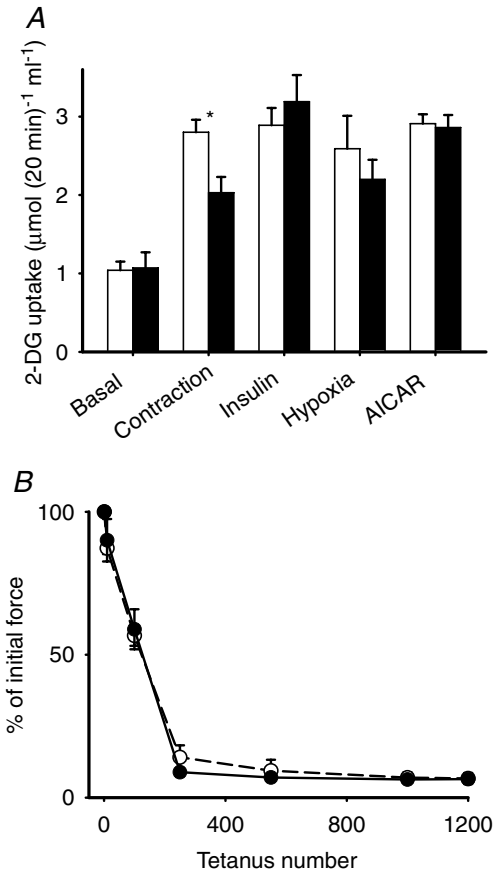

Figure 1. *N*-Acetylcysteine inhibits contraction-mediated glucose uptake in mouse EDL muscle
Values are means ± S.E.M. for 6–8 muscles (*A*) or 6 muscles (*B*). *A*, open bars, control; filled bars, NAC (20 mM). *$P < 0.05$ *versus* control values. *B*, NAC does not affect force generation during 10 min period of repeated contractions (○, control; ●, NAC). Initial force averaged 20.8 ± 1.8 mN (mg wet weight)$^{-1}$ for control conditions and 23.0 ± 1.4 mN (mg wet weight)$^{-1}$ for NAC; n.s.).

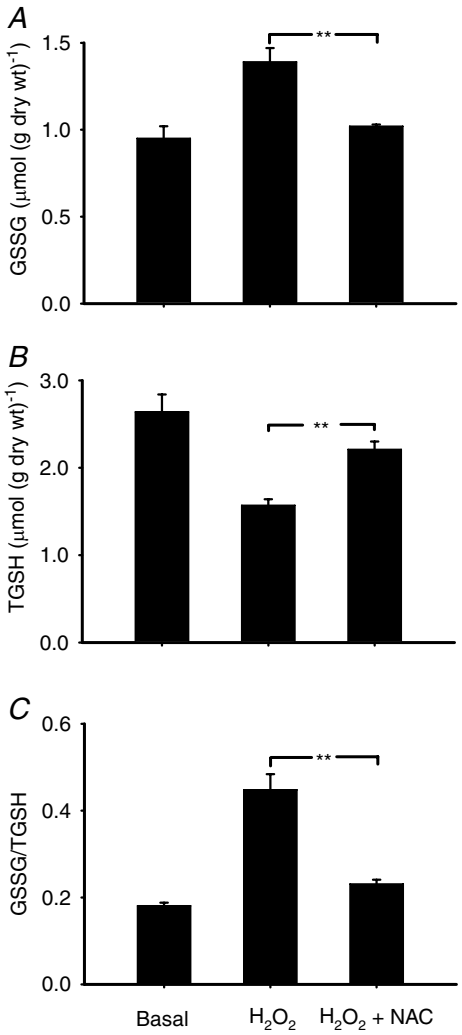

Figure 2. *N*-Acetylcysteine inhibits H_2O_2-induced oxidative stress in mouse EDL muscle
Values are means ± S.E.M. for 6 muscles. TGSH is the sum of GSH and GSSG. Muscles were untreated (basal), exposed to H_2O_2 (3 mM) or to H_2O_2 + NAC (20 mM). *A*, GSSG; *B*, TGSH; and *C*, GSSG/TGSH (the ratio is in GSH equivalents). **$P < 0.01$. Muscles in the basal group were from a separate group of mice studied at the same time as the other groups; the basal values are included in the statistical analysis and serve solely as reference values.

