Nitric Oxide is Required for the Insulin Sensitizing Effects of Contraction in Mouse Skeletal Muscle

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Running title: NO and post-contraction insulin sensitivity

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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)

Introduction

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

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

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

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

Methods

Ethical approval

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

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Animals and experimental design

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

Materials and antibodies

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

immunoblotting were purchased from Bio-Rad (Hercules, CA). RED 660 Protein Assay Reagent Kit and Neutralizer were purchased from GBiosciences (St. Louis, MO). SuperSignal West Femto Chemiluminescent Substrate was provided by Thermo Scientific (Waltham, MA). Primary antibodies for p-Akt (Ser473 and Thr308), Akt, p-TBC1D1 (Thr590, Thr596 and Ser660), TBC1D1, p-TBC1D4 (Thr642), TBC1D4 and actin used in Western Blotting were purchased from Cell Signalling Technology (Danvers, MA). HRP conjugated Goat anti-Rabbit IgG (H+L) Secondary Antibody was from Thermo Scientific (Waltham, MA).

Collection and treatment of serum

As previously reported (Gao *et al.,* 1994), a serum factor is required for an increase in insulin sensitivity after *ex vivo* rat skeletal muscle contraction, and we also found that serum alone has no effect on mouse skeletal muscle glucose uptake at rest (Levinger *et al.*, 2016).Whether serum is required during *ex vivo* contraction of mouse skeletal muscle for increases in insulin-stimulated glucose uptake has not previously been examined. After an overnight fast, blood was collected from 4 healthy men via venepuncture. The blood was allowed to clot at room temperature then centrifuged at 3,000g for 30 min. The serum was collected and frozen at -80^{o}C until use. All serum used was from the same individuals. Repeat freeze-thawing of serum was avoided.

Muscle dissection, incubation and contraction

Under deep anaesthesia, both EDL muscles were rapidly dissected. The proximal and distal tendons were tied using 5/0 silk suture with two small aluminum hooks tied to each tendon. For all incubation steps, buffer was continuously maintained at 30°C (Merry *et al.*, 2010b) and gassed with carbogen (Carbogen; BOC Gases, Australia). Muscles were pre-incubated with or without 50% human serum in *Buffer 1* [Krebs-Henseleit buffer (KHB in mM: 119 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 + 2 mM glucose + 8 mM mannitol] for 30 min. For muscle contraction, muscles were mounted in incubation chambers containing *Buffer 1* with or without serum and stimulating

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

Muscle treatment post-electrical stimulation and glucose uptake measurements

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

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

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

2003). The muscle pairs were incubated in the presence or absence of the inhibitor ODQ, or T-1032 or Rp-8-Br-PET-cGMPS during the period of 30 min of insulin and 10 min of 2-DG incubation.

Given that 120 μU/ml of insulin results in maximum insulin-stimulated glucose uptake (Hamada *et al.*, 2006), it was anticipated that both ODQ and Rp-8-Br-PET-cGMPS would attenuate the increase in insulin-stimulated glucose uptake after contraction, thus 120 μU/ml of insulin was used for ODQ and Rp-8-Br-PET-cGMPS treatments. On the other hand, given we anticipated that T-1032 would increase insulin-stimulated glucose uptake after contraction, we used a submaximal dose of insulin (60 μU/ml) (Hamada *et al.*, 2006) with T-1032 treatment to provide a greater opportunity to observe any increase in glucose uptake.

After the 10 min incubation with radioisotopic tracers, muscles were rapidly rinsed, trimmed and cut in halves and frozen in liquid nitrogen. One half was kept for immunoblotting and the other half for glucose uptake determination. The muscle for glucose uptake were homogenized in 1M NaOH at 95°C for 10 min and then neutralized by 1 M HCl followed by centrifuge. The supernatant (200μl) was added to 4 ml of liquid scintillation cocktail (PerkinElmer, Boston, MA). Radioactivity of both tracers was measured by a β scintillation counter (Tri-Carb 2910TR, PerkinElmer), and glucose uptake was calculated as previously described (Merry *et al.*, 2010a; Zhang *et al.*, 2011).

NOS activity assay

NOS activity was determined in separate EDL muscles based on the catalytic reaction of NOS converting radiolabeled L-[^{14}C] arginine to radiolabeled L-[^{14}C] citrulline, as described previously (Merry *et al.*, 2010a). NOS activity was determined from the difference between samples incubated with and without L-NAME and was expressed as picomoles of L-[^{14}C] citrulline formed per minute per milligram of muscle protein.

