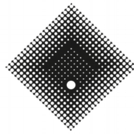

Nitric oxide is required for the insulin sensitizing effects of contraction in mouse skeletal muscle

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1 **Nitric Oxide is Required for the Insulin Sensitizing Effects of Contraction in Mouse**
2 **Skeletal Muscle**
3
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14
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23
24 Keywords: L-NMMA, muscle contraction, insulin sensitivity

Key points summary

- People with insulin resistance or type 2 diabetes can substantially increase their skeletal muscle glucose uptake during exercise and insulin sensitivity after exercise.
- Skeletal muscle nitric oxide (NO) is important for glucose uptake during exercise but how prior exercise increases insulin sensitivity is unclear.
- In this study we examined if NO is necessary for normal increases in skeletal muscle insulin sensitivity after contraction *ex vivo* in mouse muscle.
- Our study uncovers for the first time a novel role for NO in the insulin sensitizing effects of *ex vivo* contraction, which is independent of blood flow.

Abstract

The factors regulating the increase in skeletal muscle insulin sensitivity after exercise are unclear. We examined whether nitric oxide (NO) is required for the increase in insulin sensitivity after *ex vivo* contractions. Isolated C57BL/6J mouse EDL muscles were contracted for 10 min or remained at rest (basal) with or without the NO synthase (NOS) inhibition (L-NMMA; 100µM). 3.5 hrs post contraction/basal, muscles were exposed to saline or insulin (120µU/ml) with or without L-NMMA during the last 30 min. L-NMMA had no effect on basal skeletal muscle glucose uptake. The increase in muscle glucose uptake with insulin (57%) was significantly ($P<0.05$) greater after prior contraction (140% increase). NOS inhibition during the contractions had no effect on this insulin-sensitizing effect of contraction but NOS inhibition during insulin prevented the increase in skeletal muscle insulin sensitivity post-contraction. Soluble guanylate cyclase inhibition, PKG inhibition or PDE5 inhibition each had no effect on the insulin-sensitizing effect of prior contraction. In conclusion, NO is required for increases in insulin sensitivity several hours after contraction of mouse skeletal muscle via a cGMP/PKG independent pathway.

Abbreviation list:

EDL, extensor digitorum longus; eNOS, endothelial nitric oxide synthase; GLUT4, Glucose transporter type 4; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor-1; L-NMMA, the NO synthase (NOS) inhibitor N^G-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; T2D, type 2 diabetes (T2D)

61 ## Introduction

62

63 Increased physical activity is important for both the prevention and management of type 2
64 diabetes (T2D) (Wojtaszewski & Richter, 2006). After the initial insulin-independent
65 increases in glucose uptake post-contraction have worn off in 2-3 hrs (Gao *et al.*, 1994;
66 Funai *et al.*, 2010), skeletal muscle remains more sensitive to insulin for 24-48 hrs in both
67 rodents (Cartee *et al.*, 1989) and humans (Mikines *et al.*, 1988). Three to four hrs after a 60
68 min bout of single leg exercise in humans, glucose uptake during a hyperinsulinaemic
69 euglycaemic clamp ("insulin clamp") increases substantially more in the exercised leg than
70 the rested leg (Richter *et al.*, 1989; Wojtaszewski *et al.*, 2000). Importantly, acute exercise
71 increases skeletal muscle insulin sensitivity in both people with T2D and matched controls
72 (Devlin *et al.*, 1987). Although the insulin sensitizing effect of acute contraction/exercise
73 has been known for many years but the mechanisms involved are unclear.

74

75 Insulin activates insulin signalling pathways in skeletal muscle which results in GLUT-4
76 translocation to the plasma membrane and increased glucose transport. Even though there
77 are increases in insulin-stimulated glucose uptake after acute contraction or exercise, there
78 is little evidence of greater proximal insulin signalling (Wojtaszewski *et al.*, 2000;
79 Wojtaszewski & Richter, 2006). However, there are indications that more distal insulin
80 signalling may be increased by acute exercise (eg phosphorylation of Akt substrate of 160
81 KDa (AS160, also referred to as TBC1D4) (Arias *et al.*, 2007; Funai *et al.*, 2009; Treebak
82 *et al.*, 2009; Funai *et al.*, 2010; Castorena *et al.*, 2014; Kjobsted *et al.*, 2015; Sjoberg *et al.*,
83 2017). Six to 24 hrs after an acute exercise bout increases in protein expression of some of
84 key proteins such as GLUT-4 are sometimes observed (Hood, 2001). Since this introduces
85 a confounding variable, studies attempting to uncover the mechanism(s) that acute exercise
86 increases skeletal muscle insulin sensitivity are generally conducted 3-4 h after exercise
87 (Richter *et al.*, 1989; Wojtaszewski & Richter, 2006).

