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3 LOCAL HINDLIMB ANTIOXIDANT INFUSION DOES NOT AFFECT MUSCLE
4 GLUCOSE UPTAKE DURING IN SITU CONTRACTIONS IN RAT
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15 Short title: ROS and *in situ* glucose uptake during contraction
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40 **ABSTRACT**
41
42 There is evidence that ROS contribute to the regulation of skeletal muscle glucose uptake
43 during highly fatiguing *ex vivo* contraction conditions via AMPK. In this study we
44 investigated the role of ROS in the regulation of glucose uptake and AMPK signaling during
45 low-moderate intensity *in situ* hindlimb muscle contractions in rats, which is a more
46 physiological protocol and preparation. Male hooded Wistar rats were anesthetized and then
47 N-acetylcysteine (NAC) was infused into the epigastric artery (125 $mg \cdot kg \cdot hr^{-1}$) of one
48 hindlimb (contracted leg) for 15 min before this leg was electrically stimulated (0.1-ms
49 impulse at 2 Hz and 35 V) to contract at a low-moderate intensity for 15 min. The
50 contralateral leg did not receive stimulation or local NAC infusion (rest leg). NAC infusion
51 increased (P<0.05) plasma cysteine and cystine (by ~360- and 1.4-fold, respectively), and
52 muscle cysteine (by 1.5-fold, P=0.001). Although contraction did not significantly alter
53 muscle tyrosine nitration, GSH or GSSG content, S-glutathionylation of protein bands at ~250
54 and 150 kDa was increased (P<0.05) ~1.7-fold by contraction, and this increase was
55 prevented by NAC. Contraction increased (P<0.05) skeletal muscle glucose uptake 20-fold,
56 AMPK phosphorylation 6-fold, ACCβ phosphorylation 10-fold and p38 MAPK
57 phosphorylation 60-fold, and the muscle fatigued by ~30% during contraction and NAC
58 infusion had no significant effect on any of these responses. This was despite NAC preventing
59 increases in S-glutathionylation with contraction. In conclusion, unlike during highly
60 fatiguing *ex vivo* contractions, local NAC infusion during *in situ* low-moderate intensity
61 hindlimb contractions in rats, a more physiological preparation, does not attenuate increases
62 in skeletal muscle glucose uptake or AMPK signaling.
63
64 **Keywords**: Exercise, metabolism, S-glutathionylation, reactive oxygen species, AMPK
65

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66 **INTRODUCTION**

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

76 Although chronic elevation in oxidative stress (a pro-oxidant shift in cell redox status)
77 is associated with the pathophysiology of type 2 diabetes and insulin resistance (21), acute
78 treatment of isolated skeletal muscle with exogenous ROS stimulates insulin-independent
79 glucose uptake (14, 16). Acute increases in skeletal muscle ROS production occur during *in*
80 *vivo* exercise (11, 24, 40) and during *ex vivo* contractions (34, 39). Sandstrom *et al.* (39) have
81 shown that the treatment of isolated skeletal muscle *ex vivo* with the antioxidant N-
82 acetylcysteine (NAC) attenuates both the contraction-stimulated increase in oxidative stress
83 and increases in glucose uptake. Thus, there is evidence that acute increases in ROS are
84 involved in the signaling of skeletal muscle glucose uptake during *ex vivo* contractions.
85 Interestingly, Sandstrom *et al.* (39) also reported that, similar to glucose uptake, NAC
86 attenuates the contraction-stimulated increases in skeletal muscle AMPK activity.
87 Furthermore, antioxidant supplementation (allopurinol) has been shown to prevent increases
88 in p38 MAPK phosphorylation during exhaustive exercise in rats (11), and p38 MAPK has
89 been implicated in the regulation of contraction and stretch-stimulated skeletal muscle glucose

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90 uptake (6, 42). This suggests that during contraction ROS may regulate skeletal muscle
91 glucose uptake via the activation of AMPK and/or p38 MAPK.

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

105 Therefore, in this study, we determined whether local infusion of the antioxidant NAC
106 attenuates the increase in hindlimb skeletal muscle glucose uptake and AMPK signaling
107 during physiologically relevant low-moderate intensity contractions *in situ* in rats. We
108 hypothesized that low-moderate intensity contractions *in situ* would increase skeletal muscle
109 glucose uptake and AMPK signaling and that NAC infusion would attenuate these increases.

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111 **RESEARCH DESIGN AND METHODS**

112 *Animals*

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

119

120 *Materials and Antibodies*

121 Unless otherwise stated all chemicals were purchased from Sigma-Aldrich Chemicals (St.
122 Louis, MO). 2-deoxy-D-[1-^{14}C]-glucose (2DG; specific activity 1.92 TBq/mmol) was
123 purchased from Amersham life science (NSW, Australia). Primary antibodies for AMPKα,
124 p38 MAPK, α-tubulin and anti-phospho-p38 MAPK Thr^{180}/Tyr^{182}were purchased from Cell
125 Signaling Technology (Hartsfordshire, England), and 3-nitrotyrosine from Chemicon, (CA,
126 USA). Anti-phospho-ACCβ Ser^{222} and anti-phospho-AMPK Thr^{172} were purchased from
127 Upstate Biotechnology (NY, USA), and anti-glutathione was purchased from Abcam
128 (Cambridge, England). IRDye™ 800-labeled streptavidin and secondary anti-bodies IRDye™
129 800-conjugated anti-mouse and anti-rabbit IgG were purchased from Rockland, Gilbertsville,
130 PA, USA.

