Local hindlimb antioxidant infusion does not affect muscle glucose uptake during in situ contractions in rat

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Merry TL, Dywer RM, Bradley EA, Rattigan S, McConnell GK. Local hindlimb antioxidant infusion does not affect muscle glucose uptake during in situ contractions in rat. J Appl Physiol 108: 1275–1283, 2010. First published March 4, 2010; doi:10.1152/japplphysiol.01335.2009.—There is evidence that reactive oxygen species (ROS) contribute to the regulation of skeletal muscle glucose uptake during highly fatiguing exercise conditions (5). In this study we investigated the role of ROS in the regulation of glucose uptake and AMP-activated protein kinase (AMPK). Male hooded Wistar rats were anesthetized, and then more physiological protocol and preparation. Male hooded Wistar rats were anesthetized, and then N-acetylcyesteine (NAC) was infused into the epigastric artery (125 mg·kg−1·h−1) of one hindlimb (contracted leg) for 15 min before this leg was electrically stimulated (0.1-ms impulse at 2 Hz and 35 V) to contract at a low-moderate intensity for 15 min. The contralateral leg did not receive stimulation or local NAC infusion (rest leg). NAC infusion increased (P < 0.05) plasma cysteine and cystine (by ∼360- and 1.4-fold, respectively) and muscle cysteine (by 1.5-fold, P = 0.001). Although contraction did not significantly alter muscle tyrosine nitration, reduced (GSH) or oxidized glutathione (GSSG) content, S-glutathionylation of protein bands at ∼250 and 150 kDa was increased (P ∼1.7-fold by contraction, and this increase was prevented by NAC. Contraction increased (P < 0.05) skeletal muscle glucose uptake 20-fold, AMPK phosphorylation 6-fold, ACC phosphorylation 10-fold, and p38 MAPK phosphorylation 60-fold, and the muscle fatigued by ∼30% during contraction and NAC infusion had no significant effect on any of these responses. This was despite NAC preventing increases in S-glutathionylation with contraction. In conclusion, unlike during highly fatiguing exercise, local NAC infusion during in situ low-moderate intensity hindlimb contractions in rats, a more physiological preparation, does not attenuate increases in skeletal muscle glucose uptake or AMPK signaling.

exercise; metabolism; S-glutathionylation; reactive oxygen species; AMP-activated protein kinase

WHOLE BODY GLUCOSE HOMEOSTASIS is largely dependent on the transport of glucose into skeletal muscle cells (5). Insulin and contraction both regulate skeletal muscle glucose uptake by signaling the translocation of the glucose transporter protein GLUT4 to the cell surface, which facilitates the transport of glucose through the cell membrane into the cell (5). However, insulin and contraction increase skeletal muscle glucose uptake through different signaling pathways (13, 49, 51). The pathway through which contraction signals glucose uptake is not yet fully elucidated (25) but may include discrete or integrated signaling via calcium/calmodulin-dependent protein kinase (CaMK) (48), AMP-activated protein kinase (AMPK) (13), and nitric oxide (NO) (4, 37).

Although chronic elevation in oxidative stress (a pro-oxidant shift in cell redox status) is associated with the pathophysiology of type 2 diabetes and insulin resistance (21), acute treatment of isolated skeletal muscle with exogenous ROS stimulates insulin-independent glucose uptake (14, 16). Acute increases in skeletal muscle ROS production occur during in vivo exercise (11, 24, 40) and during ex vivo contractions (34, 39). Sandstrom et al. (39) have shown that the treatment of isolated skeletal muscle ex vivo with the antioxidant N-acetylcyesteine (NAC) attenuates both the contraction-stimulated increase in oxidative stress and increases in glucose uptake. Thus there is evidence that acute increases in ROS are involved in the signaling of skeletal muscle glucose uptake during ex vivo contractions. Interestingly, Sandstrom et al. (39) also reported that, similar to glucose uptake, NAC attenuates the contraction-stimulated increases in skeletal muscle AMPK activity. Furthermore, antioxidant supplementation (allopurinol) has been shown to prevent increases in p38 MAPK phosphorylation during exhaustive exercise in rats (11), and p38 MAPK has been implicated in the regulation of contraction and stretch-stimulated skeletal muscle glucose uptake (6, 42). This suggests that during contraction ROS may regulate skeletal muscle glucose uptake via the activation of AMPK and/or p38 MAPK.