88

89 Although never specifically examined, there are some findings in the literature which
90 suggest that increases in nitric oxide (NO) during contraction/exercise could be involved in

91 the increase in insulin sensitivity after contraction/exercise. Both nNOS and eNOS
92 deficient mice are insulin resistant (Shankar *et al.*, 2000) and eNOS deficient mice
93 supplemented with nitrate (NO_3), an inorganic anion abundant in vegetables which can be
94 converted *in vivo* to NO, improves glucose tolerance (Carlstrom *et al.*, 2010). In addition,
95 the content of nNOS in skeletal muscle tends to change in parallel with skeletal muscle
96 insulin sensitivity (Shankar *et al.*, 2000; Bradley *et al.*, 2007). Supporting this notion we
97 have found that endurance trained humans, who are known to be insulin sensitive, have
98 increased skeletal muscle nNOS protein (McConell *et al.*, 2007), while people with insulin
99 resistance/T2D have reduced nNOS protein levels (Bradley *et al.*, 2007). Acute and long-
100 term administration of L-Arginine, the substrate for NO formation from NOS, improves
101 insulin secretion and insulin sensitivity in healthy people and in people with diabetes (Piatti
102 *et al.*, 2001). NO also increases insulin transport in endothelial cells in skeletal muscle
103 (Wang *et al.*, 2013), and therefore presumably skeletal muscle insulin exposure. Finally,
104 we have shown that NO synthase (NOS) inhibition attenuates increases in skeletal muscle
105 glucose uptake during contraction in mice and rats (Stephens *et al.*, 2004; Ross *et al.*, 2007;
106 Merry *et al.*, 2010b) and during exercise in healthy controls and in people with T2D
107 (Bradley *et al.*, 1999; Kingwell *et al.*, 2002). Therefore, we hypothesized that NOS
108 inhibition during contraction would attenuate the increase in insulin sensitivity 3.5 hrs after
109 *ex vivo* contraction. *Ex vivo* contractions were chosen since this eliminates any potential
110 confounding effects of blood flow.
111

112 # Methods

113

114 ## Ethical approval

115 Animal care and experimental protocols and collection of human serum for this study were
116 approved by both the Animal Experimentation Ethics Committee and the Human Research
117 Ethics Committee of Victoria University and conformed to the Australian National Code of
118 Practice for the Care and Use of Animals for Scientific Purposes, as described by the
119 National Health and Medical Research Council (NHMRC) of Australia.

120 .

121 ## Animals and experimental design

122

123 12 to 14 week old male C57BL/6J mice were purchased from Animal Resources Centre
124 (Perth, WA, Australia). The mice were individually housed in groups of 2-4 and
125 maintained in an environmentally controlled animal room at 21° C with a 12:12 h light-dark
126 cycle with *ad libitum* access to standard rodent chow (Specialty Feeds, Western Australia)
127 and water. Food was removed from 8:30am to 12:30pm on the day of an experiment. After
128 mice were deeply anesthetized with pentobarbital sodium (26 G needle, 60 mg/kg
129 intraperitoneal; Rhone Merieux, Pinkenba, Queensland, Australia), mice were constantly
130 monitored for depth of anaesthesia by monitoring their plantar flexion and response to tail
131 and paw pinch. When slight reflex/response was detected, supplemented doses (1/10 of
132 original dose) of anaesthesia were administered before tissue removal. Under deeply
133 anaesthetized, the skin of the hind limbs were removed exposing the limb muscles.
134 Extensor digitorum longus (EDL) muscles were carefully excised from the mice. Following
135 the removal of muscles, whilst deeply anaesthetized, the mice were humanely killed by
136 decapitation.

137

138 ## Materials and antibodies

139 All chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO) unless
140 otherwise stated. 2-Deoxy-D-[1,2-^{3}H]-glucose and D-[1-^{14}C] Mannitol were purchased
141 from Perkin Elmer (Waltham, MA). Reagents and apparatus for SDS-PAGE and

142 immunoblotting were purchased from Bio-Rad (Hercules, CA). RED 660 Protein Assay
143 Reagent Kit and Neutralizer were purchased from GBiosciences (St. Louis, MO).
144 SuperSignal West Femto Chemiluminescent Substrate was provided by Thermo Scientific
145 (Waltham, MA). Primary antibodies for p-Akt (Ser473 and Thr308), Akt, p-TBC1D1
146 (Thr590, Thr596 and Ser660), TBC1D1, p-TBC1D4 (Thr642), TBC1D4 and actin used in
147 Western Blotting were purchased from Cell Signalling Technology (Danvers, MA). HRP
148 conjugated Goat anti-Rabbit IgG (H+L) Secondary Antibody was from Thermo Scientific
149 (Waltham, MA).
150
151 **Collection and treatment of serum**
152 As previously reported (Gao *et al.*, 1994), a serum factor is required for an increase in
153 insulin sensitivity after *ex vivo* rat skeletal muscle contraction, and we also found that
154 serum alone has no effect on mouse skeletal muscle glucose uptake at rest (Levinger *et al.*,
155 2016).Whether serum is required during *ex vivo* contraction of mouse skeletal muscle for
156 increases in insulin-stimulated glucose uptake has not previously been examined. After an
157 overnight fast, blood was collected from 4 healthy men via venepuncture. The blood was
158 allowed to clot at room temperature then centrifuged at 3,000g for 30 min. The serum was
159 collected and frozen at -80°C until use. All serum used was from the same individuals.
160 Repeat freeze-thawing of serum was avoided.
161
162
163 **Muscle dissection, incubation and contraction**
164 Under deep anaesthesia, both EDL muscles were rapidly dissected. The proximal and distal
165 tendons were tied using 5/0 silk suture with two small aluminum hooks tied to each tendon.
166 For all incubation steps, buffer was continuously maintained at 30°C (Merry *et al.*, 2010b)
167 and gassed with carbogen (Carbogen; BOC Gases, Australia). Muscles were pre-incubated
168 with or without 50% human serum in *Buffer 1* [Krebs-Henseleit buffer (KHB in mM: 119
169 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, pH 7.4) + 0.01% BSA +
170 2 mM glucose + 8 mM mannitol] for 30 min. For muscle contraction, muscles were
171 mounted in incubation chambers containing *Buffer 1* with or without serum and stimulating