131

132 *Experimental procedure*

133 An anesthetized rat model was utilized in this study as described previously (30, 37, 47).
134 Briefly, rats were anesthetized using sodium pentobarbital (1.5 μl·g bwt^{-1} i.p.) and cannulas
135 were inserted into the carotid artery and jugular veins for arterial sampling and continuous

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136 administration of anesthetic, respectively. Isotonic saline (154 mmol·l^{-1} NaCl) containing N-
137 acetylcysteine (NAC; 125 mg·kg·hr^{-1}) or isotonic saline alone was infused locally (at 1:100 of
138 the arterial flow rate) into one hindlimb (contracted leg) via the epigastric artery. Sham
139 surgery was performed on the contralateral leg (rest). NAC is a non-specific antioxidant that
140 directly scavenge ROS (2) and is deacetylated to cysteine which promotes the resynthesis of
141 reduced glutathione (GSH) (8, 41). The systemic infusion of NAC at 125 mg·kg·hr^{-1} has been
142 shown previously to elevate muscle NAC, cysteine and total GSH during exercise in humans
143 (24). After a 15 min pre-infusion, the contracted leg was electrically stimulated to contract
144 (0.1 ms impulse at 2 Hz and 35 V) for 15 min while NAC infusion continued. The knee was
145 secured by the tibiopatellar ligament and the Achilles tendon was attached to a Harvard
146 Apparatus (MA, USA) isometric transducer thereby allowing measurement of tension
147 development from the gastrocnemius-plantaris-soleus muscle group during contraction. It has
148 previously been shown that during highly fatiguing contractions *ex vivo,* ROS are involved in
149 the regulation of glucose uptake during contraction (39). Therefore this contraction protocol
150 was chosen because we have previously shown it to substantially increase muscle glucose
151 uptake and metabolic signaling while causing only a physiologically level of fatigue (37). At
152 10 min before the completion of the experiment (from t=20 to 30min) a 1.85 MBq bolus of
153 bolus of 2DG in isotonic saline was administered via the right jugular vein. Immediately
154 following the 2DG bolus, an arterial blood sample (0.5 ml) was withdrawn by an automated
155 syringe pump at 50µl·min^{-1} over 10 min. From this blood sample a plasma sample (25µl) was
156 collected to determine the average plasma specific radioactivity of 2DG. At *t*=30 min the
157 lower leg muscles (soleus, plantaris, gastrocnemius red and gastrocnemius white) from the
158 contracted and contralateral (rest) leg were rapidly dissected and freeze clamped using liquid
159 nitrogen-cooled thongs. Throughout the experimental protocol heart rate (HR), mean arterial

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160 pressure (MAP) and femoral blood flow were monitored and recorded as described previously
161 (30, 47) .
162
163 *Muscle glucose uptake*
164 The lower leg muscles (soleus, plantaris, gastrocnemius red and gastrocnemius white) were
165 ground under liquid nitrogen and 100 mg was homogenized with 1.5 ml water before free and
166 phosphorylated glucose were separated by ion exchange chromatography using anion
167 exchange resin (AG1-X8; Bio-Rad, Hercules, CA). Inorganic liquid scintillation cocktail
168 (Amersham life science) was added to samples and radioactivity was measured by a β-
169 scintillation counter (Packard TriCarb 2900TR, PerkinElmer, Boston, MA). Based on plasma
170 glucose, muscle and plasma 2DG concentrations muscle 2DG glucose uptake was calculated
171 as follows;

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

172
173
174 *NAC and Thiols*
175 *Muscle preparation:* 30 mg of ground muscle sample was rapidly homogenized on ice in 300
176 µl of 0.42 M PCA, immediately following homogenization PCA was neutralized with 40 µl of
177 2.5 M K_2CO_3. Samples were then centrifuged at 13,000 g for 5 min at 4°C and the supernatant
178 was recovered.
179 *HPLC method for plasma and muscle sample:* For analysis of reduced thiols (reduced NAC,
180 GSH and cysteine), 10 µl of distilled H_2O was added to 50 µl of sample, and for the
181 determination of total thiols (TNAC, TGSH and total cysteine) 10 µl of tributylphosphine
182 solution diluted 1:10 was added to 50 µl of sample to oxidize reduced thiols. All samples were
183 then incubated for 30 min on ice and 25 µl of 4-Fluoro-7-sulfamoylbenzofurazan (ADB-F; 5
184 $mg \cdot ml^{-1}$ in borate buffer: 0.2 M boric acid, 2 mM sodium EDTA, pH 8.0) was added.

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185 Following 10 min incubation at 50°C, 10 µl of 2 M PCA was added and samples were
186 centrifuged at 13,000 g for 5 min. A 40 µl aliquot of the supernatant was then injected onto a
187 reverse-phase HPLC Gemini column (5µm C18 110Å, phenomenex®) with 0.1 M sodium
188 acetate buffer (pH 4.0) in 10% methanol as a mobile phase, at a flow rate of 1.5 $ml \cdot min^{-1}$ and
189 detection wavelength of 386 nm excitation and 516 nm emission. All HPLC values obtained
190 for thiols were measured against standards. Oxidized thiols (NAC, GSSG, cystine) were
191 calculated from the difference between the measured reduced and total thiols.