Although ROS appear to be essential for normal increases in skeletal muscle glucose uptake and AMPK signaling during ex vivo contractions (39), it remains to be determined whether ROS are involved in regulating contraction-stimulated glucose uptake and AMPK signaling using intact and more physiological models. This is important because ex vivo models rely on diffusion gradients for substrate delivery and clearance (1), unlike in vivo exercise where muscle and capillary blood flow are involved in the regulation of skeletal muscle glucose uptake (31, 47). Furthermore, unlike in vivo, ex vivo models of muscle contraction generally involve nonuniform delivery of oxygen to all muscle fibers, an absence of antioxidant systems surrounding muscle (such as that supplied by blood flow), and supramaximal highly fatiguing stimulation protocols (1), which are all likely to artificially inflate oxidative stress and alter ROS signaling (32). As such, it may be that during these highly fatiguing ex vivo contraction conditions, ROS preferentially regulate skeletal muscle glucose uptake and AMPK activity.

Therefore, in this study, we determined whether local infusion of the antioxidant NAC attenuates the increase in hindlimb skeletal muscle glucose uptake and AMPK signaling during physiologically relevant low-moderate intensity contractions in situ in rats. We hypothesized that low-moderate intensity contractions in situ would increase skeletal muscle glucose uptake more physiological protocol and preparation. Male hooded Wistar rats were anesthetized, and then N-acetylcyesteine (NAC) was infused into the epigastric artery (125 mg·kg−1·h−1) of one hindlimb (contracted leg) for 15 min before this leg was electrically stimulated (0.1-ms impulse at 2 Hz and 35 V) to contract at a low-moderate intensity for 15 min. The contralateral leg did not receive stimulation or local NAC infusion (rest leg). NAC infusion increased (P < 0.05) plasma cysteine and cystine (by ∼360- and 1.4-fold, respectively) and muscle cysteine (by 1.5-fold, P = 0.001). Although contraction did not significantly alter muscle tyrosine nitration, reduced (GSH) or oxidized glutathione (GSSG) content, S-glutathionylation of protein bands at ∼250 and 150 kDa was increased (P ∼1.7-fold by contraction, and this increase was prevented by NAC. Contraction increased (P < 0.05) skeletal muscle glucose uptake 20-fold, AMPK phosphorylation 6-fold, ACC phosphorylation 10-fold, and p38 MAPK phosphorylation 60-fold, and the muscle fatigued by ∼30% during contraction and NAC infusion had no significant effect on any of these responses. This was despite NAC preventing increases in S-glutathionylation with contraction. In conclusion, unlike during highly fatiguing exercise, local NAC infusion during in situ low-moderate intensity hindlimb contractions in rats, a more physiological preparation, does not attenuate increases in skeletal muscle glucose uptake or AMPK signaling.

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uptake and AMPK signaling and that NAC infusion would attenuate these increases.

RESEARCH DESIGN AND METHODS

Animals

Male hooded Wistar rats weighing 238 ± 2 g and ∼8 wk of age were maintained in an environmentally controlled room at 21°C with 12:12-h light-dark cycle at the University of Tasmania. Rats were given ad libitum access to standard rodent chow and water. The present study was approved by the University of Tasmania Ethics Committee and conformed to the guidelines for the care and use of experimental animals, as described by the National Health and Medical Research Council (Australia).