in the presence of NAC (Fig. 4). The increase we detected was modest, but is in line with earlier findings in isolated rat diaphragm bundles (about 30% increase after 1 h of repeated contractions; Reid *et al.* 1992). Repeated current pulses may generate molecular species that can affect our measurements of ROS formation. Therefore, control experiments were performed where muscle bundles were first incubated with 1 μM tetrodotoxin for 5 min to abolish action potentials and force generation (Belluardo *et al.* 2001) and then exposed to repeated tetanic stimulations. Under these conditions, there was no significant effect of stimulation on CM-H_2DCF fluorescence (89 ± 6% of prestimulation value, n.s.; $n = 5$). This demonstrates that the increase in fluorescence seen with stimulation (Fig. 4) did not result from an artefact associated with field stimulation. Then 1 mM H_2O_2 was added to the muscle preparation at the end of each experiment and always gave a robust increase in fluorescence (136 ± 6%, $P < 0.001$; $n = 15$). Thus NAC attenuated endogenous ROS formation during contraction as measured by two independent methods: glutathione status and CM-H_2DCF fluorescence. Finally, it should be noted that the effects of contraction on glutathione status and CM-H_2DCF fluorescence were markedly smaller than those seen with the addition of exogenous H_2O_2, which indicates that the degree of ROS production during contraction was limited and did not reach high/pathological levels (see Discussion).

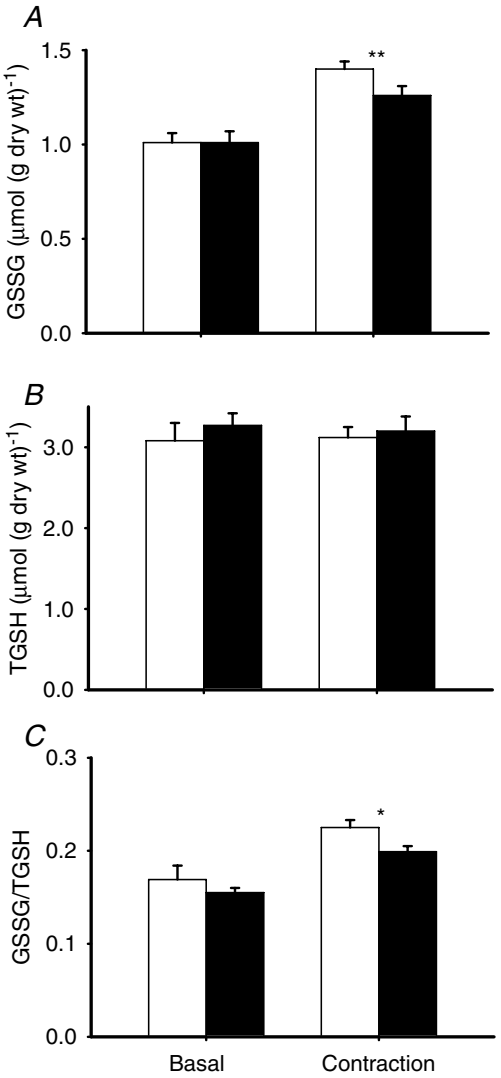

Figure 3. *N*-Acetylcysteine inhibits contraction-induced oxidative stress in mouse EDL muscle
Values are means ± S.E.M. for 6 muscles. TGSH, sum of GSH and GSSG. Unfilled bars, basal; filled bars, NAC (20 mM). *A*, GSSG; *B*, TGSH; and *C*, GSSG/TGSH (the ratio is in GSH equivalents). $^*P < 0.05$; $^{**}P < 0.01$ *versus* control values.

N-Acetylcysteine and AMPK

The results thus far indicated that ROS/H_2O_2 (or their derivatives) produced during contraction resulted in an accelerated glucose transport that was presumably mediated by the Glut-4 transport protein. To investigate the pathway through which ROS were working, we measured AMPK activity following contraction in the absence and presence of NAC. *N*-Acetylcysteine had no significant effect on AMPK activity in resting muscle (Fig. 5). Contraction resulted in almost a 10-fold increase in AMPK activity and about 50% of the increase was blocked by NAC. Thus NAC inhibited contraction-mediated activation of AMPK and 2-DG uptake to the same relative extent. We verified that exogenous H_2O_2 activates AMPK in mouse EDL muscles (basal, 5.9 ± 1.6 nmol (g dry weight)$^{-1}$ min^{-1}; and in the presence of 3 mM H_2O_2, 18.2 ± 4.0 nmol (g dry weight)$^{-1}$ min^{-1}; $n = 6$; $P < 0.05$). It is noteworthy that

