Downstream mechanisms of nitric oxide-mediated skeletal muscle glucose uptake during contraction

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Merry TL, Lynch GS, McConell GK. Downstream mechanisms of nitric oxide-mediated skeletal muscle glucose uptake during contraction. Am J Physiol Regul Integr Comp Physiol 299: R1656–R1665, 2010. First published October 13, 2010; doi:10.1152/ajpregu.00433.2010.—There is evidence that nitric oxide (NO) is required for the normal increases in skeletal muscle glucose uptake during contraction, but the mechanisms involved have not been elucidated. We examined whether NO regulates glucose uptake during skeletal muscle contractions via cGMP-dependent or cGMP-independent pathways. Isolated extensor digitorum longus (EDL) muscles from mice were stimulated to contract ex vivo, and potential NO signaling pathways were blocked by the addition of inhibitors to the incubation medium. Contraction increased (P < 0.05) NO synthase (NOS) activity (≈40%) and dichlorofluorescein (DCF) fluorescence (a marker of oxidant levels; −95%), which was prevented with a NOS inhibitor Nω-monomethyl-L-arginine (L-NMMA), and antioxidants [nonspecific antioxidant, N-acetylcysteine (NAC); thiol-reducing agent, DTT], respectively. L-NMMA and NAC both attenuated glucose uptake during contraction by −50% (P < 0.05), and their effects were not additive. Neither the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, which prevents the formation of cGMP, the cGMP-dependent protein (PKG) inhibitor Rp-8-bromo-β-phenyl-1,N2-ethenoguanosine 3’,5’-cyclic monophosphorothioate sodium salt nor white light, which breaks S-nitrosylated bonds, affects glucose uptake during contraction; however, DTT attenuated (P < 0.05) contraction-stimulated glucose uptake (by 70%). NOS inhibition and antioxidant treatment reduced contraction-stimulated increases in protein S-glutathionylation and tyrosine nitration (P < 0.05), without affecting AMPK or p38 MAPK phosphorylation. In conclusion, we provide evidence to suggest that NO and ROS are highly interrelated molecules (9), and their effects were not additive. Neither L-NMMA and NAC both attenuated glucose uptake during contraction by −50% (P < 0.05), and their effects were not additive. Neither the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, which prevents the formation of cGMP, the cGMP-dependent protein (PKG) inhibitor Rp-8-bromo-β-phenyl-1,N2-ethenoguanosine 3’,5’-cyclic monophosphorothioate sodium salt nor white light, which breaks S-nitrosylated bonds, affects glucose uptake during contraction; however, DTT attenuated (P < 0.05) contraction-stimulated glucose uptake (by 70%). NOS inhibition and antioxidant treatment reduced contraction-stimulated increases in protein S-glutathionylation and tyrosine nitration (P < 0.05), without affecting AMPK or p38 MAPK phosphorylation. In conclusion, we provide evidence to suggest that NO and ROS are both involved in the regulation of glucose uptake during skeletal muscle contractions ex vivo, and given their close relationship, it is possible that they interact to this end.

Studies investigating the mechanisms through which NO regulates skeletal muscle glucose uptake have focused largely on NO donor-stimulated skeletal muscle glucose uptake, rather than that produced endogenously during contraction (19, 21, 50, 52–54). The prevention of NO donor-mediated increases in skeletal muscle cyclic guanosine monophosphate (cGMP) formation and cGMP-dependent protein kinase (PKG) activity, through the inhibition of guanylate cyclase (GC), prevents NO donor-induced increases in glucose uptake at rest (54). Similarly, the cGMP analog, 8-bromo-cGMP, can increase resting skeletal muscle glucose uptake (52), suggesting that NO mediates glucose uptake in skeletal muscle via the traditional NO/cGMP/PKG signaling pathway (48). Like NO-donors, contraction-stimulated increases in skeletal muscle NO production raises muscle cGMP concentration (27), and therefore, cGMP is likely to contribute to the regulation of skeletal muscle glucose uptake during contraction. However, NO often exerts its effects independent of the cGMP-PKG signaling pathway, via various posttranslational signaling events, such as the addition of a NO group (S-nitrosylation) or glutathione (S-glutathionylation) to cysteine sulphydryls on proteins, or peroxynitrite nitration of protein tyrosine residuals (tyrosine nitration) (13, 48, 55). Importantly, all of these signaling events require, or have the potential to be modulated by ROS (9, 30). Therefore, the mechanism(s) through which NO stimulates skeletal muscle glucose uptake during contraction is yet to be investigated. Furthermore, whether reactive oxygen and nitrogen (such as NO) species regulate skeletal muscle glucose uptake through a similar pathway during contraction has not been examined.