192

193 *Muscle signaling*

194 300 mg of ground muscle was homogenized (10 $\mu l \cdot mg^{-1}$ tissue) in ice-cold lysis buffer (50
195 mM Tris-HCl at pH 7.5 containing 1 mM EDTA, 10% v/v glycerol, 1% v/v Triton X-100, 50
196 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM DTT, 1 mM PMSF and 5 $\mu l.ml^{-1}$ Protease Inhibitor
197 Cocktail). Lysates were then incubated for 20 min on ice and centrifuged at 13,000 g for 20
198 min at 4°C. For analysis of S-glutathionylation, a marker of oxidative stress (9), muscle was
199 extracted under non-reducing conditions with lysis and sample buffer (1.5 M Tris-HCL, pH
200 6.8, 30% glycerol, 10% SDS, 0.6 M DTT, 0.0012% bromophenol blue) containing no DTT,
201 and 5 mM and 10 mM of N-Ethylmaleimide, respectively, to alkylate free thiol groups.
202 Protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce,
203 Rockford, Il) with BSA as the standard. Total protein was diluted 1:3 in sample buffer, heated
204 for 10 min at 100°C (with the exception of samples used for 3-nitrotyrosine analysis, which
205 were heated at 37°C for 10 min) and stored at -20°C before 80 µg of total protein was
206 separated by SDS-PAGE, transferred to PVDF membrane, and blocked in PBS containing 5%
207 nonfat milk for 1 h at room temperature. Membranes were incubated over night at 4°C with
208 primary antibody for glutathione and phosphorylation-specific primary antibodies for ACCβ
209 Ser^{222} and p38 MAPK Thr^{180}/Tyr^{182} before binding was detected with rabbit IgG secondary

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210 antibody. Direct fluorescence was detected and quantified using the Odyssey infrared imaging
211 system (LICOR Biosciences, Lincoln, NB). Membranes were then stripped (2% SDS (w/v) in
212 25 mM Glycine, pH 2.0) and re-probed with primary antibodies for ACCβ (streptavidin) and
213 p38 MAPK to determine total protein levels. However, for AMPKα Thr^{172} phosphorylation,
214 membranes were first probed with a AMPKα primary antibody before being stripped and re-
215 probed with a AMPKα Thr^{172} phosporylation-specific antibody as we find that AMPKα Thr^{172}
216 phosphorylation cannot be effectively stripped. Protein phosphorylation was expressed
217 relative to the total protein abundance of the protein of interest or α-tubulin.
218
219 *Statistical Analysis*
220 All data are expressed as means ± SEM. Results were analyzed by SPSS statistical package
221 using two-factor ANOVA as well as two-factor repeated measures ANOVA. Because NAC
222 infusion started pre-contraction, the repeated measure ANOVA's were partitioned to assess
223 the effect of NAC at rest (0 to 15 min) and during exercise (15 to 30 min). If the ANOVA
224 revealed a significant interaction, specific differences between mean values were located
225 using the Fisher's least significance difference test. The level of significance was set at $P <$
226 0.05.
227
228

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229 **RESULTS**

230 *Force development:* Peak contraction force decreased by ~30% by the end of the 15 min
231 contraction period (P<0.001; Figure 1). Local NAC infusion did not affect initial peak
232 contraction force (P=0.56) or the rate of fatigue (P=0.65, Figure 1).

233

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

240

241 *Heart rate and blood pressure:* Local NAC infusion did not affect resting heart rate (325±38
242 bpm). Heart rate was only measured during the first 5 min of contraction, and was not
243 increased significantly from rest at this point (data not shown). Resting mean arterial pressure
244 (MAP) was not affected by local NAC infusion (Figure 3A), however local NAC infusion
245 attenuated the contraction induced increase in MAP by ~12 mmHg at *t*=20 min (P<0.05,
246 Figure 3A) suggesting some systemic affects of NAC infusion.

247

248 *Leg blood flow and vascular resistance:* Leg blood flow remained unchanged during the pre-
249 contraction infusion period (Figure 3B). Contraction increased femoral blood flow to the
250 contracted leg by ~400% (P<0.001; Figure 3B). Local NAC infusion did not affect the
251 contraction-induced increase in leg blood flow (P=0.78; Figure 3B). Pre-contraction vascular
252 resistance was reduced in NAC rest leg compared with saline infused rest leg (P=0.02, Figure
253 3C), however resting vascular resistance was not affected by local NAC infusion in contracted

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254 leg (P=0.19, Figure 3C). During contraction, vascular resistance was increased in the rest leg,
255 due to sympathetic outflow contributing to blood flow redistribution (45), and reduced in the
256 contracted leg (P<0.05, Figure 3C). Although NAC infusion appeared to attenuate (by ~60%
257 at *t*=20 min) vascular resistance in the resting leg during contraction, NAC did not
258 significantly alter vascular resistance during contraction (P=0.09 for interaction).

259

260 *Muscle and plasma thiols:* Muscle glutathione levels were measured as marker of cellular
261 oxidative state (29) since under conditions of oxidative stress reduced glutathione (GSH) is more
262 rapidly oxidized to oxidized glutathione (GSSG). Figure 4A, C and E show that muscle GSH,
263 GSSG, and GSSG/GSH ratio were not significantly affected by contraction or local NAC
264 infusion. NAC is rapidly deacylated to produce cysteine (10), and like NAC, cysteine can directly
265 scavenge ROS (2, 8). Therefore, cysteine levels were measured as a marker of NAC-enhanced
266 antioxidant defenses. Local NAC infusion increased muscle cysteine similarly by 50% in the rest
267 and contracted leg (P=0.001, Figure 4B). There was a tendency for local NAC infusion to increase
268 muscle cystine (P=0.07), and NAC infusion tended to increase the affect of contraction on muscle
269 cystine (P=0.08; Figure 4D). Contraction did not affect muscle cysteine or cystine concentrations
270 (Figure 4B and D). Local NAC infusion increased (P<0.05) plasma cysteine and cystine 360- and
271 1.4-fold, respectively (Figure 4F).