172 platinum electrodes (Zultek Engineering, Australia), and stimulated for 10 min with the
173 following parameters (12 V, train durations: 350 ms at a frequency of 60 Hz, 12
174 contractions/min) (Merry *et al.*, 2010b). Non-contracted muscles were treated the same as
175 contracted muscles except that they were not stimulated to contract. Muscles were
176 incubated in the presence or absence of the NOS inhibitor L-NMMA (100µM; (Merry *et*
177 *al.*, 2010a)) during the pre-incubation and contraction periods (See Fig. 1).

178

179 **Muscle treatment post-electrical stimulation and glucose uptake measurements**

180 Immediately after electrical stimulation, all muscles (regardless of whether the previous
181 incubation was with or without L-NMMA) were transferred to a vial containing *buffer 1* for
182 a 1-min wash. Muscles were then transferred to other baths containing *buffer 1* for 3 hrs
183 with the -buffer changed every 30 min.

184 After 3 hrs all muscles were incubated with *Buffer 2* containing 2 mM pyruvate +8 mM
185 mannitol with or without insulin for 30 min. For glucose uptake analysis, all muscles were
186 incubated for 10 min with *buffer 3* containing 2 mM 2-Deoxy-D-[1,2-^{3}H]-glucose (2-DG,
187 0.256µCi/ml) and 16 mM D-[1-^{14}C] Mannitol (0.166µCi/ml), and insulin, if it was present
188 during the previous incubation with *buffer 2*. For some muscle pairs L-NMMA (100µM)
189 was also present during this incubation.

190 To determine whether NO during insulin exposure was acting through the NO/cGMP/PKG
191 pathway, the GC inhibitor 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ, which
192 blocks the NO-mediated increase in cGMP, 10 µM (Merry *et al.*, 2010a)), or the
193 phosphodiesterase type 5 inhibitor (T-1032, which inhibits cGMP breakdown and therefore
194 raises cGMP levels, 27 µM (Mahajan *et al.*, 2003)) or the cGMP-dependent protein kinase
195 (PKG) inhibitor (Rp-8-Br-PET-cGMPS, 5 µM (Merry *et al.*, 2010a)) were used to block of
196 the NO/cGMP/PKG pathway (Fig. 3). The concentrations of ODQ and Rp-8-Br-PET-
197 cGMPS used in this study were based on our previous studies using isolated *ex vivo*
198 muscles (Merry *et al.*, 2010a; Merry *et al.*, 2010b). In addition, the PDE 5 inhibitor T1032
199 was used in our study rather than another PDE-5 inhibitor, zaprinast, since zaprinast has
200 been shown in our previous study to have no inhibitory effect on insulin-mediated glucose
201 uptake by muscles *in vivo,* while T-1032 showed the inhibitory effects (Mahajan *et al.*,

202 2003). The muscle pairs were incubated in the presence or absence of the inhibitor ODQ,
203 or T-1032 or Rp-8-Br-PET-cGMPS during the period of 30 min of insulin and 10 min of 2-
204 DG incubation.
205 Given that 120 μU/ml of insulin results in maximum insulin-stimulated glucose uptake
206 (Hamada *et al.*, 2006), it was anticipated that both ODQ and Rp-8-Br-PET-cGMPS would
207 attenuate the increase in insulin-stimulated glucose uptake after contraction, thus 120
208 μU/ml of insulin was used for ODQ and Rp-8-Br-PET-cGMPS treatments. On the other
209 hand, given we anticipated that T-1032 would increase insulin-stimulated glucose uptake
210 after contraction, we used a submaximal dose of insulin (60 μU/ml) (Hamada *et al.*, 2006)
211 with T-1032 treatment to provide a greater opportunity to observe any increase in glucose
212 uptake.
213 After the 10 min incubation with radioisotopic tracers, muscles were rapidly rinsed,
214 trimmed and cut in halves and frozen in liquid nitrogen. One half was kept for
215 immunoblotting and the other half for glucose uptake determination. The muscle for
216 glucose uptake were homogenized in 1M NaOH at 95°C for 10 min and then neutralized by
217 1 M HCl followed by centrifuge. The supernatant (200μl) was added to 4 ml of liquid
218 scintillation cocktail (PerkinElmer, Boston, MA). Radioactivity of both tracers was
219 measured by a β scintillation counter (Tri-Carb 2910TR, PerkinElmer), and glucose uptake
220 was calculated as previously described (Merry *et al.*, 2010a; Zhang *et al.*, 2011).
221
222 **NOS activity assay**
223 NOS activity was determined in separate EDL muscles based on the catalytic reaction of
224 NOS converting radiolabeled L-[^{14}C] arginine to radiolabeled L-[^{14}C] citrulline, as
225 described previously (Merry *et al.*, 2010a). NOS activity was determined from the
226 difference between samples incubated with and without L-NAME and was expressed as
227 picomoles of L-[^{14}C] citrulline formed per minute per milligram of muscle protein.
228
229 **Sample Preparation and Immunoblotting**
230 Sample preparation for immunoblotting was initially described by Murphy RM (Murphy,
231 2011). Briefly, 10 20-μm thickness muscle sections were homogenized with 100μl of