The purpose of this study was to investigate the mechanism(s) through which NO regulates glucose uptake during skeletal muscle contractions ex vivo. We tested the hypothesis that NO increases skeletal muscle glucose uptake during con-
tions via cGMP-dependent and -independent pathways that involve signaling through interaction with ROS.

MATERIALS AND METHODS

Animals. This study used male C57BL/6 mice at 12–14 wk. Mice were maintained in an environmentally controlled room at 21°C with a 12:12-h light-dark cycle (light 0630–1830) and had 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. 2-Deoxy-[γ-14C]-glucose and 2-deoxy-[1,2-3H]-glucose were purchased from Sigma-Aldrich Chemicals (Piscataway, NJ). Primary antibodies for AMPKα, p38 MAPK, α-tubulin, and anti-phospho-p38 MAPK Thr180/Tyr182 were purchased from Cell Signaling Technology (Hartfordshire, UK) and nNOS from BD Transduction Laboratories (Sydney, NSW, Australia). Anti-phospho-ACC Ser271 and anti-phospho-AMPK Thr172 were purchased from Upstate Biotechnology (Lake Placid, NY), anti-nitrotyrosine from Chemicon (Temecula, CA), and anti-glutathione from Abcam (Cambridge, UK). IRDye 800-labeled streptavidin and secondary antibodies IRDye 800-conjugated anti-mouse and anti-rabbit IgG were purchased from Rockland (Gilbertsville, PA). Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO).

Muscle dissection, incubation, and contraction protocol. The EDL muscles were carefully excised from mice that were anesthetized deeply with pentobarbital sodium (60 mg/kg ip; Rhone Merieux, Pinkenba, Queensland, Australia). EDL muscles were used in this study since nNOS is more highly expressed in muscle containing primarily glycolytic fibers (26, 32), and NO appears to play a greater role in regulating glucose uptake in EDL muscles than in soleus primarily glycolytic fibers (26, 32), and NO appears to play a greater role in regulating glucose uptake in EDL muscles than in soleus muscles (which contain primarily oxidative muscle fibers) of mice when contracted ex vivo (32). The proximal tendon was tied to a force transducer (PanLab, Barcelona, Spain) using 5/0 silk suture, and the muscles were suspended vertically in incubation chambers (Radnoti, Monrovia, CA) that were filled with Ringer solution (in mM): 118.5 NaCl, 24.7 NaHCO3, 4.74 KCl, 1.18 MgSO4, 1.18 KH2PO4, 2.5 CaCl2, 8 mannitol, 2 sodium pyruvate, and 0.01% BSA, with pH 7.4, maintained at 30°C and continuously oxygenated with 95% O2 and 5% CO2 (Carbogen; BOC Gases, South Melbourne, Victoria, Australia). Ringer solution was maintained at 30°C since this temperature has previously been reported to be optimal for maintaining muscle function and measuring glucose uptake ex vivo (22). However, it is acknowledged that this may have reduced oxidant activity compared with physiological temperature (37°C) (2).