272

273 *Muscle glucose uptake:* The contracted leg had a muscle glucose uptake ~20-fold greater than
274 the rest leg (P<0.001; Figure 5A). Local NAC infusion did not affect muscle glucose uptake
275 in the rest or in the contracted leg (P>0.05, Figure 5A).

276

277 *Muscle S-glutathionylation and tyrosine nitration:* Contraction significantly (P<0.05)
278 increased muscle S-glutathionylation of protein bands at ~250 and 150 kDa in the contracted
279 saline infused leg ~1.7-fold and NAC infusion prevented this increase (P<0.05; Figure 5B).

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280 Neither contraction nor NAC affected protein S-glutathionylation of any other visible protein
281 bands. Muscle tyrosine nitration was not significantly affected by contraction or NAC
282 infusion (Figure 5C).
283
284 *AMPKα, ACCβ and p38 MAPK phosphorylation:* Contraction increased ($P<0.05$)
285 phosphorylation of AMPK (6-fold) and ACCβ (10-fold) (Figure 6A and B) and this increase
286 was not affected by local NAC infusion (Figure 6A and B). Similarly, contraction increased
287 ($P=0.002$) p38 MAPK phosphorylation (by ~60-fold) and this increase was not affected by
288 local NAC infusion ($P>0.05$; Figure 6C).
289

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290 **DISCUSSION**

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

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

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

337 It has been previously reported that the treatment of isolated mouse EDL muscles with
338 NAC attenuates increases in *ex vivo* contraction-induced oxidative stress and glucose uptake

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339 (39). The authors propose that because NAC also inhibited AMPK phosphorylation and
340 activity, ROS activate AMPK during contraction and this contributes to the activation of
341 skeletal muscle glucose uptake during *ex vivo* contractions (18, 39). Indeed, we have also
342 found that NAC attenuates increases in glucose uptake during *ex vivo* contractions in mouse
343 EDL and soleus muscles (26). However, here we report that local NAC infusion did not
344 affect the increase in skeletal muscle glucose uptake, AMPK phosphorylation, or the
345 phosphorylation of the major downstream target of AMPK, ACCβ during *in situ* contractions
346 (Figure 5 and 6). This suggests that the low levels of oxidative stress associated with
347 physiologically relevant contraction intensities is not involved in the regulation of skeletal
348 muscle glucose uptake or AMPK signaling.

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

361 Interestingly, NAC had a small but significant affect on blood pressure during
362 contraction. This suggests that NAC or ROS may have been exerting some systemic affects
363 during contraction, which is worthy of further investigation. However, it is unlikely that these

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364 small systemic effects can account for the finding that ROS are not involved in the regulation
365 of skeletal muscle glucose uptake during low-moderate intensity *in situ* contractions. It is
366 possible, however, that during exhaustive endurance exercise, or under the normal *ex vivo*
367 conditions of supra-maximal stimulation, non-uniform oxygen delivery (due to oxygen
368 diffusion limitations), and reduced antioxidant capacity (in the absence of anti-oxidant
369 systems found in blood) oxidative stress would be greatly elevated (33) and play a role in the
370 regulation of skeletal muscle glucose uptake via the activation of AMPK. Indeed it is during
371 high intensity exercise, which substantially elevates ROS production (32), when glucose
372 uptake (36) and AMPK activation (7) are greatest, and it is only at high concentrations that
373 exogenous ROS (H_2O_2; 3 mM) activate AMPK (14, 16, 46). Therefore, experimental
374 investigation is required to assess the role of ROS and oxidative stress in signaling glucose
375 uptake and AMPK activity during high intensity exercise in intact preparations with blood
376 flow. However, the contraction intensity required to increase skeletal muscle ROS levels to a
377 large enough extent to activate AMPK and increase glucose uptake as seen in *ex vivo*
378 preparations (39), is not likely to be physiologically realistic to humans. Therefore, the
379 hypothesis that ROS regulate skeletal muscle glucose uptake during contraction may be an
380 artifact of non-physiological *ex vivo* contraction conditions, and our results suggest that
381 mechanisms other than ROS regulate skeletal muscle glucose uptake during normal
382 submaximal muscle contractions.

383 It has been shown that the phosphorylation of p38 MAPK is increased by exogenous
384 ROS (19) and that the attenuation of exercise-induced oxidative stress by the xanthine oxidase
385 inhibitor, allopurinol, attenuates increases in p38 MAPK phosphorylation during exhaustive
386 exercise (11). Indeed, there is some evidence that the inhibition of p38 MAPK attenuates
387 glucose uptake during contraction (42), and ROS signaling via p38 MAPK may be involved
388 in regulating skeletal muscle stretch-induced glucose uptake (6). However, we show here that

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389 contraction can increase skeletal muscle p38 MAPK phosphorylation without significant
390 alterations in the GSSG/GSH ratio, and that local NAC infusion, which prevented S-
391 glutathionylation, does not affect p38 MAPK phosphorylation during contraction. This
392 suggests that during low-moderate intensity contractions ROS are not involved in the
393 regulation of p38 MAPK signaling. The inconsistency between the results of Gomez-Cabrera
394 et al. (11) and ours may be related to the use of a xanthine oxidase specific inhibition, rather
395 than the use of a general antioxidant such as NAC and requires further investigation.
396 Furthermore, the role of p38 MAPK in regulating skeletal muscle glucose uptake during
397 contraction, and potential interactions with ROS at high exercise intensities is worthy of
398 further investigation since some isoforms of p38 MAPK appear to play a greater role in
399 regulating glucose up than others (15, 42).