Materials and Antibodies

Unless otherwise stated all chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). 2-Deoxy-d-[1-14C]glucose (2DG; specific activity 1.92 TBq/mmol) was purchased from Amersham life science (NSW, Australia). Primary antibodies for AMPKα, p38 MAPK, α-tubulin, and anti-phospho-p38 MAPK Thr180/Tyr182 were purchased from Cell Signaling Technology (Hartfordshire, UK), and 3-nitrotyrosine from Chemicon. Anti-phospho-ACCβ Ser272 and anti-phospho-AMPK Thr172 were purchased from Upstate Biotechnology, and anti-glutathione was purchased from Abcam (Cambridge, UK). IRDye 800-conjugated anti-mouse and anti-rabbit IgG were purchased from Rockland (Gilbertsville, PA).

Experimental Procedure

An anesthetized rat model was utilized in this study as described previously (30, 37, 47). Briefly, rats were anesthetized using pentobarbital sodium (1.5 μl/g body wt) and cannulas were inserted into the carotid artery and jugular veins for arterial sampling and continuous administration of anesthetic, respectively. Isotonic saline (154 mmol/l NaCl) containing NAC (125 mg·kg⁻¹·h⁻¹) or isotonic saline alone was infused locally (at 1:100 of the arterial flow rate) into one hindlimb (contracted leg) via the epigastric artery. Sham surgery was performed on the contralateral leg (rest). NAC is a nonspecific antioxidant that directly scavenge ROS (2) and is deacetylated to cysteine, which promotes the resynthesis of reduced glutathione (GSH) (8, 41). The systemic infusion of NAC at 125 mg·kg⁻¹·h⁻¹ has been shown previously to elevate muscle NAC, cysteine, and total GSH during exercise in humans (24). After a 15-min preinfusion, the contracted leg was electrically stimulated to contract (0.1-ms impulse at 2 Hz and 35 V) for 15 min while NAC infusion continued. The knee was secured by the tibiotarillar ligament, and the Achilles tendon was attached to a Harvard Apparatus isometric transducer, thereby allowing measurement of tension development from the gastrocnemius-plantaris-soleus muscle group during contraction. It has previously been shown that during highly fatiguing contractions ex vivo, ROS are involved in the regulation of glucose uptake during contraction (39). Therefore this contraction protocol was chosen because we have previously shown it to substantially increase muscle glucose uptake and metabolic signaling while causing only a physiological level of fatigue (37). At 10 min before the completion of the experiment (from t = 20 to 30 min), a 1.85-MBq bolus of 2DG in isotonic saline was administered via the right jugular vein. Immediately following the 2DG bolus, an arterial blood sample (0.5 ml) was withdrawn by an automated syringe pump at 50 μl/min over 10 min. From this blood sample a plasma sample (25 μl) was collected to determine the average plasma specific radioactivity of 2DG. At t = 30 min the lower leg muscles (soleus, plantaris, gastrocnemius red, and gastrocnemius white) from the contracted and contralateral (rest) leg were rapidly dissected and freeze-clamped using liquid nitrogen-cooled tongs. Throughout the experimental protocol, heart rate (HR), mean arterial pressure (MAP), and femoral blood flow were monitored and recorded as described previously (30, 47).

Muscle Glucose Uptake

The lower leg muscles (soleus, plantaris, gastrocnemius red, and gastrocnemius white) were ground under liquid nitrogen, and 100 mg was homogenized with 1.5 ml water before free and phosphorylated glucose were separated by ion-exchange chromatography using anion-exchange resin (AG1-X8; Bio-Rad, Hercules, CA). Inorganic liquid scintillation cocktail (Amersham Life Science) was added to samples, and radioactivity was measured by a β-scintillation counter (Packard TriCarb 2900TR, Perkin-Elmer, Boston, MA). Based on plasma glucose, muscle, and plasma 2DG concentrations, muscle 2DG glucose uptake (R’g) was calculated as follows:

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R'g = \frac{\text{muscle } ^{3}H \text{2DG6-P (dpm/g) } \times \text{average plasma } ^{3}H \text{2DG (dpm/ml) } \times 10 \text{ (min)}}{\text{plasma } \text{glucose (μg/ml)}}
\]

where 2DG6-P is 2-deoxy-d-glucose-6-phosphate.