Muscles were stimulated with square wave electrical pulses generated by a Grass S48 stimulator, amplified by an EP500B power amplifier (Audio Assemblies, Cambellfield, Victoria, Australia) and delivered to two platinum plate electrodes that thrombosed the muscle preparation but did not touch it. Optimal muscle length (L0) was determined from isometric twitch contractions (47), and forces were recorded on a PowerLab running Chart 5.0 software (AD Instruments, Castle Hill, NSW, Australia). Separate muscles were incubated for 40 min in the presence or absence of the NOS inhibitor Nω-monomethyl-arginine [l-NMMA; 100 μM (18)], the nonspecific antioxidant N-acetylcysteine [NAC; 20 mM, (18, 44)], l-NMMA + NAC, the GC inhibitor H8-[1,2,4]oxadiazolo-[4,3-a]quinoline-1-one [ODQ, which prevents the formation of cGMP, 10 μM, (16)], the PKG inhibitor Rp-8-bromo-β-phenyl-1-N2-ethenoguanosine 3′,5′-cyclic monophosphorothioate sodium salt [Rp-8-Br-PET-cGMPS, 5 μM (14)], the thiol-reducing agent dithiothreitol [DTT; 10 mM (1)], the peroxynitrite scavenger urate [10 mM (40, 46)] or white light (dissection lamp 10 cm from incubating muscle; Novaflax, World Precision Instruments, Sarasota, FL) to break S-nitrosylated bonds (6, 7). Light from the dissection lamp diffused through the isolated muscle, and, therefore, is likely to break any S-nitrosylation bonds that may form, unlike indirect exposure to white light from laboratory lighting (24). Contracted muscles were then stimulated (pulse durations: 600 ms at a frequency of 60 Hz for 25 contractions/min) during the final 10 min of incubation. This stimulation protocol has been previously shown to be sufficient to recruit all motor units and elicit maximum force (29). Noncontracted (resting) muscles were incubated in the presence or absence of the NO donor diethylenetriamine/NO (Deta/NO, 500 μM) for 40 min. At the conclusion of the incubation/contraction protocol, the muscles were quickly cut from the sutured attachments and rapidly frozen in liquid nitrogen for future biochemical analyses. Separate muscles were used for glucose uptake, immunoblotting, NOS activity, and oxidant levels.

Glucose uptake measurements. Glucose uptake was determined during the final 5 min of contraction and over 5 min of recovery in contracted muscles (22, 32). In noncontracted (resting) muscles, glucose uptake was determined during the final 10 min of incubation.

To measure glucose uptake, the muscle incubation buffer was exchanged for one containing 1 mM 2-deoxy-[γ-14C]-glucose (0.128 μCi/ml) and 8 mM 2-deoxy-[1-14C]-mannitol (0.083 μCi/ml). After incubation, the muscles were washed in ice-cold Ringer solution and snap frozen in liquid nitrogen. To calculate muscle 2-deoxy-[γ-14C]-glucose uptake, muscles were digested for 10 min at 80°C in 1 M NaOH, neutralized with 1 M HCl and spun at 13,000 g for 2 min. The supernatant was recovered and radioactivity was measured using a β-scintillation counter (Packard TriCarb 2900TR, PerkinElmer, Boston, MA) (49).

Immunoblotting. Half of a frozen EDL muscle was homogenized (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 Na2PO4, 100 μM DTT, 1 mM PMSF, and 5 μl/ml protease inhibitor cocktail), incubated for 20 min on ice and spun at 13,000 g for 20 min at 4°C. The supernatant was diluted 1:3 in sample buffer (1.5 M Tris·HCl, pH 6.8, 30% glycerol, 10% SDS, 0.6 M DTT, 0.0012% bromophenol blue), heated for 10 min at 37°C, and then stored in the freezer at −20°C. Protein concentration was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Muscles examined for S-glutathionylation were extracted and run under non-reducing conditions with homogenization and sample buffer containing 5 mM and 10 mM of N-ethylmaleimide, respectively. Total protein (80 μg) was separated using SDS-PAGE, transferred to PVDF membrane and blocked in 5% nonfat milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies for nitrotyrosine, glutathione, and phosphorylation-specific antibodies for ACCβ Ser271 and, p38 MAPK Thr180/Tyr182, nNOS, and α-tubulin to determine total protein levels. However, for AMPK, membranes were first probed with AMPKα primary antibody before being stripped and reprobed with AMPKα Thr172 phosphorylation-specific antibody since in our hands, we find that AMPKα Thr172 cannot be stripped effectively. Where protein phosphorylation was measured, phosphorylation was expressed relative to the total protein abundance of the protein of interest. Positive controls were run for nitrotyrosine and glutathione blots. Treatment of tibialis anterior muscles with 2.5 mM of ONOO− and 5 mM of H2O2 increased tyrosine nitration and S-glutathionylation, respectively (data not shown).