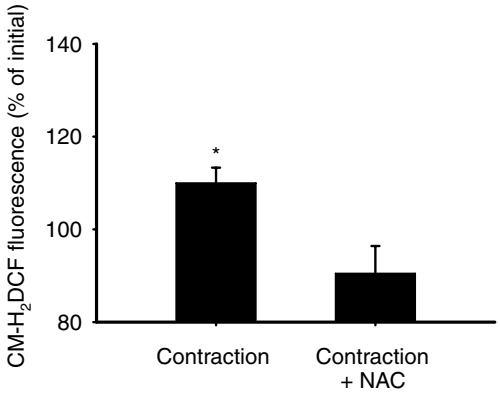

Figure 4. *N*-Acetylcysteine inhibits contraction-mediated ROS formation in bundles of mouse EDL muscle fibres
Values are means ± S.E.M. Measurements of CM-H_2DCF fluorescence were performed in muscle fibres that underwent 10 min of repeated contractions in the absence (contraction, $n = 6$) or presence of 20 mM NAC (contraction + NAC, $n = 4$). Data are expressed as a percentage of the pretreatment value. $^*P < 0.05$ *versus* precontraction value.

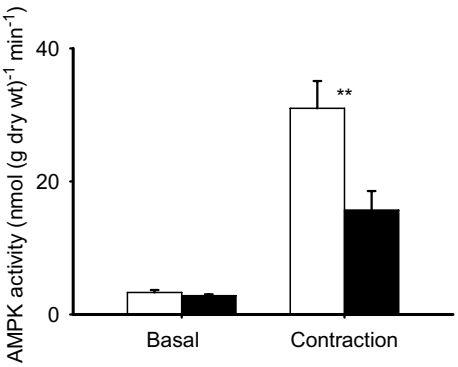

Figure 5. *N*-Acetylcysteine inhibits contraction-mediated activation of AMPK in mouse EDL muscle
Values are means ± S.E.M. for $n = 6$ (basal) or $n = 5$ muscles (contraction). Open bars, control; filled bars, NAC (20 mM). Muscles were stimulated for 10 min with repeated contractions. $^{**}P < 0.01$ *versus* control values.

the increase in AMPK activity induced by H_2O_2 under basal conditions (~12 nmol (g dry weight)$^{-1}$ min^{-1}) is similar to that blocked by NAC during contraction (~15 nmol (g dry weight)$^{-1}$ min^{-1}; Fig. 5).

Since AMPK activity is modulated by phosphorylation, we also determined the extent of AMPK phosphorylation after contraction in the absence and presence of NAC. Consistent with the changes in AMPK activity, contraction resulted in almost a 10-fold increase in AMPK phosphorylation (Fig. 6*A* and *B*). *N*-Acetylcysteine did not affect AMPK phosphorylation in the basal state, but diminished the degree of phosphorylation after contraction by about 50%, which is in agreement with the effects of NAC on AMPK activity after repeated contractions. Acetyl CoA carboxylase participates in the control of fatty acid oxidation and is a substrate for AMPK. Therefore, we also measured ACC phosphorylation, which should inactivate the enzyme. Repeated contractions also increased ACC phosphorylation, but the increase was not affected by NAC (Fig. 6*C* and *D*). *N*-Acetylcysteine did not affect expression of either total AMPK or total ACC expression in the basal state or after contraction (data not shown). Taken together, the results are consistent with the idea that NAC was blocking a H_2O_2-mediated activation of AMPK during repeated contractions.

Hydrogen peroxide and 2-DG uptake

N-Acetylcysteine is a general antioxidant and thus does not indicate the species of ROS that may mediate glucose transport during contraction. However, the results thus far were consistent with the involvement of H_2O_2. To assess the role of H_2O_2 more directly, two strategies were employed: (1) use of another antioxidant, ebselen; and (2) use of muscles overexpressing SOD2. Ebselen is a glutathione peroxidase mimetic that removes H_2O_2 in the presence of GSH (Cotgreave *et al.* 1987). Thus ebselen should enhance H_2O_2 removal and would therefore be expected to inhibit contraction-mediated glucose uptake. With respect to SOD2 overexpression, we assumed that these muscles would exhibit increased H_2O_2 production (hence increased glucose uptake) during contraction, owing to increased conversion of superoxide to H_2O_2. Consistent with this assumption is the observation that when superoxide production in mitochondria isolated from skeletal muscle is accelerated, the accumulation of superoxide is decreased in mice overexpressing SOD2 *versus* wild type (Silva *et al.* 2005).