232 solubilizing buffer (0.125 M Tris–Cl [pH 6.8], 4% w/v SDS, 10% glycerol, 10 mM EGTA,
233 0.1 M DTT (dithiothreitol) and protease inhibitor cocktail). Protein concentration was
234 determined by a Red 660 assay kit (G-Biosciences, St. Louis, MO). Proteins (5 μg loaded
235 per well) were separated with 10% SDS–PAGE gels, then transferred to PVDF for 120 min
236 at 100 V. Following transfer, the membrane was blocked with 5% (w/v) skim milk powder
237 dissolved in TBST (Tris-Buffered Saline, 0.1% Tween-20) at room temperature for 1 h.
238 The primary antibodies were diluted in 5% (w/v) BSA in TBST and applied and incubated
239 overnight at 4°C. After a1 h incubation with secondary antibody at room temperature,
240 images were exposed to SuperSignal West Femto Chemiluminescent Substrate and
241 VersaDoc™ Imaging System and densitometry was performed using the Quantity One
242 software (Bio-Rad Laboratories, Hercules, CA, USA). All phosphorylation data is
243 presented relative to the total protein of the protein of interest.
244
245 **Statistical analysis**
246 All data are expressed as means ± SEM. Statistical testing was performed with SPSS
247 statistical package 22 or Graph Pad Prism 6. For multiple comparisons, one-way ANOVA
248 and two-way ANOVA with or without repeat measurement (between factor: insulin and
249 treatment condition – for glucose uptake and protein expression) were used. Tukey's post
250 hoc test or Fisher's LSD testwas performed when ANOVA revealed significance. The
251 Statistical significance was accepted at $p \leq 0.05$.

252 ## Results

253

254 **The effect of serum exposure during *ex vivo* contraction on mouse skeletal muscle**
255 **insulin sensitivity 3.5 hrs post-contraction**

256 First we examined the effect of serum on mouse skeletal muscle insulin-stimulated glucose
257 uptake post *ex vivo* contraction with slight modifications to that which has been previously
258 described (Funai *et al.*, 2010) (Fig. 1A). It has been previously shown during an insulin
259 dose response (0, 60, 120 and 20,000 µU/ml) that glucose uptake in isolated mouse skeletal
260 EDL from sedentary mice is maximal at 120 µU/ml and tends (P=0.08) to be increased at
261 the submaximal dose of 60 µU/ml (Hamada *et al.*, 2006). In addition, Kjobsted et.al
262 recently reported that submaximal insulin (100 µU/ml) and to a greater extent maximal
263 insulin (10,000 µU/ml), enhance glucose uptake *ex vivo* in isolated EDL muscle from wild
264 type mice 3 hours after *in situ* contraction (Kjobsted *et al.*, 2017).

265 We anticipated that L-NMMA would attenuate the insulin-stimulated glucose uptake after
266 prior contraction. Therefore, 120 µU/ml of insulin was used in our study except where
267 indicated. Our data showed that electrical stimulated contraction in serum-free buffer did
268 not increase basal (no insulin) or 120µU/ml insulin-stimulated skeletal muscle glucose
269 uptake in mouse EDL measured 3.5 hrs post electrical stimulation (Fig.1B). In contrast,
270 stimulation of glucose uptake by insulin was markedly enhanced ($p<0.05$) 3.5 hrs post *ex*
271 *vivo* contractile activity in muscles stimulated to contract while immersed in 50% human
272 serum in buffer 1 (Fig. 1B). Therefore, 50% human serum in buffer 1 was used for all
273 experiments, which differs to the 100% serum used previously in rats (Gao *et al.*, 1994;
274 Funai *et al.*, 2010).

275

276 **NOS inhibition during insulin exposure blocks the increase in the insulin-stimulated**
277 **glucose uptake after contraction**

278 As we have shown that NO synthase (NOS) inhibition attenuates the increase in skeletal
279 muscle glucose uptake during contraction in mice and rats (Stephens *et al.*, 2004; Ross *et*
280 *al.*, 2007; Merry *et al.*, 2010b) and during exercise in healthy controls and in people with