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Oxidant levels and NOS activity. The fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) was used to measure oxidant levels in muscle sections. Immediately following contraction or incubation at rest, muscles were rapidly mounted in OCT and frozen in isopentane cooled in liquid nitrogen. DCFH-DA (5 μM) was added to 10 μm EDL muscle cross sections, covered with a coverslip, and allowed to dry overnight at room temperature. The oxidized derivative of DCFH-DA, 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 determined using Zeiss LSM-510 acquisition and analysis software. We measured DCF fluorescence as an index of intracellular oxidant levels in accordance with previous studies (34). All procedures were carried out in low light to avoid photo-oxidation. NOS activity was determined in separate EDL 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 using SPSS statistical package using one-factor ANOVA (treatment) and one-factor repeated-measures ANOVA for contraction forces (treatment and time). If the ANOVA revealed a significant treatment effect, or a treatment by time interaction, specific contrasts were analyzed using SPSS statistical package using one-factor ANOVA (treatment) and one-factor repeated-measures ANOVA for contraction forces (treatment and time). The level of significance was set at P < 0.05.

RESULTS

Muscle contraction. Treatment of muscles with the peroxynitrite scavenger, urate, reduced peak tetanic force (normalized to muscle mass) by ~37% (P = 0.02; Fig. 1C). Peak force was not affected by any other treatment (P > 0.05; Fig. 1, A and C). Peak force was obtained within the first 10 s of the contraction protocol and declined thereafter (P < 0.001; Fig. 1, B and D). The rate of decline from peak force was not affected by any treatment (P > 0.05; Fig. 1, B and D).

Oxidant levels and NOS activity. Basal oxidant levels in muscles were increased ~95% with contraction (Fig. 2, A and B). Urate tended (P = 0.059) to attenuate this increase (Fig. 2B), and the nonspecific antioxidant, NAC, and the thioredoxin-reducing agent, DTT, prevented contraction-stimulated increases in oxidant activity (P > 0.05 vs. basal; Fig. 2B). Contraction-increased NOS activity in EDL muscles by ~40% and the NOS inhibitor, L-NMMA, reduced NOS activity during contraction to ~35% of basal activity (Fig. 2C).

Contraction and deta/NO-stimulated glucose uptake. Contraction-increased muscle glucose uptake by 1.8–2.3-fold (P < 0.05, Figs. 3, A and B, and 4). NAC and L-NMMA attenuated the increase in glucose uptake during contraction, similarly, by ~50% (P < 0.05) and cotreatment of L-NMMA with NAC did not have an additive effect on the magnitude of the reduction (Fig. 3A). The GC inhibitor, ODQ, which prevents cGMP formation (16), did not affect contraction-stimulated glucose uptake (Fig. 3B) but prevented the 1.5-fold increase (P = 0.01) in glucose uptake stimulated by the NO donor Deta/NO (Fig. 3C). Furthermore, muscle glucose uptake during contraction in the presence (1.84 ± 0.12 μmol·g⁻¹·h⁻¹) and absence (1.84 ± 0.12 μmol·g⁻¹·h⁻¹) of DTT, NAC, and L-NMMA was not significantly different (P > 0.05, Figs. 3A, B, and C).

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MAPK Thr180/Tyr182 phosphorylation was increased and this increase was prevented by L-NMMA and NAC (Fig. 2). The approximately three-fold contraction-stimulated increase in S-glutathionylation and tyrosine nitration of a protein band at 37 kDa was prevented by NAC but unaffected by l-NMMA (Fig. 5A). L-NMMA and NAC both attenuated the contraction-stimulated increase in S-glutathionylation at ~37 kDa (P < 0.05; Fig. 5A). Contraction increased tyrosine nitration of a protein band at ~37 kDa by 1.7-fold (P = 0.01), and this increase was prevented by l-NMMA and NAC (Fig. 5B). Neither contraction nor treatment significantly affected protein S-glutathionylation or tyrosine nitration of any other visible protein bands.