NAC and Thiols

Muscle preparation. Thirty milligrams of ground muscle sample was rapidly homogenized on ice in 300 μl of 0.42 M PCA; immediately following homogenization PCA was neutralized with 40 μl of 2.5 M K₂CO₃. Samples were then centrifuged at 13,000 g for 5 min at 4°C and the supernatant was recovered.

HPLC method for plasma and muscle sample. For analysis of reduced thiols (reduced NAC, GSH, and cysteine), 10 μl of distilled H₂O was added to 50 μl of sample, and for the determination of total thiols (TNAC, TGSH, and total cysteine) 10 μl of tributylphosphine solution diluted 1:10 was added to 50 μl of sample to oxidize reduced thiols. All samples were then incubated for 30 min on ice and 25 μl of 4-fluoro-7-sulfamoylbenzofurazan (ABF-5; 5 mg/ml in borate buffer; 0.2 M boric acid, 2 mM sodium EDTA, pH 8.0) was added. Following 10 min incubation at 50°C, 10 μl of 2 M PCA was added and samples were centrifuged at 13,000 g for 5 min. A 40-μl aliquot of the supernatant was then injected onto a reverse-phase HPLC Gemini column (5 μm C₁₈, 110 Å, Phenomenex) with 0.1 M sodium acetate buffer (pH 4.0) in 10% methanol as a mobile phase, at a flow rate of 1.5 ml/min and detection wavelength of 386 nm excitation and 516 nm emission. All HPLC values obtained for thiols were measured against standards. Oxidized thiols (NAC, GSSG, cysteine) were calculated from the difference between the measured reduced and total thiols.

Muscle Signaling

Three-hundred milligrams of ground muscle was homogenized (10 μl/mg tissue) in ice-cold lysis buffer (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). Lysates were then incubated for 20 min on ice and centrifuged at 13,000 g for 20 min at 4°C. For analysis of S-glutathionylation, a marker of oxidative stress (9), muscle was extracted under nonreducing conditions with lysis and sample buffer (1.5 M Tris-HCl, pH 6.8, 30% glycerol, 10% SDS, 0.6
MDTT, 0.0012% bromophenol blue) containing no DTT, and 5 mM and 10 mM of N-ethylmaleimide, respectively, to alkylate free thiol groups. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) with BSA as the standard. Total protein was diluted 1:3 in sample buffer, heated for 10 min at 100°C (with the exception of samples used for 3-nitrotyrosine analysis, which were heated at 37°C for 10 min), and stored at −20°C before 80 μg of total protein was separated by SDS-PAGE, transferred to PVDF membrane, and blocked in PBS containing 5% nonfat milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibody for glutathione and phosphorylation-specific primary antibodies for ACCβ Ser222 and p38 MAPK Thr180/Tyr182 before binding was detected with rabbit IgG secondary antibody. Direct fluorescence was detected and quantified using the Odyssey infrared imaging system (LICOR Biosciences, Lincoln, NE). Membranes were then stripped [2% SDS (wt/vol) in 25 mM glycine, pH 2.0] and reprobed with primary antibodies for ACCβ (streptavidin) and p38 MAPK 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 α-tubulin.

Statistical Analysis

All data are expressed as means ± SE. Results were analyzed by SPSS statistical package using two-factor ANOVA as well as two-factor repeated-measures ANOVA. Because NAC infusion started precontraction, the repeated-measures ANOVAs were partitioned to assess the effect of NAC at rest (0–15 min) and during exercise (15–30 min). If the ANOVA revealed a significant interaction, specific differences between mean values were located using the Fisher’s least significance difference test. The level of significance was set at P < 0.05.