281 T2D (Bradley *et al.*, 1999; Kingwell *et al.*, 2002), in order to examine whether NO is
282 required for the increase in insulin sensitivity post *ex viv*o contraction (Fig. 2A), muscles
283 were treated with the NO synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA,
284 100 μM) either 1) during the period of the pre-incubation (30 min) and the muscle
285 contraction (10 min) (NOS inhibition during contraction), or 2) during vehicle or the
286 120μU/ml insulin incubation (30 min) and 2-DG tracer incubation (10 min); NOS
287 inhibition during contraction). In the absence of insulin skeletal muscle glucose uptake was
288 similar (P> 0.05) 3.5 hrs after no contraction, contraction, NOS inhibition during
289 contraction and NOS inhibition during insulin (Fig. 2B). This indicates that the effect of
290 prior contraction had worn off. Contraction significantly (P<0.01) increased insulin-
291 stimulated glucose uptake 3.5 hrs post-contraction and this increase was not affected by
292 NOS inhibition during the pre-incubation and contraction periods (Fig. 2B). Surprisingly,
293 NOS inhibition during insulin (and 2-DG tracer) incubation prevented the increase in
294 insulin-stimulated glucose uptake in response to prior contraction (Fig. 2B). The
295 incremental (delta) increase in insulin-stimulated glucose uptake (insulin-stimulated
296 glucose uptake minus basal glucose uptake) was significantly higher in the contraction and
297 the contraction plus NOS inhibition during contraction groups than the non contraction and
298 contraction plus NOS inhibition during insulin groups(Fig. 2B).
299
300
301 **NOS activity**
302 NOS activity was significantly reduced by NOS inhibition during insulin treatment to a
303 level significantly below the basal state (Fig. 2C). NOS activity has a tendency to increase
304 in the NOS inhibition during contraction group although this was not significant (P=0.08)
305 (Fig. 2C).
306
307 **The NO-mediated insulin-sensitizing effect of prior contraction does not involve**
308 **cGMP/PKG downstream signaling**
309 Since NO signalling involves activation of the soluble form of guanylate cyclase to produce
310 cGMP, the NO/cGMP/PKG signalling pathway is generally considered to be the major

downstream target of NO (Stamler & Meissner, 2001) (Fig. 3A). To explore the mechanism(s) that NO acts to increase insulin-stimulated skeletal muscle glucose uptake post contraction, and specifically whether this NO signalling is through cGMP/PKG, the soluble guanylate cyclase (sGC) inhibitor ODQ (which blocks the NO-mediated increase in cGMP), the PDE 5 inhibitor T1032 (which inhibits cGMP breakdown and therefore raises cGMP levels) and the cGMP-dependent protein kinase (PKG) inhibitor Rp-8-Br-PET-cGMPS were applied to block this pathway as per our previous studies (Mahajan *et al.*, 2003; Merry *et al.*, 2010a; Merry *et al.*, 2010b). We found that the insulin sensitizing effects of prior contraction were not affected by the presence of these inhibitors during insulin incubation 3.5 hrs post-contraction (Fig. 3B).

To exclude the possibility that there was a physical interaction between insulin and the inhibitors which may have prevented them having an effect on insulin-stimulated glucose uptake, the resting muscles were co-incubated with or without L-NMMA, ODQ or T1032 with insulin for 30 min, then were incubated with [^{3}H]-2-deoxyglucose and [^{14}C]-mannitol for 10 min to measure glucose uptake (Fig. 3C). As can be seen in Fig. 3D, there was no difference between insulin and insulin plus any of these inhibitors, indicating that no physical interaction could explain the effect of L-NMMA and the lack of effect of these other agents.

Insulin signalling

There was little Akt Thr308 and Akt Ser473 phosphorylation in the absence of insulin and no significant differences between the treatments (Fig. 4). Insulin significantly ($P<0.001$) increased phosphorylation of Akt at both Thr308 and Ser473 with no differences observed between the four treatments (Fig. 4B-C). Insulin significantly increased phosphorylation of TBC1D1 at Thr590 ($P<0.01$) and Thr596 ($P<0.001$) but not at Ser660 with no greater insulin-stimulated phosphorylation at these sites 3.5hrs following prior contraction (Fig. 5A-D). Although TBC1D4 Thr642 phosphorylation *per se* did significantly increase with insulin (data not shown, $P<0.05$), given the variability of the total TBC1D4 data (data not shown, $P>0.05$), this increase was not significant when TBC1D4 Thr642 phosphorylation was presented relative to the total TBC1D4 (Fig. 5E-F). NOS inhibition either during

341 contraction or during insulin had no significant effect on TBC1D1 or TBC1D4
342 phosphorylation at the sites that we examined (Fig. 5).

343 ## Discussion

344

345 We report that in mouse muscle, as has been shown in rat muscle, *ex vivo* contraction
346 increases insulin sensitivity several hours after contraction. In contrast to our hypothesis,
347 NOS inhibition during contraction had no effect on insulin-stimulated glucose uptake 3.5
348 hrs later. However, remarkably, NOS inhibition during the insulin treatment 3.5 hrs after
349 contraction prevented the insulin sensitizing effect of the prior contraction. Our results also
350 suggest that nitric oxide's effects on insulin sensitivity after contraction may not act via the
351 classic NO/cGMP/PKG signalling pathway. Given that the measurements were conducted
352 in isolated muscles, these observed effects of NOS inhibition cannot be due to alterations in
353 other confounders such as blood flow so must relate to muscle effects per se.