Contraction-stimulated AMPK, ACCβ, p38 MAPK, and nNOS phosphorylation. Contraction increased muscle AMPKα Thr172 phosphorylation 4.5-fold (P = 0.004), and this increase was not affected by any treatment (Fig. 6A). The approximately twofold contraction-stimulated increase (P = 0.02) in ACCβ Ser214 phosphorylation was attenuated by urate but not affected by any other treatment (Fig. 6B). The approximately three-fold increase (P < 0.001) in muscle nNOS Ser1412 phosphorylation during contraction was not affected by any treatment (Fig. 6C). p38 MAPK Thr180/Tyr182 phosphorylation was increased approximately three-fold (P = 0.04) with contraction, and this increase was prevented by white light and urate, but not affected by any other treatment (Fig. 6D).

Basal glucose uptake, resting tension, and AMPK signaling. Resting glucose uptake in EDL muscles was reduced ~25% by W16590 AND CONTRACTION MEDIATED GLUCOSE UPTAKE

DISCUSSION

The major findings of this study were that 1) the attenuation of increases in both NO activity and ROS levels during skeletal muscle contractions ex vivo by l-NMMA and NAC, respectively, attenuates glucose uptake; 2) cotreatment of l-NMMA and NAC does not have an additive affect on the attenuation of skeletal muscle glucose uptake during contraction; 3) the guanylate cyclase inhibitor, ODQ, attenuates NO donor-stimulated glucose uptake, but neither ODQ nor the PKG inhibitor, Rp-8-Br-PET-cGMPS, affects skeletal muscle glucose uptake during contraction; 4) l-NMMA and NAC attenuate contraction-stimulated increases in muscle protein S-glutathionylation and tyrosine nitration at ~37 kDa without affecting AMPK or p38 MAPK phosphorylation; and 5) the thiol-reducing agent (DTT) and peroxynitrite scavenger (urate) attenuate contraction-stimulated increases in skeletal muscle...
glucose uptake; however, urate appeared to have several non-specific effects, which confound interpretation of these data and are discussed in detail below. Taken together, these findings suggest that NOS-derived oxidants regulate skeletal muscle glucose uptake during ex vivo contractions via a cGMP-independent pathway. In addition, these data suggest that ROS and NO may be involved in the regulation of skeletal muscle glucose uptake during contractions ex vivo through a similar pathway that does not require AMPK or p38 MAPK phosphorylation but may involve signaling through peroxynitrite and S-glutathionylation. However, whether a causal relationship exists between skeletal muscle glucose uptake during contraction and peroxynitrite and S-glutathionylation signaling now needs to be examined.

Previously, we have shown that the inhibition of NOS attenuates glucose uptake during muscle contractions (8, 25, 32, 42). In support of these findings, we show here that preventing increases in NOS activity during ex vivo contractions (Fig. 2C) of mouse EDL muscles attenuates increases in glucose uptake (Fig. 3A). The mechanism(s) through which NO stimulates skeletal muscle glucose uptake during contraction has not been examined previously, although inhibition of GC with LY-83584 has been shown to prevent NO donor-stimulated increases in cGMP formation, glucose uptake, and PKG activity in rat skeletal muscles at rest (54). We have confirmed these findings by showing that the specific GC inhibitor, ODQ (16), prevented the increase in resting muscle glucose uptake stimulated by the NO donor, Deta/NO (Fig. 3C). However, surprisingly, ODQ did not affect skeletal muscle glucose uptake during contraction (Fig. 3B). We were unable to measure skeletal muscle cGMP formation following contraction. Since cGMP is broken down extremely quickly by cGMP-specific phosphodiesterase (PDE) in the absence of PDE inhibitors (5), we believe we did not freeze muscle fast enough following contraction to see an increase in cGMP content. However, Lau et al. (27) freeze-clamped muscles following contraction and reported that contraction increases muscle cGMP levels to a similar extent as NO donors. Since ODQ inhibited NO donor-stimulated glucose uptake, which requires cGMP signaling (53), we are confident that ODQ prevented any increases in cGMP formation that may have occurred during contraction. In addition, we report that the PKG inhibitor (Rp-8-Br-PET-cGMPS) also does not affect glucose uptake during contraction. This suggests that in contrast to NO donor-stimulated glucose uptake in resting muscle (52–54), during skeletal muscle contraction NO regulates glucose uptake through a cGMP-PKG-independent pathway.