RESULTS

Force Development

Peak contraction force decreased by ~30% by the end of the 15-min contraction period (P < 0.001; Fig. 1). Local NAC infusion did not affect initial peak contraction force (P = 0.56) or the rate of fatigue (P = 0.65, Fig. 1).

Plasma and Muscle NAC

Local NAC infusion resulted in plasma NAC and reduced NAC concentration of 19.1 ± 2.6 and 3.1 ± 1.1 μM, respectively (Fig. 2A). Local NAC infusion increased muscle NAC in the contracted leg to a greater extent than the rest leg (P = 0.03, Fig. 2B). Similar concentrations of reduced NAC were found in the muscle of the rest and contracted legs (P = 0.16; Fig. 2B). NAC was not detected in the plasma or muscle during saline infusion (data not shown).

Heart Rate and Blood Pressure

Local NAC infusion did not affect resting heart rate (325 ± 38 beats/min). Heart rate was only measured during the first 5 min of contraction and was not increased significantly from rest at this point (data not shown). Resting MAP was not affected by local NAC infusion (Fig. 3A); however, local NAC infusion attenuated the contraction-induced increase in MAP by ~12 mmHg at t = 20 min (P < 0.05, Fig. 3A), suggesting some systemic affects of NAC infusion.

Leg Blood Flow and Vascular Resistance

Leg blood flow remained unchanged during the precontraction infusion period (Fig. 3B). Contraction increased femoral
blood flow to the contracted leg by ~400% \((P < 0.001; \text{Fig. 3B})\). Local NAC infusion did not affect the contraction-induced increase in leg blood flow \((P = 0.78; \text{Fig. 3B})\). Precontraction vascular resistance was reduced in NAC rest leg compared with saline infused rest leg \((P = 0.02, \text{Fig. 3C})\); however, resting vascular resistance was not affected by local NAC infusion in contracted leg \((P = 0.19, \text{Fig. 3C})\). During contraction, vascular resistance was increased in the rest leg, due to sympathetic outflow contributing to blood flow redistribution \((45)\), and reduced in the contracted leg \((P < 0.05, \text{Fig. 3C})\). Although NAC infusion appeared to attenuate \((\sim 60\% \text{ at } t = 20 \text{ min})\) vascular resistance in the resting leg during contraction, NAC did not significantly alter vascular resistance during contraction \((P = 0.09 \text{ for interaction})\).

**Muscle and Plasma Thiols**

Muscle glutathione levels were measured as marker of cellular oxidative state \((29)\) since under conditions of oxidative stress reduced glutathione \((\text{GSH})\) is more rapidly oxidized to oxidized glutathione \((\text{GSSG})\). Figure 4, \(A, C, \) and \(E\), shows that muscle GSH, GSSG, and GSSG/GSH ratio were not significantly affected by contraction or local NAC infusion. NAC is rapidly decylated to produce cysteine \((10)\), and like NAC, cysteine can directly scavenge ROS \((2, 8)\). Therefore, cysteine levels were measured as a marker of NAC-enhanced antioxidant defenses. Local NAC infusion increased muscle cysteine similarly by 50% in the rest and contracted leg \((P = 0.001, \text{Fig. 4B})\). There was a tendency for local NAC infusion to increase muscle cysteine \((P = 0.07)\), and NAC infusion tended to increase the affect of contraction on muscle cysteine \((P = 0.08; \text{Fig. 4D})\). Contraction did not affect muscle cysteine or cystine concentrations \((\text{Fig. 4, B and D})\). Local NAC infusion increased \((P < 0.05)\) plasma cysteine and cystine 360- and 1.4-fold, respectively \((\text{Fig. 4F})\).

**Muscle Glucose Uptake**

The contracted leg had a muscle glucose uptake ~20-fold greater than the rest leg \((P < 0.001; \text{Fig. 5A})\). Local NAC infusion did not affect muscle glucose uptake in the rest or in the contracted leg \((P > 0.05, \text{Fig. 5A})\).