354

355 Several previous studies in rats (Gao *et al.*, 1994; Funai *et al.*, 2010) have reported that *ex*
356 *vivo* muscle contraction increases skeletal muscle insulin-stimulated glucose uptake ~3 hrs
357 later, which is consistent with human exercise studies (Richter *et al.*, 1989; Wojtaszewski *et*
358 *al.*, 2000). Our results extend these findings to mice which is important because this means
359 that studies with genetically modified mice are now possible. As has been shown in rats
360 (Gao *et al.*, 1994; Funai *et al.*, 2010), we found in mice that it was necessary to include
361 serum during the *ex vivo* muscle contractions in order to observe the insulin sensitizing
362 effects of contraction. Furthermore, we found that a mixture of 50% serum with 50% KHB
363 buffer rather than 100% serum as used in rats was sufficient to induce greater insulin-
364 stimulated glucose uptake ~3 hrs after *ex vivo* contraction in mouse skeletal muscle (Fig. 1).

365

366 NOS inhibition during contraction in mice and during exercise in humans attenuates the
367 increase in glucose uptake during contraction/exercise (Bradley *et al.*, 1999; Kingwell *et*
368 *al.*, 2002; Ross *et al.*, 2007; Merry *et al.*, 2010a; Merry *et al.*, 2010b). As such, we
369 hypothesized that NOS inhibition during contraction would attenuate the increase in insulin
370 sensitivity 3.5 hrs after contraction. However, our hypothesis was not confirmed as NOS
371 inhibition during contraction had no effect on later insulin sensitivity. We have found
372 previously that addition of L-arginine can overcome the inhibitory effects of NOS

373 inhibition during contraction (Hong *et al.*, 2015). Therefore, it is possible that the effects of
374 the NOS inhibitor were somewhat nullified by the presence of serum during contraction
375 because L-arginine is present in healthy human serum at a concentration of ~100 µM.
376
377 Importantly, NOS inhibition during insulin incubation blocked the increase in insulin
378 sensitivity in response to earlier contraction (Fig.2B). The mechanism(s) involved are
379 unclear at this stage. The relationship between skeletal muscle, NO production, NOS
380 activity, diabetes, exercise and insulin sensitivity are complex. Insulin has been shown to
381 increase nNOS phosphorylation in C_2C_{12} muscle cells and in mouse skeletal muscle
382 (Hinchee-Rodriguez *et al.*, 2013) and skeletal muscle NOS activity increases during a
383 euglycaemic hyperinsulinaemic clamp in healthy humans (Kashyap *et al.*, 2005). Therefore,
384 it is possible that insulin activates increases in skeletal muscle NO production to increase
385 glucose uptake and that the NOS inhibitor then prevented this effect. Indeed, in line with
386 the prevention of the contraction-stimulated increase in insulin sensitivity, NOS activity
387 was significantly reduced in the presence of NOS inhibition during insulin treatment (Fig.
388 2C).
389
390 Most studies in rodents and humans find little effect of prior exercise or contraction on
391 proximal insulin signalling (Wojtaszewski *et al.*, 2000; Hamada *et al.*, 2006; Funai *et al.*,
392 2010; Castorena *et al.*, 2014). In line with this, we found there was no difference in insulin-
393 stimulated Akt phosphorylation with or without prior *ex vivo* contraction (Fig. 4). Despite
394 unaltered proximal signalling, some studies have reported greater downstream insulin
395 signalling at the level of TBC1D4 3 hrs after exercise in rats and humans (Funai *et al.*,
396 2009; Treebak *et al.*, 2009; Castorena *et al.*, 2014). Although previous studies found
397 increases in mouse EDL TBC1D4 Thr642 phosphorylation with insulin (Chen *et al.*, 2011;
398 Kjobsted *et al.*, 2015; Kjobsted *et al.*, 2017), in the current study we found no significant
399 increase in TBC1D4 Thr642 phosphorylation with insulin when TBC1D4 Thr642
400 phosphorylation was presented relative to the total TBC1D4. However, TBC1D4 Thr642
401 phosphorylation *per se* did increase with insulin but given variability with total TBC1D4,

402 this effect was lost when TBC1D4 Thr642 phosphorylation was divided by total TBC1D4
403 (Fig. 5F).
404
405 It has now been shown in our human study that skeletal muscle pTCB1D4 Thr704
406 (pTCB1D4 Thr711in mice) is increased 4 hours after exercise (Sjoberg *et al.*, 2017). In
407 addition, the increase in pTCB1D4 Thr704 during a euglycemic hyperinsulinemic clamp is
408 greater in previously exercised muscle than in non-exercised muscle in humans (Sjoberg *et*
409 *al.*, 2017). It is not known if similar responses of pTCB1D4 Thr711 are observed in mice
410 as unfortunately an antibody for TBC1D4 704/711 phosphorylation was not commercially
411 available when we conducted this study. Future mouse studies should examine this site.
412
413 It is important to note that Funai et al (Funai *et al.*, 2010) reported additive effects of prior
414 *in vivo* exercise and *ex vivo* contraction on insulin stimulated glucose uptake, suggesting
415 that *in vivo* exercise and *ex vivo* contraction may enhance insulin sensitivity by different
416 mechanisms. Along these lines, we recently found that NOS inhibition in humans
417 overcomes the greater insulin sensitivity in a leg that exercise 4 hrs earlier compared with a
418 rested leg. In that study (Sjoberg *et al.*, 2017), like in this study, NOS inhibition had no
419 effect on insulin signalling in either the contracted on non-contracted muscle. However, in
420 that study it appeared that the reduction in blood flow with NOS inhibition, especially in
421 microvascular blood flow, was the major reason for the NOS inhibition, like in the current
422 study, overcoming/preventing the increased insulin sensitivity due to earlier exercise.
423 However, in the current study there is no blood flow component. These results support the
424 suggestion that *in vivo* exercise and *ex vivo* contraction may enhance insulin sensitivity by
425 different mechanisms, with both involving NO. Further research is required to clarify this.
426
427 Akt, TBC1D1 and TBC1D4 phosphorylation were not affected by NOS inhibition during
428 insulin treatment and therefore do not appear to account for the observed effects of NOS
429 inhibition preventing the increase in insulin sensitivity after contraction. The mechanisms
430 responsible for this remarkable effect of NOS inhibition on insulin-stimulated glucose
431 uptake after contraction are not clear. Recent evidence indicates that the cytoskeleton is