Sample Preparation and Immunoblotting

Sample preparation for immunoblotting was initially described by Murphy RM (Murphy, 2011). Briefly, 10 20-μm thickness muscle sections were homogenized with 100μl of

solubilizing buffer (0.125 M Tris–Cl [pH 6.8], 4% w/v SDS, 10% glycerol, 10 mM EGTA, 0.1 M DTT (dithiothreitol) and protease inhibitor cocktail). Protein concentration was determined by a Red 660 assay kit (G-Biosciences, St. Louis, MO). Proteins (5 µg loaded per well) were separated with 10% SDS–PAGE gels, then transferred to PVDF for 120 min at 100 V. Following transfer, the membrane was blocked with 5% (w/v) skim milk powder dissolved in TBST (Tris-Buffered Saline, 0.1% Tween-20) at room temperature for 1 h. The primary antibodies were diluted in 5% (w/v) BSA in TBST and applied and incubated overnight at 4°C. After a1 h incubation with secondary antibody at room temperature, images were exposed to SuperSignal West Femto Chemiluminescent Substrate and VersaDoc^{TM} Imaging System and densitometry was performed using the Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). All phosphorylation data is presented relative to the total protein of the protein of interest.

Statistical analysis

All data are expressed as means ± SEM. Statistical testing was performed with SPSS statistical package 22 or Graph Pad Prism 6. For multiple comparisons, one-way ANOVA and two-way ANOVA with or without repeat measurement (between factor: insulin and treatment condition – for glucose uptake and protein expression) were used. Tukey's post hoc test or Fisher's LSD testwas performed when ANOVA revealed significance. The Statistical significance was accepted at $p \leq 0.05$.

Results

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

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

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

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

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

T2D (Bradley *et al.*, 1999; Kingwell *et al.*, 2002), in order to examine whether NO is required for the increase in insulin sensitivity post *ex vivo* contraction (Fig. 2A), muscles were treated with the NO synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA, 100 µM) either 1) during the period of the pre-incubation (30 min) and the muscle contraction (10 min) (NOS inhibition during contraction), or 2) during vehicle or the 120µU/ml insulin incubation (30 min) and 2-DG tracer incubation (10 min); NOS inhibition during contraction). In the absence of insulin skeletal muscle glucose uptake was similar ($P > 0.05$) 3.5 hrs after no contraction, contraction, NOS inhibition during contraction and NOS inhibition during insulin (Fig. 2B). This indicates that the effect of prior contraction had worn off. Contraction significantly ($P<0.01$) increased insulin-stimulated glucose uptake 3.5 hrs post-contraction and this increase was not affected by NOS inhibition during the pre-incubation and contraction periods (Fig. 2B). Surprisingly, NOS inhibition during insulin (and 2-DG tracer) incubation prevented the increase in insulin-stimulated glucose uptake in response to prior contraction (Fig. 2B). The incremental (delta) increase in insulin-stimulated glucose uptake (insulin-stimulated glucose uptake minus basal glucose uptake) was significantly higher in the contraction and the contraction plus NOS inhibition during contraction groups than the non contraction and contraction plus NOS inhibition during insulin groups(Fig. 2B).

NOS activity

NOS activity was significantly reduced by NOS inhibition during insulin treatment to a level significantly below the basal state (Fig. 2C). NOS activity has a tendency to increase in the NOS inhibition during contraction group although this was not significant ($P=0.08$) (Fig. 2C).

The NO-mediated insulin-sensitizing effect of prior contraction does not involve cGMP/PKG downstream signaling

Since NO signalling involves activation of the soluble form of guanylate cyclase to produce cGMP, the NO/cGMP/PKG signalling pathway is generally considered to be the major

311 downstream target of NO (Stamler & Meissner, 2001) (Fig. 3A). To explore the
312 mechanism(s) that NO acts to increase insulin-stimulated skeletal muscle glucose uptake
313 post contraction, and specifically whether this NO signalling is through cGMP/PKG, the
314 soluble guanylate cyclase (sGC) inhibitor ODQ (which blocks the NO-mediated increase in
315 cGMP), the PDE 5 inhibitor T1032 (which inhibits cGMP breakdown and therefore raises
316 cGMP levels) and the cGMP-dependent protein kinase (PKG) inhibitor Rp-8-Br-PET-
317 cGMPS were applied to block this pathway as per our previous studies (Mahajan *et al.*,
318 2003; Merry *et al.*, 2010a; Merry *et al.*, 2010b). We found that the insulin sensitizing
319 effects of prior contraction were not affected by the presence of these inhibitors during
320 insulin incubation 3.5 hrs post-contraction (Fig. 3B).
321 To exclude the possibility that there was a physical interaction between insulin and the
322 inhibitors which may have prevented them having an effect on insulin-stimulated glucose
323 uptake, the resting muscles were co-incubated with or without L-NMMA, ODQ or T1032
324 with insulin for 30 min, then were incubated with [^{3}H]-2-deoxyglucose and [^{14}C]-mannitol
325 for 10 min to measure glucose uptake (Fig. 3C). As can be seen in Fig. 3D, there was no
326 difference between insulin and insulin plus any of these inhibitors, indicating that no
327 physical interaction could explain the effect of L-NMMA and the lack of effect of these
328 other agents.
329
330 **Insulin signalling**