**Muscle S-Glutathionylation and Tyrosine Nitration**

Contraction significantly \((P < 0.05)\) increased muscle S-glutathionylation of protein bands at ~250 and 150 kDa in the contracted saline infused leg ~1.7-fold, and NAC infusion prevented this increase \((P < 0.05; \text{Fig. 5B})\). Neither contraction nor NAC affected protein S-glutathionylation of any other visible protein bands. Muscle tyrosine nitration was not significantly affected by contraction or NAC infusion \((\text{Fig. 5C})\).

**AMPKα, ACCβ, and p38 MAPK Phosphorylation**

Contraction increased \((P < 0.05)\) phosphorylation of AMPK \((6\text{-fold})\) and ACCβ \((10\text{-fold})\) \((\text{Fig. 6, A and B})\), and this increase was not affected by local NAC infusion \((\text{Fig. 6, A and B})\). Similarly, contraction increased \((P = 0.002)\) p38 MAPK phosphorylation \((\sim 60\text{-fold})\), and this increase was not affected by local NAC infusion \((P > 0.05; \text{Fig. 6C})\).

**DISCUSSION**

The major finding of this study was that local infusion of the antioxidant NAC does not attenuate the increase in hindlimb skeletal muscle glucose uptake or AMPK signaling during in situ contractions in rats. Although our low-moderate stimulation protocol did not affect muscle tyrosine nitration, GSH, or GSSG contents, it significantly increased S-glutathionylation of protein bands at ~250 and 150 kDa, and these increases in
glutathionylation were prevented by NAC infusion. Therefore, because our low-moderate contraction protocol also substantially increased skeletal muscle glucose uptake and metabolic signaling, and resulted in a physiological degree of fatigue, this study provides evidence to suggest ROS are not essential for the regulation of skeletal muscle glucose uptake or AMPK signaling during low-moderate intensity contractions in situ.

Surprisingly, and in contrast to several studies in humans and rats (11, 24, 40) we were unable to detect a change in muscle GSH or GSSG following contraction. Interestingly, however, we found that S-glutathionylation of protein bands at ~250 and 150 kDa was increased during contraction. We are currently conducting experiments to determine the nature of these proteins. S-glutathionylation is the addition of glutathione to protein cysteine residues, which is enhanced during times of nitrosative and oxidative stress (9). Although contraction increases nitrosative stress (3, 37), the increase in S-glutathionylation during contraction was likely the result of oxidative stress because NAC infusion abolished these increases. This suggests that ROS production increased during our contraction protocol causing a small oxidative shift in cell redox that was not large enough to substantially deplete antioxidant defenses and therefore alter glutathione levels, but sufficient to increase S-glutathionylation, which NAC prevented.

It is likely that no measurable changes in muscle glutathione status were seen in this study because of the low-moderate intensity (0.1-ms impulse at 2 Hz and 35 V), but physiologically relevant, short-duration (15 min) stimulation protocol we employed. In support, previously Sahlin et al. (38) has shown that muscle glutathione levels are not affected by submaximal short-duration (20 min) dynamic exercise in humans. Like glutathione status, muscle tyrosine nitration was also not affected by the low-moderate intensity contraction protocol (Fig. 5B). Nitric oxide and superoxide interact to form peroxynitrite, which at high concentrations promotes protein tyrosine nitration (12). Tyrosine nitration is generally associated with deleterious inhibitory effects on muscle signaling (28), and therefore it is not surprising that if any peroxynitrite was produced during contraction it did not increase tyrosine nitration. It is, however, important to acknowledge that the stimulation intensity employed is physiologically comparable to submaximal exercise in humans as it substantially increases muscle glucose uptake (Fig. 5) and metabolic signaling (Fig. 6) without being highly fatiguing (Fig. 1). Conversely, the majority of those studies reporting a reduction in muscle GSH or an increase in GSSG/GSH ratio following contraction either employ exercise...
to exhaustion (11, 40) or prolonged high to strenuous exercise (70% peak oxygen consumption) (24, 44, 52), which results in greater increases in ROS production and depletion of antioxidant defenses (17, 27, 33). It is during such exercise that NAC can attenuate muscle GSH depletion (24, 43) by promoting its resynthesis (8, 41). Therefore, because our contraction protocol did not deplete GSH, there was no requirement for increased GSH resynthesis and thus NAC did not affect muscle GSH/GSSG content. However, NAC prevented exercise-stimu-