432 important for skeletal muscle glucose uptake in response to both contraction and insulin (Su
433 *et al.*, 2005; Wang, 2011; Sylow *et al.*, 2013a) and given that skeletal muscle nNOS is
434 associated with the cytoskeleton (Percival *et al.*, 2010), it is possible that this could be
435 playing a role. Depolymerization of the actin cytoskeleton decreases glucose uptake (Sylow
436 *et al.*, 2013b) and rearrangement of the actin cytoskeleton by Rac1 (Ras-related C3
437 botulinum toxin substrate 1), a small Rho family GTPase, is necessary for insulin-
438 stimulated GLUT4 translocation in L6 myotubes (Ueda *et al.*, 2008). In addition, Rac1 and
439 its downstream target, PAK1, are activated by contraction/exercise in human and mouse
440 skeletal muscle and insulin-stimulated GLUT4 translocation is impaired in skeletal muscle
441 from Rac1 knockout mice (Sylow *et al.*, 2013a; Sylow *et al.*, 2013b). Inhibition of Rac1 or
442 Rac1 knockout reduces both contraction-stimulated and insulin-stimulated glucose uptake
443 in mouse muscle (Sylow *et al.*, 2013a; Sylow *et al.*, 2013b). There is also some evidence of
444 interactions between Rac1 and NO, including in C_2C_{12} muscle cells (Su *et al.*, 2005; Cheng
445 *et al.*, 2006; Godfrey & Schwarte, 2010). Follow up studies should examine whether NOS
446 inhibition during insulin exposure attenuates increases in pPAK1 after prior *ex vivo* skeletal
447 muscle contraction.
448
449 The cGMP/PKG pathway, which is present in skeletal muscle, is generally considered to
450 be the major downstream signaling pathway of NO (Stamler & Meissner, 2001). However,
451 modification of cGMP/PKG signalling with the soluble guanylate cyclase inhibitor ODQ
452 (guanylate cyclase produces cGMP in response to NO), the PDE 5 inhibitor T1032 (PDE5
453 breaks down cGMP) and the cGMP-dependent protein kinase (PKG) inhibitor Rp-8-Br-
454 PET-cGMPS, had no significant effect on the insulin-sensitizing effects of prior contraction
455 in mouse muscle *ex vivo* (Fig. 3A-B). These results suggest that NO increases skeletal
456 muscle insulin sensitivity post-contraction via cGMP/PKG independent mechanism(s). This
457 is similar to what we have found previously during *ex vivo* contractions where L-NMMA
458 attenuates the increase in skeletal muscle glucose uptake during *ex vivo* contractions but
459 there is no effect of inhibition of sGC or PKG (Merry *et al.*, 2010a). Moreover, Wang et al.
460 (Wang *et al.*, 2013) found in endothelial cells and Kaddai et al (Kaddai *et al.*, 2008) found

461 in adipocytes that the stimulatory effect of NO donors on insulin transport was not through
462 cGMP/PKG but through S-nitrosylation.
463
464 The alternatively-spliced isoform of nNOS, nNOSμ, is the primary source of skeletal
465 muscle NO during contraction in mouse muscle (Silvagno *et al.*, 1996) and in contracting
466 muscle cells (Hirschfield *et al.*, 2000). Indeed, it has been shown contraction increases
467 cGMP during *ex vivo* skeletal muscle contraction in normal mice and eNOS KO mice but
468 not in nNOSμ KO mice (Lau *et al.*, 2000). Therefore, it is possible that in the current study
469 skeletal muscle NO production was from nNOSμ. Follow up studies should examine
470 whether the increase in insulin sensitivity after *ex vivo* contraction is attenuated in nNOSμ
471 mouse muscle. In addition, studies with NOS inhibition in humans could be conducted to
472 determine if NO production plays a role in the insulin sensitizing effects of exercise in
473 humans. We have infused local NOS inhibitors into the femoral artery of humans during
474 exercise in studies examining the role of NO in glucose uptake during exercise (Bradley *et*
475 *al.*, 1999). Similar methods could be used with infusion of a NOS inhibitor during insulin
476 several hours after acute exercise. It has been shown that 4 hours after single leg exercise
477 there is 50% or greater increases in insulin-stimulated glucose uptake into the exercised leg
478 compared with the rested leg (Richter *et al.*, 1989).
479
480 Due to technical difficulties and the small muscle mass we were unable to measure soluble
481 guanylate cyclase activity to confirm the efficacy of ODQ or to measure PKG activity to
482 confirm the efficacy of Rp-8-Br-PET-cGMP in our study. It should be considered,
483 however, that we have found that the same concentration of ODQ used in the current study
484 prevents NO donor stimulated increases in glucose uptake in EDL muscle (Merry *et al.*,
485 2010a).
486
487 In conclusion, we have shown that NO is required for normal increases in insulin sensitivity
488 several hours after *ex vivo* contraction of mouse muscle. NOS inhibition during contraction
489 had no effect on insulin sensitivity 3.5 hrs later but, remarkably, NOS inhibition during
490 insulin exposure post-contraction prevented the increases in insulin sensitivity following *ex*