Ebselen significantly increased basal 2-DG uptake (Fig. 7*A*). Despite this increase, 2-DG uptake was still significantly lower after contraction in the presence of ebselen. Thus the contraction-mediated 2-DG uptake

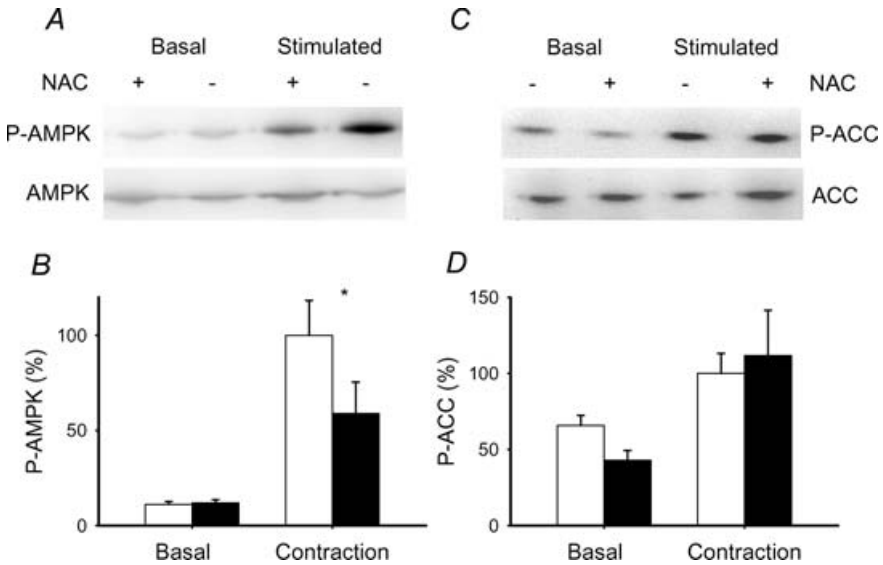

Figure 6. *N*-Acetylcysteine inhibits contraction-mediated phosphorylation of AMPK but not ACC in mouse EDL muscle
A, representative immunoblots of phosphorylated AMPK (P-AMPK) and total AMPK (AMPK). *B*, mean ± S.E.M. values for P-AMPK ($n = 4$). Open bars, control; filled bars, NAC (20 mM). The mean value of the stimulated controls is set to 100% and all the other values are expressed as a percentage of this value. Muscles were stimulated for 10 min with repeated contractions. $^{*}P < 0.05$ *versus* control values. *C*, representative immunoblots of phosphorylated ACC (P-ACC) and total ACC (ACC). *D*, mean ± S.E.M. values for P-ACC ($n = 4$). See *B* for additional details.

was diminished by ebselen by almost 60%, which is similar to the inhibition seen with NAC. Ebselen resulted in a small decrease in initial force (control, 15.6 ± 1.4 mN (mg wet weight)$^{-1}$; ebselen, 13.0 ± 0.9 mN (mg wet weight)$^{-1}$; $P < 0.05$). However, the fatigue profile was not significantly affected during the repeated contractions (Fig. 7*B*).

Overexpression of SOD2 did not significantly affect the basal rate of 2-DG uptake (Fig. 7*C*), probably owing to the low rate of ROS production in the basal state. In contrast, 2-DG uptake was significantly higher (∼25%) after a series of repeated contractions in SOD2 overexpressing *versus* wild-type muscles. This increase was not associated with alterations in force production (Fig. 7*D*). It might be argued that the increase in contraction-mediated 2-DG uptake is a consequence of an adaptive increase in the capacity of the glucose transport system and thus all interventions that result in Glut-4 translocation will result in higher values in SOD2 overexpressing muscles. We therefore stimulated 2-DG uptake with insulin. In contrast to the effect of SOD2 overexpression on contraction-mediated 2-DG uptake, insulin-mediated 2-DG uptake was not significantly altered (WT, 2.72 ± 0.31 μmol (20 min)$^{-1}$ (ml intracellular water)$^{-1}$; SOD2, 2.51 ± 0.24 μmol (20 min)$^{-1}$ (ml intracellular water)$^{-1}$; $n = 6$).