400 A small oxidative shift in cell redox is required for optimal skeletal muscle contraction
401 force, however, high levels of oxidative stress appear to contribute to the development of
402 muscular fatigue (33). NAC treatment has been shown to attenuate fatigue during longer
403 duration and/or more strenuous exercise/muscle contractions (20, 35) than used in the current
404 study by attenuating the exercise induced increase in oxidative stress (24, 43). Since our
405 contraction protocol did not alter skeletal muscle glutathione levels this suggests that the level
406 of oxidative stress was not sufficient to negatively impact on muscle function, and therefore
407 may explain why NAC did not affect the rate of fatigue during contraction (Figure1).

408 In conclusion, this study shows for the first time that local infusion of the antioxidant
409 NAC during *in situ* hindlimb contractions in rats does not attenuate increases in skeletal
410 muscle glucose uptake or AMPK signaling. Although our low-moderate intensity *in situ*
411 muscle contraction protocol, which is more relevant to normal submaximal exercise than
412 tetanic contractions associated with *ex vivo* studies, did not alter muscle GSH/GSSG levels or
413 tyrosine nitration, it did increase protein S-glutathionylation indicating small increases in

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414 muscle oxidative stress. NAC prevented the increases in S-glutathionylation during
415 contraction, but did not affect the large increases in skeletal muscle glucose uptake (20-fold)
416 or phosphorylation of AMPK and p38 MAPK. These results suggest that, unlike during
417 highly fatiguing *ex vivo* contraction conditions, ROS do not regulate skeletal muscle glucose
418 uptake or metabolic signaling during physiologically relevant, low-fatiguing, skeletal muscle
419 contractions *in situ* in rat.
420

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565 FIGURE LEGENDS
566
567 **Figure 1.** Force production of the hindlimb of rats when locally infused with NAC N-acetyl-
568 L-cysteine (NAC) or saline during 15 min of *in situ* contractions (see Methods for details).
569 Data are means ± SEM, n=4 per group, §P<0.05 for time.
570
571 **Figure 2.** Plasma (A) and, rest and contracted leg muscle (B) N-acetylcysteine (NAC) content
572 during *in situ* hindlimb contractions (taken at *t*=30 min) in rats receiving local NAC infusion
573 into the contracted leg. Data are means ± SEM, n=6-8 per group, #P<0.05 vs rest leg.
574
575 **Figure 3.** Effect of N-acetylcysteine (NAC) or saline infusion on mean arterial pressure (A),
576 and rest and contracted leg femoral blood flow (B), and vascular resistance (C) at rest and
577 during *in situ* hindlimb contractions in rats. Data are means ± SEM, n=6-8 per group, §P<0.05
578 for time, #P<0.05 for condition (rest vs contraction), *P<0.05 vs saline.
579
580 **Figure 4.** Effect of local N-acetylcysteine (NAC) or saline infusion on rest and contracted leg
581 muscle reduced glutathione (GSH) (A), cysteine (B), oxidized glutathione (GSSG) (C),
582 cystine (D) and GSSG/GSH ratio (E) following 15 min of *in situ* hindlimb contractions in the
583 contacted leg of rats, and plasma cysteine and cystine levels during contraction (at *t*=30 min)
584 (F). Data are means ± SEM, n=6-8 per group, *P<0.05 for treatment (NAC vs saline).
585
586 **Figure 5.** Effect of local N-acetylcysteine (NAC) or saline infusion on rest and contracted leg
587 muscle glucose uptake (A), S-glutathionylation (B) and tyrosine nitration (C) following 15
588 min of *in situ* hindlimb contractions in the contacted leg of rats. Data are means ± SEM, n=6
589 per group, #P<0.05 for condition (rest vs contraction), ‡P<0.05 vs saline of same condition,
590 †P<0.05 vs rest of same treatment.

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591

592 **Figure 6.** Effect of local N-acetylcysteine (NAC) or saline infusion on rest and contracted leg

593 AMPK Thr^{172} (A), ACCβ Ser^{222} (B) and p38 MAPK Thr^{180}/Tyr^{182} (C) phosphorylation

594 following 15 min of *in situ* hindlimb contractions in the contacted leg of rats. Data are means

595 ± SEM, n=6 per group, #P<0.05 for condition (rest vs contraction).