491 *vivo* contraction. Although we found NOS inhibition during insulin treatment post-
492 contraction had no effect on Akt, TBC1D1 or TBC1D4 phosphorylation at the sites that we
493 examined, future mouse studies should examine other sites of TBC1D4 phosphorylation,
494 especially the increase in pTCB1D4 Thr704 in response to insulin in humans (pTCB1D4
495 Thr711 in mice) is greater ~5 hrs after exercise. Finally, given that blocking soluble
496 guanylate cyclase and PKG during insulin exposure had no effect on the increase in insulin
497 sensitivity after contraction, this suggests that NO acts independently of the cGMP/PKG
498 pathway to increase insulin sensitivity after contraction.
499

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703

704

705 ## Additional information section

706

707 **Competing interests**

708 The authors declare no conflicts of interest, financial or otherwise.

709

710 **Author contributions**

711 XZ and GKM were responsible for the conception and design of the study. XZ, DH and
712 YHH conducted the experiments. XZ, DH, SR and GKM contributed to analysis of data.
713 AZ and AH contributed to set up *ex vivo* contraction apparatus. XZ and GKM wrote the
714 first version of the manuscript. All contributed to the review and edition of the manuscript.
715 All authors have approved the final version of the manuscript and agree to be accountable
716 for all aspects of the work. All persons designated as authors qualify for authorship, and all
717 those who qualify for authorship are listed.

718

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725

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728

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731
Figures and Legends

732 Figure 1. Effect of *ex vivo* muscle contraction with and without serum on insulin
733 sensitivity of glucose uptake. Insulin 120µU/ml. **A**. Experimental design. **B**. 2-DG
734 uptake. Mean ± SEM, n=3-4 per group, *P<0.05 vs no insulin, #p<0.05 vs no serum.
735 White bars: no insulin; Black bars: insulin.

736

737 Figure 2. NOS inhibition during insulin exposure prevents the increase in insulin-
738 stimulated glucose uptake and NOS activity 3.5 hrs post-contraction in mouse skeletal
739 EDL muscles. **A**. Experimental design. **B**. The effect of NOS inhibition (L-NMMA;
740 100µM)) during contraction and during insulin (120µU/ml) exposure on glucose uptake
741 3.5 hrs after *ex vivo* contraction. Mean ± SEM, N=6-12 *P<0.05 vs no insulin treatment;
742 #P<0.05 vs rest plus insulin group and vs contraction and then NOS inhibition during
743 insulin group. **C**. NOS activity of EDL muscles in the presence of insulin. Mean ± SEM,
744 n=6 per group. #P<0.05 vs rest and vs contraction and then NOS inhibition during
745 contraction group.

746

747

748 Figure 3. Agents modifying the cGMP/PKG pathway had no effect on insulin-stimulated
749 glucose uptake 3.5 hrs after contraction. Soluble guanylate cyclase (sGC) inhibition by
750 ODQ (10 µM), PDE5 inhibition by T-1032 (27 µM), and PKG inhibition by Rp-8-Br-
751 PET-cGMPS (5 µM). 120µU/ml of insulin was used in all experiments except in T-1032
752 treatment where 60µU/ml was used. **A**. Relationship of the inhibitors used with the
753 cGMP/PKG pathway. **B**. 2-DG glucose uptake. Mean ± SEM, n=4-6 per group. #P<0.05
754 vs rest. White bars: vehicle; Black bars: inhibitor. **C**. Experimental design to examine
755 any possible physical interaction between insulin and the inhibitors used. The inhibitors
756 (L-NMMA, ODQ and T1032) were incubated with insulin for 30 min. **D**. No physical
757 interaction between insulin and the examined inhibitors. Mean ± SEM, n=4-6, * P<0.05
758 vs no insulin.

759

760 Figure 4. Akt phosphorylation 3.5 hrs after *ex vivo* contraction in mouse skeletal muscle.
761 N = 6 per group. Insulin (120μU/ml). All values are shown as means ± SEM; * $P < 0.05$
762 or ** $P<0.01$ or *** $P<0.001$ vs no insulin.

763

764 Figure 5. TBC1D1 and TBC1D4 phosphorylation in response to insulin 3.5 hrs after *ex*
765 *vivo* contraction in mouse skeletal muscle. Insulin (120μU/ml). N = 6 in each group. All
766 values are shown as means ± SEM; * $P < 0.05$ or ** $P<0.01$ or *** $P<0.001$ vs no
767 insulin.