Interestingly, NO has been shown to regulate glucose uptake in adipocytes through a cGMP-independent mechanism possibly involving S-nitrosylation (23). However, S-nitrosylation does not appear to be involved in regulating skeletal muscle glucose uptake during contraction because in the current study, white light, which breaks S-nitrosylation bonds (6, 7), did not affect the increase in contraction-stimulated glucose uptake (Fig. 4). However, further work is needed to ensure that white light prevents the formation of all S-nitrosyl bonds during contraction to confirm that S-nitrosylation is not involved in regulating skeletal muscle glucose uptake during contraction. This is particularly important because S-nitrosylation can have a direct effect on ROS signaling via S-glutathionylation (30).

In agreement with previous studies, we found that contraction increases oxidant levels in skeletal muscle (39, 44) and that this increase can be prevented by antioxidant treatment (Fig. 2). DCF fluorescence was measured as a marker of

**Fig. 3.** 2-deoxy-glucose uptake in EDL muscles during the final 10 min of basal incubation or 10 min of contraction in the presence or absence of L-NMMA, NAC, L-NMMA + NAC (A), ODQ (B), and when treated with diethylenetriamine/NO (Deta/NO), and Deta/NO + ODQ (C). Data are expressed as means ± SE; n = 6–12 per group. *P < 0.05 vs. basal; #P < 0.05 vs. contraction; †P < 0.05 vs. Deta/NO.

**Fig. 4.** 2-deoxy-glucose uptake in EDL muscles during the final 10 min of basal incubation or 10 min of contraction in the presence or absence of white light, DTT, or urate. Data are expressed as means ± SE; n = 6–10 per group. *P < 0.05 vs. basal; #P < 0.05 vs. contraction.
muscle ROS levels, and contraction-induced increases in DCF fluorescence were prevented by the antioxidants NAC and DTT (Fig. 2). This suggests that although DCFH can be oxidized to DCF by reactive nitrogen species (33), as well as by ROS (34), in our hands, contraction-induced increases in DCFH oxidation are likely to have resulted from an increase in intracellular ROS levels. The peroxynitrite (ONOO\(^-\)) scavenger, urate (40), tended to attenuate contraction-induced increases in oxidant levels, suggesting that ONOO\(^-\) is formed during contraction of muscles. ONOO\(^-\) formation has previously been reported in contracted muscle cells (36). Interpretation of the biological significance of these findings may be complicated by the measurement of DCF fluorescence in muscle sections following contraction, and it would be preferable to measure ROS production in intact muscles during contraction. Nonetheless, our results for oxidant levels are supported by NAC attenuating contraction-induced increases in S-glutathionylation and tyrosine nitration (Fig. 5).

We observed that the antioxidants NAC, DTT, and urate attenuated muscle contraction-stimulated glucose uptake ex vivo (Figs. 3A and 4). It must be acknowledged, however, that it is not known whether DTT or urate inhibition of increases in glucose uptake is specific to contraction-stimulated increases. Regardless, these data support previous findings that the antioxidants NAC, ebselen, catalase, and MnTBAP attenuate stretch (11) and contraction-stimulated increases in skeletal muscle glucose uptake (44). Furthermore, Sandstrom et al. (44) showed that the increases in skeletal muscle glucose uptake and AMPK signaling during contraction were attenuated in parallel by NAC treatment, while Chambers et al. (11) provided some evidence that ROS may regulate stretch-stimulated skeletal muscle glucose uptake via p38 MAPK (p38). In contrast, we found that the antioxidants NAC and DTT attenuated contraction-stimulated glucose uptake independent p38 phosphorylation, and NAC, DTT, and urate did not affect AMPK phosphorylation during contraction (Fig. 6). The lack of p38 effect in our current study suggests that stretch and contraction stimulate glucose uptake through separate pathways. However, the discrepancy between Sandstrom et al. (44) and our finding in regard to the effects of NAC on AMPK is more difficult to discern but is consistent with our previous findings (32). The discrepancy may relate to Sandstrom et al. (44) using a longer precontraction NAC incubation (30 vs. 60 min), cooler incubation temperatures (25°C vs. 30°C), or a more strenuous contraction protocol than was employed in the present study.