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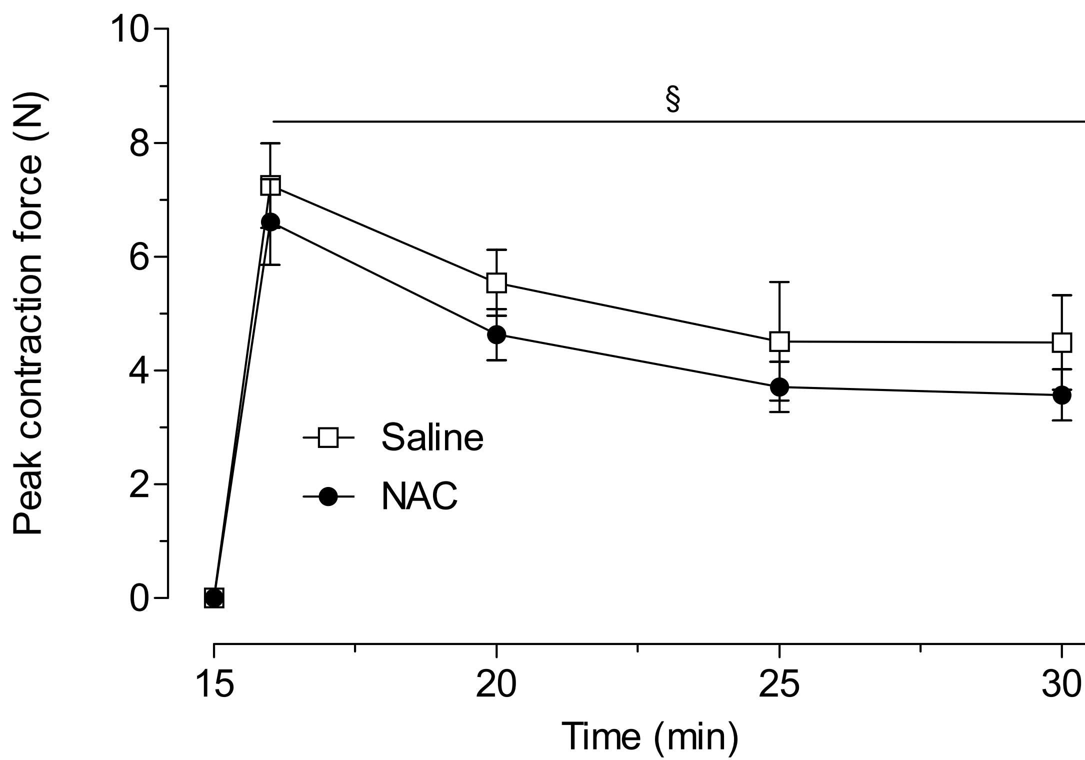

Figure 1.

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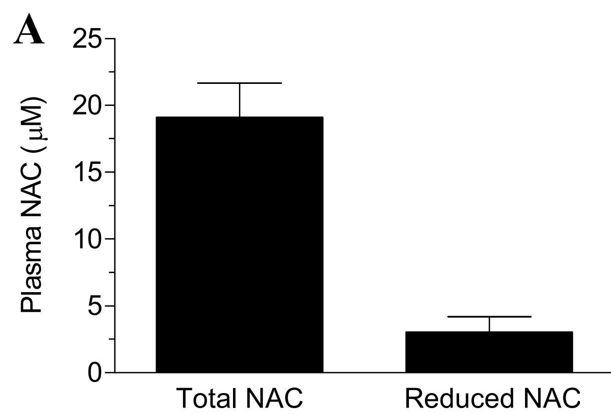

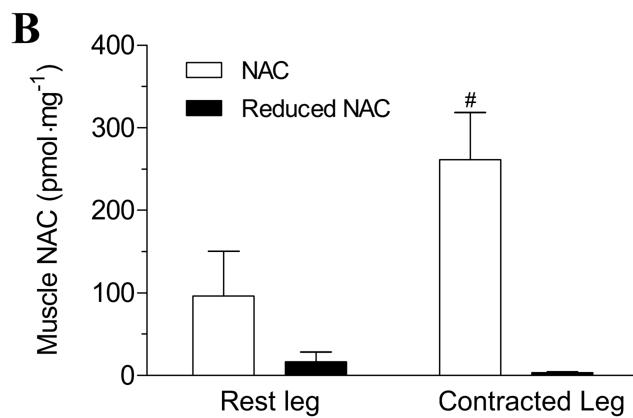

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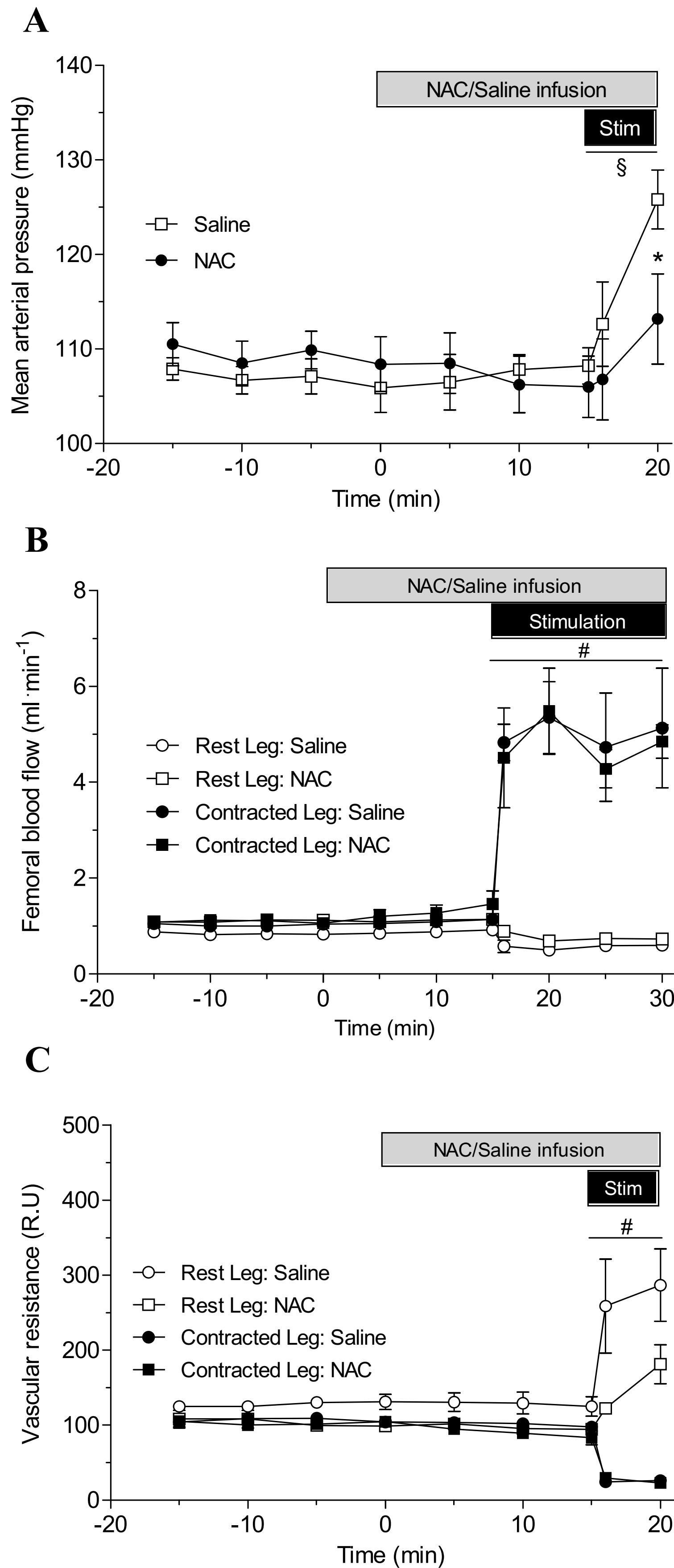