The data from the NAC, ebselen and SOD2 experiments are consistent with the idea that H_2O_2, or its derivatives, plays a significant in role in contraction-mediated glucose transport. In line with this idea, we determined that exogenous H_2O_2 also stimulates 2-DG uptake in mouse EDL muscles (basal, 0.82 ± 0.31 μmol (20 min)$^{-1}$ (ml intracellular water)$^{-1}$; in presence of 3 mM H_2O_2, 2.10 ± 0.31 μmol (20 min)$^{-1}$ (ml intracellular water)$^{-1}$; $P < 0.001$).

Discussion

The major findings of the present study are: (1) anti-oxidants diminish contraction-mediated 2-DG uptake,

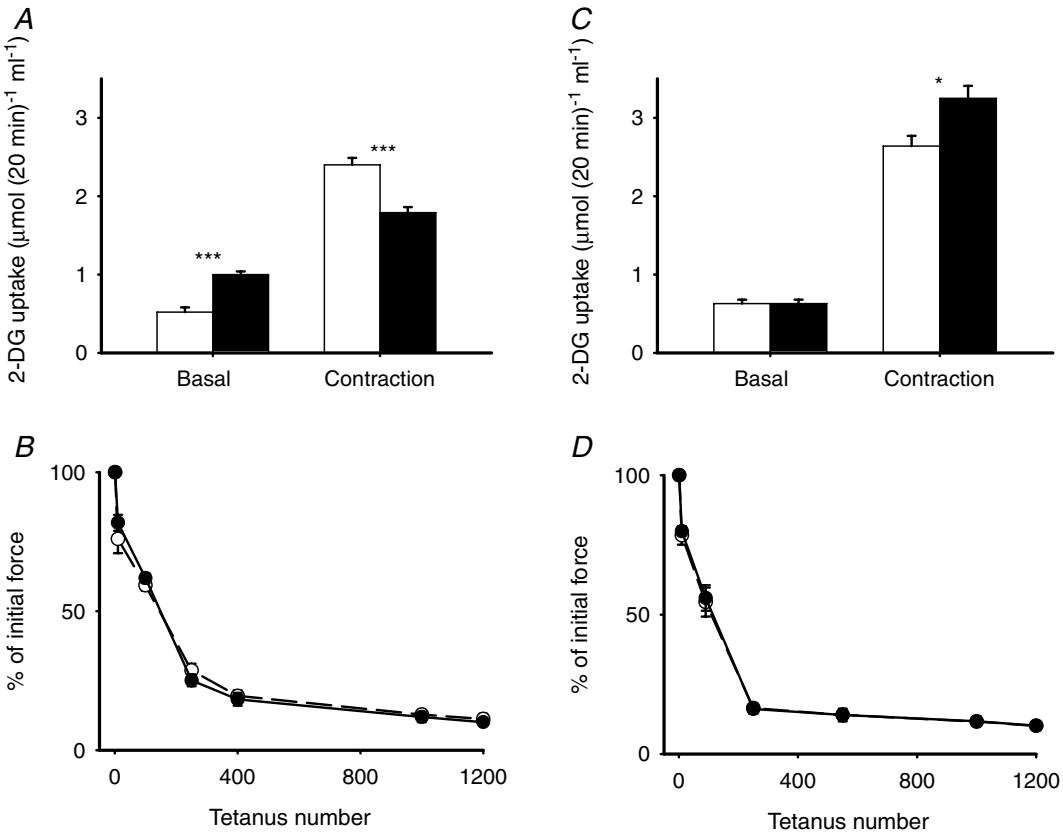

Figure 7. Ebselen inhibits and overexpression of SOD2 enhances contraction-mediated glucose uptake in mouse EDL muscle

Values are means ± S.E.M. for 6–9 muscles. *A*, open bars, control; filled bars, ebselen (30 μM). ***$P < 0.001$ *versus* control values. *B*, ebselen does not affect the decline in force during repeated contractions (○, control; ●, ebselen). *C*, open bars, wild type; filled bars, SOD2 overexpression. *$P < 0.05$ *versus* wild type. *D*, SOD2 overexpression does not affect force generation during repeated contractions (○, control; ●, SOD2 overexpression). Initial force averaged 14.9 ± 0.8 mN (mg wet weight)$^{-1}$ for wild type and 16.0 ± 1.3 mN (mg wet weight)$^{-1}$ for SOD2 overexpression (n.s.).