Fig. 5. S-glutathionylation (A) and tyrosine nitration (B) in extensor digitorum longus (EDL) muscles after 40-min incubation or when stimulated to contract during the final 10 min of incubation in the presence or absence of L-NMMA and NAC. Data are expressed as means ± SE; n = 8 per group. *P < 0.05 vs. basal; #P < 0.05 vs. contraction.
Fig. 6. AMPK Thr\textsuperscript{172} phosphorylation (A), ACCβ Ser\textsuperscript{211} phosphorylation (B), and nNOS Ser\textsuperscript{1412} phosphorylation (C), and p38 MAPK kinase Thr\textsuperscript{180}/Tyr\textsuperscript{182} phosphorylation (D) in EDL muscles following 40 min of incubation or when stimulated to contract during the final 10 min of incubation in the presence or absence of L-NMMA, NAC, NAC+L-NMMA, ODQ, white light, DTT, or urate. Data are expressed as means ± SE; n = 8 per group. *P < 0.05 vs. basal; #P < 0.05 vs. contraction.
tion of protein bands at muscle glucose uptake, S-glutathionylation and tyrosine nitration, and it is possible that this may play a role in the regulation of skeletal muscle glucose uptake during contraction; however, a causal relationship needs to be established. Interestingly, tyrosine nitration is promoted by high ONOO\(^{-}\) concentrations and normally associated with irreversible pathophysiological inhibition of protein phosphorylation (35). To reconcile these two views of ONOO\(^{-}\), it is possible that during intense ex vivo contractions, higher than normal physiological levels of ONOO\(^{-}\) are formed and cause tyrosine nitration. On the other hand, we would suggest that during more physiological exercise, lower concentrations of ONOO\(^{-}\), which do not cause tyrosine nitration, are produced and can cause transient and reversible phosphotyrosine signaling that can alter protein activities (35). It is, therefore, important that future studies identify whether relationships exist between protein tyrosine nitration, ONOO\(^{-}\) signaling, S-glutathionylation, and glucose uptake during contraction, and to identify the specific protein targets.

Incubation of the skeletal muscle with urate, an ONOO\(^{-}\) scavenger (40, 46), reduced initial contraction force and inhibited muscle signaling (ACCB and p38) during contraction, independent of NOS inhibition (Fig. 6). This indicates that urate is having effects on skeletal muscle independent of ONOO\(^{-}\) scavenging. Furthermore, urate stimulated an increase in resting muscle passive tension (Fig. 7C), which is likely to have caused the increase in resting muscle AMPK phosphorylation and glucose uptake (Fig. 7C), which is likely to have caused the increase in resting muscle AMPK phosphorylation and glucose uptake (Fig. 7C). Although skeletal muscle glucose uptake during contraction may not be dependent on force production per se (45), the various effects of urate on the muscle likely confound interpretation of ONOO\(^{-}\) effects on skeletal muscle glucose uptake during contraction. It was notable, however, that the increase in resting muscle glucose uptake with urate was similar to that seen after the combination of urate and contraction (Fig. 4); suggesting that urate prevented any further increase in skeletal muscle glucose uptake during contraction. Therefore, investigating the role of ONOO\(^{-}\) in regulating skeletal muscle glucose uptake during contraction requires further attention.

In conclusion, this study provides the first evidence to suggest that that during ex vivo contractions NOS-derived oxidants regulate skeletal glucose uptake through a cGMP-PKG-independent pathway that does not involve p38 MAPK or AMPK signaling. Furthermore, we provide evidence that NO and ROS may regulate skeletal muscle glucose uptake during contraction ex vivo through a similar pathway, with some suggestion that signaling through peroxynitrite and S-glutathionylation signalizing may be involved.