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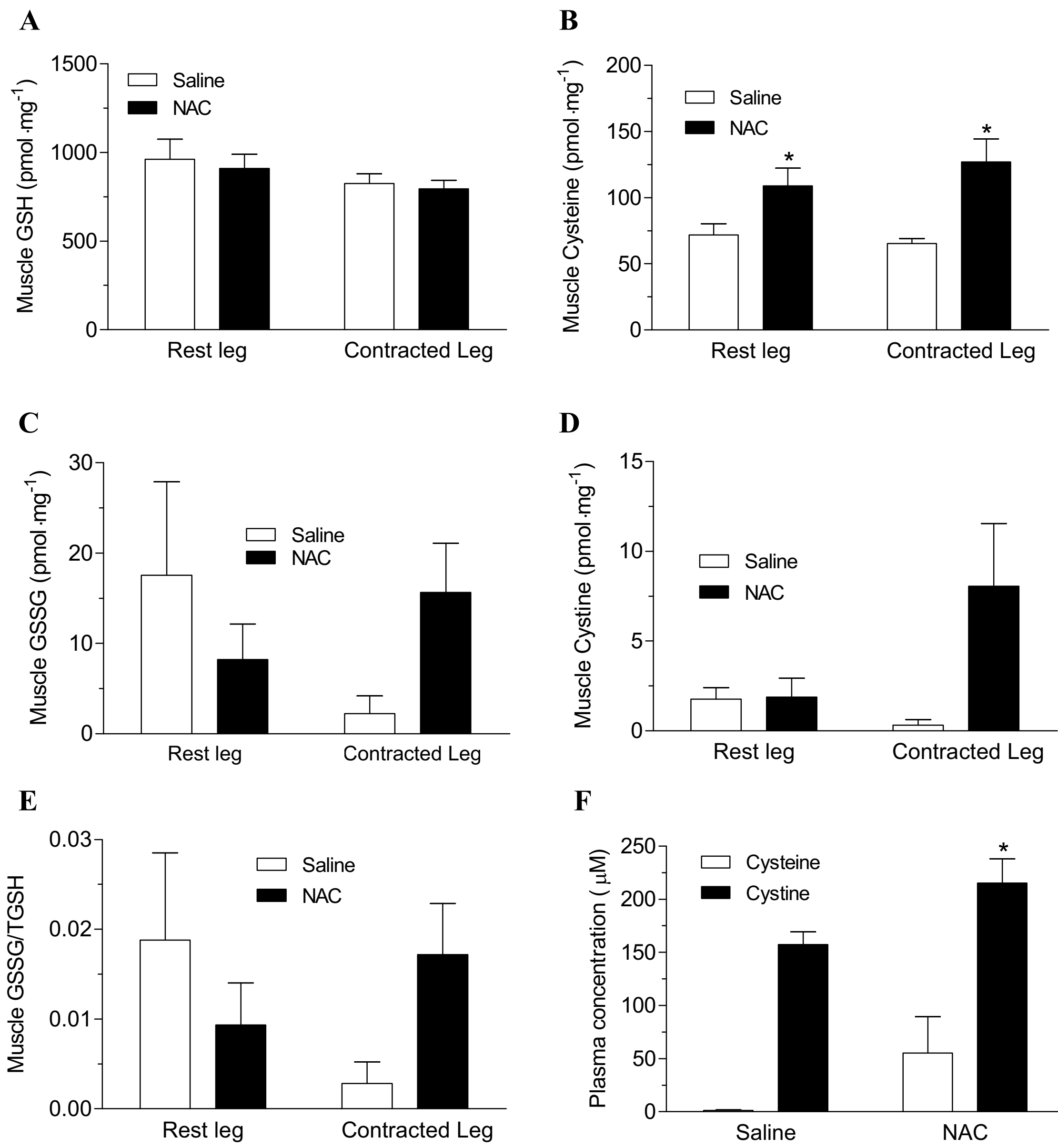