but not insulin-, hypoxia- or AICAR-mediated 2-DG uptake; (2) overexpression of SOD2 increases contraction-mediated 2-DG uptake; (3) NAC diminishes contraction-mediated activation and phosphorylation of AMPK; and (4) antioxidants diminish contraction-mediated 2-DG uptake and activation/phosphorylation of AMPK to the same relative extent. These findings support the idea that endogenous ROS production plays an important role in contraction-mediated glucose transport and that the ROS effect is mediated via an AMPK-dependent pathway.

While this study was in progress, it was reported that exogenous H_2O_2, as well as a superoxide-generating system, accelerated glucose transport in isolated resting rat epitrochlearis muscles by a mechanism that involved the activation of the $\alpha 1$ isoform of AMPK (Toyoda *et al.* 2004). Moreover, the effects of H_2O_2 and the superoxide-generating system on glucose transport and AMPK activity, as well as glutathione status, were partly blocked by NAC. These results are consistent with the findings in the present study, which indicate that endogenous ROS production is involved in activating glucose transport during contraction. Thus, of the various ROS components, H_2O_2, or its derivatives, is most likely to be the activating factor during contraction.

The possibility that reactive nitrogen species (RNS) also participate in contraction-mediated glucose transport was not addressed in the present study. Indeed, there is considerable evidence that RNS are produced during muscle contraction (Murrant & Reid, 2001). Specifically, increased NO production has been proposed to mediate contraction-stimulated glucose transport (Balon & Nadler, 1997; Roberts *et al.* 1997). However, other studies indicate that NO-mediated glucose transport occurs via a pathway that is distinct from the contraction-mediated pathway (Etgen *et al.* 1997; Higaki *et al.* 2001).

The idea that ROS are involved in contraction-mediated glucose transport may not be intuitive. This is because oxidative stress may play a role in insulin resistance and the pathogenesis of diabetes (Bonnefont-Rousselot, 2002; Houstis *et al.* 2006). Indeed, it is well known that excessive levels of ROS have deleterious effects on cell function and viability, whereas low/physiological levels of ROS are requisite for various signalling pathways and optimal cell function (Murrant & Reid, 2001; Goldstein *et al.* 2005). The present results implicating endogenously produced ROS in contraction-mediated glucose transport support the idea that a limited ROS production is requisite for normal physiological function.

An interesting finding was that NAC significantly blocked contraction- but not hypoxia-mediated glucose transport. These two stimuli are believed to activate glucose transport by similar but not identical pathways (Wojtaszewski *et al.* 1998). It is generally believed that mitochondria are the primary source of ROS produced in eukaryotic cells and that 2–5% of electron flux through the respiratory chain escapes to produce superoxide anions (Murrant & Reid, 2001). Thus an increase in mitochondrial respiration would be expected to result in a proportional increase in superoxide production. There is also evidence that ROS can be produced in extramitochondrial sites and under anaerobic conditions (Murrant & Reid, 2001; Zuo & Clanton, 2005). Our results are consistent with the idea that more ROS are produced during conditions associated with high rates of mitochondrial respiration.

The findings that NAC partly inhibited contraction-mediated activation of AMPK and that exogenous H_2O_2 activated AMPK raise the question of the mechanism whereby ROS results in AMPK activation. AMPK is usually considered to be activated by increases in AMP and decreases in ATP and phosphocreatine (PCr; Ponticos *et al.* 1998). While this manuscript was under review, evidence was presented from vascular endothelium cells that mitochondrially derived ROS activated AMPK by a mechanism independent of nucleotide concentrations (Quintero *et al.* 2006).