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Fig. 7. 2-Deoxy-glucose uptake in EDL muscles during the final 10 min of basal incubation in the presence or absence of L-NMMA (100 μM), NAC (20 mM), DTT, and urate (A). EDL muscle AMPK Thr\(^{172}\) phosphorylation following 40 min of basal incubation in the presence or absence of urate (B). Resting (passive) tension of EDL muscles during the final 10 min of basal incubation in the presence or absence of L-NMMA, NAC, DTT, or urate (C). Data are means ± SE; n = 4–5 per group. *P < 0.05 vs. basal.

skeletal muscle glucose uptake. Superoxide and NO interact to form ONOO\(^{-}\), which can cause the tyrosine nitration of proteins (46). Indeed, tyrosine nitration increased with contraction, and this increase was prevented by NAC treatment and NOS inhibition (Fig. 5). Furthermore, ONOO\(^{-}\) promotes protein S-glutathionylation (30); oxidative or nitrosative stress induced reversible formation of mixed disulfides between protein sulfide groups and glutathione (13), which is becoming increasingly recognized as the redox equivalent of phosphor- ylation in regulating protein signaling (13, 30). As such, we found that contraction-stimulated S-glutathionylation is attenuated by a general antioxidant (NAC).

Interestingly, the contraction-induced increase in skeletal muscle glucose uptake, S-glutathionylation and tyrosine nitration of protein bands at ~37 kDa was attenuated by NOS inhibition and NAC treatment (Figs. 3A, 4 and 5). This suggests that during contraction, ROS and NO interact to alter protein S-glutathionylation and tyrosine nitration, and it is possible that this may play a role in the regulation of skeletal muscle glucose uptake during contraction; however, a causal relationship needs to be established. Interestingly, tyrosine nitration is promoted by high ONOO\(^{-}\) concentrations and normally associated with irreversible pathophysiological inhibition of protein phosphorylation (35). To reconcile these two views of ONOO\(^{-}\), it is possible that during intense ex vivo contractions, higher than normal physiological levels of ONOO\(^{-}\) are formed and cause tyrosine nitration. On the other hand, we would suggest that during more physiological exercise, lower concentrations of ONOO\(^{-}\), which do not cause tyrosine nitration, are produced and can cause transient and reversible phosphotyrosine signaling that can alter protein activities (35). It is, therefore, important that future studies identify whether relationships exist between protein tyrosine nitration, ONOO\(^{-}\) signaling, S-glutathionylation, and glucose uptake during contraction, and to identify the specific protein targets.

Incubation of the skeletal muscle with urate, an ONOO\(^{-}\) scavenger (40, 46), reduced initial contraction force and inhibited muscle signaling (ACCB and p38) during contraction, independent of NOS inhibition (Fig. 6). This indicates that urate is having effects on skeletal muscle independent of ONOO\(^{-}\) scavenging. Furthermore, urate stimulated an increase in resting muscle passive tension (Fig. 7C), which is likely to have caused the increase in resting muscle AMPK phosphorylation and glucose uptake (Fig. 7C). Although skeletal muscle glucose uptake during contraction may not be dependent on force production per se (45), the various effects of urate on the muscle likely confound interpretation of ONOO\(^{-}\) effects on skeletal muscle glucose uptake during contraction. It was notable, however, that the increase in resting muscle glucose uptake with urate was similar to that seen after the combination of urate and contraction (Fig. 4); suggesting that urate prevented any further increase in skeletal muscle glucose uptake during contraction. Therefore, investigating the role of ONOO\(^{-}\) in regulating skeletal muscle glucose uptake during contraction requires further attention.

In conclusion, this study provides the first evidence to suggest that that during ex vivo contractions NOS-derived oxidants regulate skeletal glucose uptake through a cGMP-PKG-independent pathway that does not involve p38 MAPK or AMPK signaling. Furthermore, we provide evidence that NO and ROS may regulate skeletal muscle glucose uptake during contraction ex vivo through a similar pathway, with some suggestion that signaling through peroxynitrite and S-glutathionylation signaling may be involved.

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