Fig. 5. Effect of local NAC or saline infusion on rest and contracted leg muscle glucose uptake (A), S-glutathionylation (B), and tyrosine nitration (C) following 15 min of in situ hindlimb contractions in the contacted leg of rats. Data are means ± SE; n = 6 per group. #P < 0.05 for condition (rest vs. contraction). †P < 0.05 vs. saline of same condition. ‡P < 0.05 vs. rest, of same treatment.

Fig. 6. Effect of local NAC or saline infusion on rest and contracted leg AMPK Thr172 (A), ACCβ Ser279 (B), and p38 MAPK Thr180/Tyr182 (C) phosphorylation following 15 min of in situ hindlimb contractions in the contacted leg of rats. Data are means ± SE; n = 6 per group. #P < 0.05 for condition (rest vs. contraction).
lated muscle S-glutathionylation, providing evidence that it was having GSH-independent antioxidant effects in the muscle (2, 8).

It has been previously reported that the treatment of isolated mouse extensor digitorum longus (EDL) muscles with NAC attenuates increases in ex vivo contraction-induced oxidative stress and glucose uptake (39). The authors propose that because NAC also inhibited AMPK phosphorylation and activity, ROS activate AMPK during contraction and this contributes to the activation of skeletal muscle glucose uptake during ex vivo contractions (18, 39). Indeed, we have also found that NAC attenuates increases in glucose uptake during ex vivo contractions in mouse EDL and soleus muscles (26). However, here we report that local NAC infusion did not affect the increase in skeletal muscle glucose uptake, AMPK phosphorylation, or the phosphorylation of the major downstream target of AMPK, ACCβ during in situ contractions (Fig. 5 and 6). This suggests that the low levels of oxidative stress associated with physiologically relevant contraction intensities is not involved in the regulation of skeletal muscle glucose uptake or AMPK signaling.

It has been proposed that muscle glucose uptake during contraction is differentially regulated in muscles containing predominantly oxidative and predominantly glycolytic type fibers (50). The hindlimb muscle sample was estimated to contain only a small proportion of oxidative type fibers (~14%) (22). It is likely that ROS would play a greater signaling role in glycolytic fibers because they have lower levels of endogenous antioxidant enzymes (23). Furthermore, ROS are involved in the regulating contraction-stimulated glucose uptake in muscle containing predominantly glycolytic and oxidative fibers ex vivo (26, 39). Therefore, the finding that NAC infusion did not affect hindlimb muscle glucose uptake during contraction is unlikely to be a result of fiber-type composition of the sampled muscle. Alternatively, however, it is possible that the NAC-derived increases in muscle antioxidant defenses [NAC and cysteine content (2, 8)] were insufficient to prevent all ROS signaling, and therefore the signaling of glucose uptake during contraction.