Incubation of isolated rat skeletal muscle with ROS (1 mM H_2O_2 or a superoxide-generating system) also did not result in significant changes in the total tissue AMP/ATP ratio (Toyoda *et al.* 2004). Similarly, perfusion of isolated rat hearts with 300 μM H_2O_2, which was sufficient to significantly activate AMPK, did not significantly alter

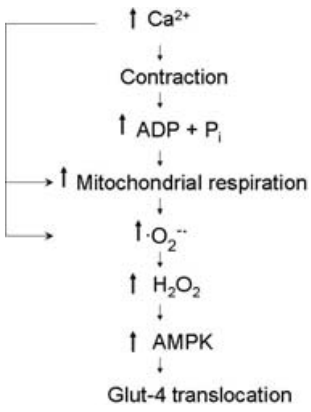

Figure 8. Scheme for ROS-mediated glucose transport during muscle contraction
Following the release of Ca^{2+} from the sarcoplasmic reticulum, actomyosin interaction occurs, resulting in muscle contraction, ATP breakdown and increases in ADP and inorganic phosphate (P_i). ADP and P_i stimulate mitochondrial respiration, which can also be stimulated by increases in Ca^{2+} that activate mitochondrial dehydrogenases. Increased respiration results in superoxide anion ($O_2^{\cdot -}$) formation through NADH dehydrogenase and semiquinone components; $O_2^{\cdot -}$ formation can also occur by extramitochondrial mechanisms (e.g. via a Ca^{2+}-mediated activation of phospholipase A2). Superoxide anion is then dismutated by superoxide dismutase to H_2O_2, which results in increased LKB1-mediated phosphorylation and activation of AMPK, followed by a translocation of Glut-4 to the surface membrane.

the AMP/ATP ratio (Leon *et al.* 2004). Furthermore, if oxidants induce decreases in high-energy phosphates, then antioxidants should block such decreases. However, antioxidants do not affect the changes in high-energy phosphates (ATP, PCr and inorganic phosphate) that occur in the isolated rat diaphragm during repeated contractions, hypoxia or contractions in the presence of hypoxia (Wright *et al.* 2005). Consistent with these data is the present finding that NAC does not interfere with AMP-mediated activation of AMPK (AICAR experiment).

It was recently demonstrated that an important upstream kinase in the AMPK cascade is LKB1 and, based on current evidence, LKB1-mediated phosphorylation and activation of AMPK are not sensitive to AMP. Moreover, blocking LKB1 activity resulted in an inability of H_2O_2 to activate AMPK (Woods *et al.* 2003). We therefore suggest that the activation of AMPK by endogenously produced ROS during contraction does not occur via alterations in high-energy phosphates. Other modes of activation of AMPK that are independent of changes in adenine nucleotides include hyperosmotic shock and exposure to metformin (Fryer *et al.* 2002). Thus, it appears that contraction-mediated activation of AMPK can occur by at least two mechanisms: one that is NAC insensitive and involves changes in high-energy phosphates and one that is NAC sensitive and independent of changes in high-energy phosphates.

A scheme describing the steps whereby we suggest that endogenously produced ROS result in the activation of AMPK and glucose transport is provided in Fig. 8. The scheme indicates that Ca^{2+}, in addition to initiating contraction, can participate in ROS production at different sites in the cell. Hydrogen peroxide-mediated activation of AMPK probably occurs via LKB1 (see above). However, previously presented data indicate that H_2O_2 does not directly activate LKB1 (Woods *et al.* 2003). Rather, it appears that H_2O_2 enhances the substrate suitability of AMPK for LKB1.

Another interesting finding was that the contraction-mediated phosphorylation of ACC was not affected by NAC. Acetyl CoA carboxylase is a substrate for AMPK and therefore ACC phosphorylation during contraction is often considered to reflect increased AMPK activity, since the increase in the phoshorylation state of the two proteins is well correlated during contraction (Park *et al.* 2002). The dissociation between the changes in AMPK and ACC phosphorylation following contraction in the presence of NAC is not the first evidence for such dissociation during contraction. Recently, it was demonstrated in skeletal muscle from adenylate kinase-deficient mice (which lack the ability to produce AMP) that repeated contractions did not result in phosphorylation of AMPK, whereas phosphorylation of ACC was comparable to that of wild-type mice (Hancock *et al.* 2006). Other instances of dissociation between ACC and AMPK phosphorylation have also been observed (see Hancock *et al.* 2006 for references). Thus, factors other than AMPK can control ACC phosphorylation during contraction and these factors are not NAC sensitive.

A complete understanding of the mechanisms involved in contraction-mediated glucose transport is still lacking. To our knowledge, this study provides the first evidence in support of the idea that endogenously produced ROS play a significant role in contraction-mediated glucose transport in fast-twitch muscle.

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