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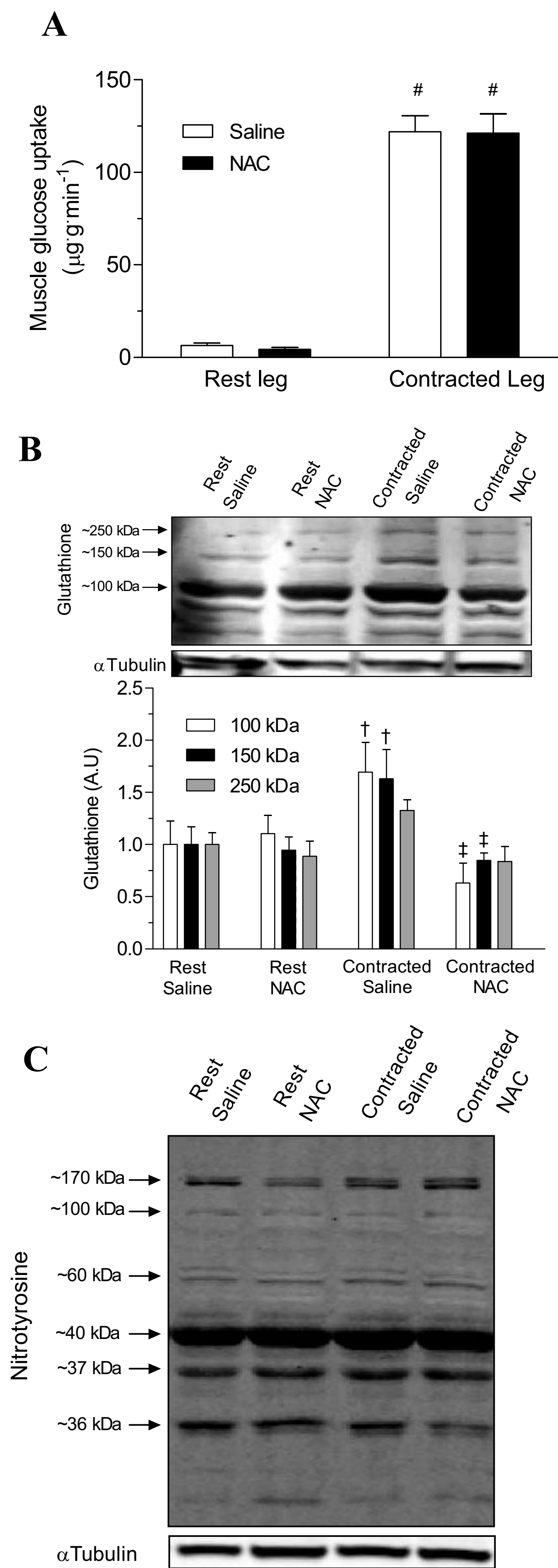

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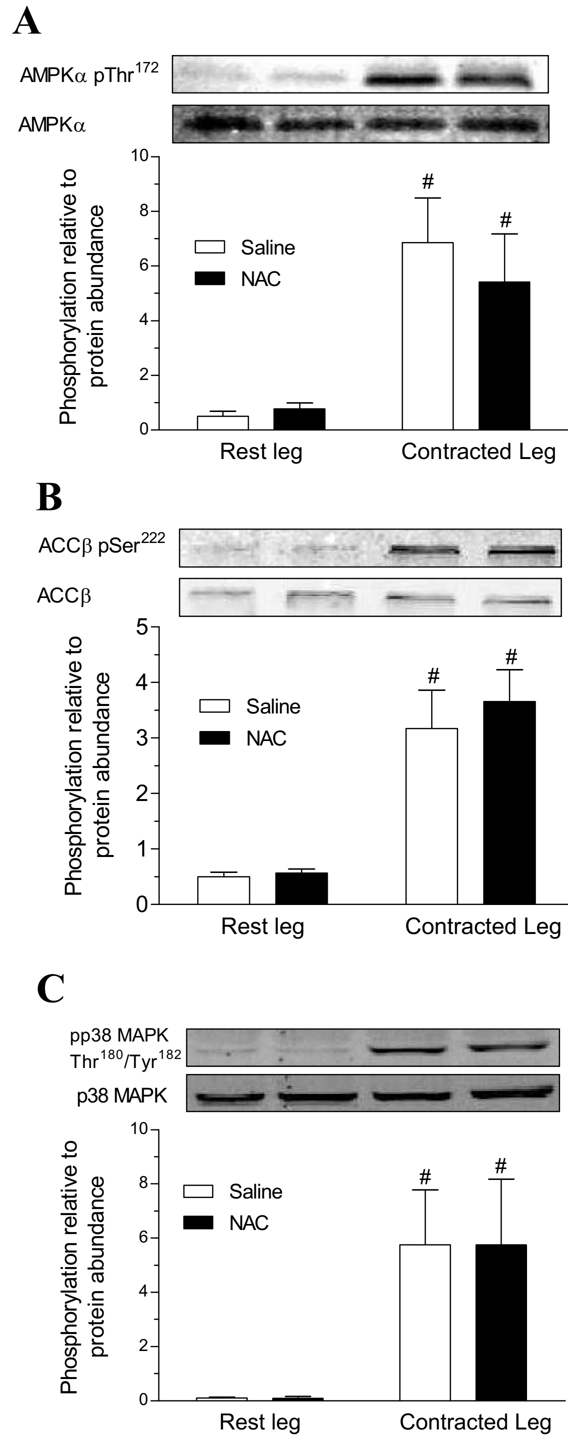

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