Interestingly, NAC had a small but significant affect on blood pressure during contraction. This suggests that NAC or ROS may have been exerting some systemic affects during contraction, which is worthy of further investigation. However, it is unlikely that these small systemic effects can account for the finding that ROS are not involved in the regulation of skeletal muscle glucose uptake during low-moderate intensity in situ contractions. It is possible, however, that during exhaustive endurance exercise, or under the normal ex vivo conditions of supramaximal stimulation, nonuniform oxygen delivery (due to oxygen diffusion limitations), and reduced antioxidant capacity (in the absence of antioxidant systems found in blood), oxidative stress would be greatly elevated (33) and play a role in the regulation of skeletal muscle glucose uptake via the activation of AMPK. Indeed it is during high-intensity exercise, which substantially elevates ROS production (32), when glucose uptake (36) and AMPK activation (7) are greatest, and it is only at high concentrations that exogenous ROS (H2O2; 3 mM) activate AMPK (14, 16, 46). Therefore, experimental investigation is required to assess the role of ROS and oxidative stress in signaling glucose uptake and AMPK activity during high-intensity exercise in intact preparations with blood flow. However, the contraction intensity required to increase skeletal muscle ROS levels to a large enough extent to activate AMPK and increase glucose uptake as seen in ex vivo preparations (39) is not likely to be physiologically realistic to humans. Therefore, the hypothesis that ROS regulate skeletal muscle glucose uptake during contraction may be an artifact of nonphysiological ex vivo contraction conditions, and our results suggest that mechanisms other than ROS regulate skeletal muscle glucose uptake during normal submaximal muscle contractions.

It has been shown that the phosphorylation of p38 MAPK is increased by exogenous ROS (19) and that the attenuation of exercise-induced oxidative stress by the xanthine oxidase inhibitor, allopurinol, attenuates increases in p38 MAPK phosphorylation during exhaustive exercise (11). Indeed, there is some evidence that the inhibition of p38 MAPK attenuates glucose uptake during contraction (42), and ROS signaling via p38 MAPK may be involved in regulating skeletal muscle stretch-induced glucose uptake (6). However, we show here that contraction can increase skeletal muscle p38 MAPK phosphorylation without significant alterations in the GSSG/GSH ratio, and that local NAC infusion, which prevented S-glutathionylation, does not affect p38 MAPK phosphorylation during contraction. This suggests that during low-moderate intensity contractions, ROS are not involved in the regulation of p38 MAPK signaling. The inconsistency between the results of Gomez-Cabrera et al. (11) and ours may be related to the use of a xanthine oxidase-specific inhibition, rather than the use of a general antioxidant such as NAC, and requires further investigation. Furthermore, the role of p38 MAPK in regulating skeletal muscle glucose uptake during contraction, and potential interactions with ROS at high exercise intensities, is worthy of further investigation since some isoforms of p38 MAPK appear to play a greater role in regulating glucose uptake than others (15, 42).

A small oxidative shift in cell redox is required for optimal skeletal muscle contraction force; however, high levels of oxidative stress appear to contribute to the development of muscular fatigue (33). NAC treatment has been shown to attenuate fatigue during longer duration and/or more strenuous exercise/muscle contractions (20, 35) than used in the present study by attenuating the exercise-induced increase in oxidative stress (24, 43). Since our contraction protocol did not alter skeletal muscle glutathione levels, this suggests that the level of oxidative stress was not sufficient to negatively impact on muscle function and therefore may explain why NAC did not affect the rate of fatigue during contraction (Fig. 1).

In conclusion, this study shows for the first time that local infusion of the antioxidant NAC during in situ hindlimb contractions in rats does not attenuate increases in skeletal muscle glucose uptake or AMPK signaling. Although our low-moderate intensity in situ muscle contraction protocol, which is more relevant to normal submaximal exercise than tetanic contractions associated with ex vivo studies, did not alter muscle GSH/GSSG levels or tyrosine nitration, it did increase protein S-glutathionylation, indicating small increases in muscle oxidative stress. NAC prevented the increases in S-glutathionylation during contraction but did not affect the large increases in skeletal muscle glucose uptake (20-fold) or phosphorylation of AMPK and p38 MAPK. These results suggest that, unlike during highly fatiguing ex vivo contraction conditions, ROS do not regulate skeletal muscle glucose uptake or metabolic sig-
naling during physiologically relevant, low-fatiguing, skeletal muscle contractions in situ in rat.

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