331 There was little Akt Thr308 and Akt Ser473 phosphorylation in the absence of insulin and
332 no significant differences between the treatments (Fig. 4). Insulin significantly ($P<0.001$)
333 increased phosphorylation of Akt at both Thr308 and Ser473 with no differences observed
334 between the four treatments (Fig. 4B-C). Insulin significantly increased phosphorylation of
335 TBC1D1 at Thr590 ($P<0.01$) and Thr596 ($P<0.001$) but not at Ser660 with no greater
336 insulin-stimulated phosphorylation at these sites 3.5hrs following prior contraction (Fig.
337 5A-D). Although TBC1D4 Thr642 phosphorylation *per se* did significantly increase with
338 insulin (data not shown, $P<0.05$), given the variability of the total TBC1D4 data (data not
339 shown, $P>0.05$), this increase was not significant when TBC1D4 Thr642 phosphorylation
340 was presented relative to the total TBC1D4 (Fig. 5E-F). NOS inhibition either during

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

Discussion

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

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

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

inhibition during contraction (Hong *et al.*, 2015). Therefore, it is possible that the effects of the NOS inhibitor were somewhat nullified by the presence of serum during contraction because L-arginine is present in healthy human serum at a concentration of ~100 μM.

Importantly, NOS inhibition during insulin incubation blocked the increase in insulin sensitivity in response to earlier contraction (Fig.2B). The mechanism(s) involved are unclear at this stage. The relationship between skeletal muscle, NO production, NOS activity, diabetes, exercise and insulin sensitivity are complex. Insulin has been shown to increase nNOS phosphorylation in C_2C_{12} muscle cells and in mouse skeletal muscle (Hinchee-Rodriguez *et al.*, 2013) and skeletal muscle NOS activity increases during a euglycaemic hyperinsulinaemic clamp in healthy humans (Kashyap *et al.*, 2005). Therefore, it is possible that insulin activates increases in skeletal muscle NO production to increase glucose uptake and that the NOS inhibitor then prevented this effect. Indeed, in line with the prevention of the contraction-stimulated increase in insulin sensitivity, NOS activity was significantly reduced in the presence of NOS inhibition during insulin treatment (Fig. 2C).

Most studies in rodents and humans find little effect of prior exercise or contraction on proximal insulin signalling (Wojtaszewski *et al.*, 2000; Hamada *et al.*, 2006; Funai *et al.*, 2010; Castorena *et al.*, 2014). In line with this, we found there was no difference in insulin-stimulated Akt phosphorylation with or without prior *ex vivo* contraction (Fig. 4). Despite unaltered proximal signalling, some studies have reported greater downstream insulin signalling at the level of TBC1D4 3 hrs after exercise in rats and humans (Funai *et al.*, 2009; Treebak *et al.*, 2009; Castorena *et al.*, 2014). Although previous studies found increases in mouse EDL TBC1D4 Thr642 phosphorylation with insulin (Chen *et al.*, 2011; Kjobsted *et al.*, 2015; Kjobsted *et al.*, 2017), in the current study we found no significant increase in TBC1D4 Thr642 phosphorylation with insulin when TBC1D4 Thr642 phosphorylation was presented relative to the total TBC1D4. However, TBC1D4 Thr642 phosphorylation *per se* did increase with insulin but given variability with total TBC1D4,

this effect was lost when TBC1D4 Thr642 phosphorylation was divided by total TBC1D4 (Fig. 5F).

It has now been shown in our human study that skeletal muscle pTCB1D4 Thr704 (pTCB1D4 Thr711in mice) is increased 4 hours after exercise (Sjoberg *et al.*, 2017). In addition, the increase in pTCB1D4 Thr704 during a euglycemic hyperinsulinemic clamp is greater in previously exercised muscle than in non-exercised muscle in humans (Sjoberg *et al.*, 2017). It is not known if similar responses of pTCB1D4 Thr711 are observed in mice as unfortunately an antibody for TBC1D4 704/711 phosphorylation was not commercially available when we conducted this study. Future mouse studies should examine this site.

It is important to note that Funai et al (Funai *et al.*, 2010) reported additive effects of prior *in vivo* exercise and *ex vivo* contraction on insulin stimulated glucose uptake, suggesting that *in vivo* exercise and *ex vivo* contraction may enhance insulin sensitivity by different mechanisms. Along these lines, we recently found that NOS inhibition in humans overcomes the greater insulin sensitivity in a leg that exercise 4 hrs earlier compared with a rested leg. In that study (Sjoberg *et al.*, 2017), like in this study, NOS inhibition had no effect on insulin signalling in either the contracted on non-contracted muscle. However, in that study it appeared that the reduction in blood flow with NOS inhibition, especially in microvascular blood flow, was the major reason for the NOS inhibition, like in the current study, overcoming/preventing the increased insulin sensitivity due to earlier exercise. However, in the current study there is no blood flow component. These results support the suggestion that *in vivo* exercise and *ex vivo* contraction may enhance insulin sensitivity by different mechanisms, with both involving NO. Further research is required to clarify this.

Akt, TBC1D1 and TBC1D4 phosphorylation were not affected by NOS inhibition during insulin treatment and therefore do not appear to account for the observed effects of NOS inhibition preventing the increase in insulin sensitivity after contraction. The mechanisms responsible for this remarkable effect of NOS inhibition on insulin-stimulated glucose uptake after contraction are not clear. Recent evidence indicates that the cytoskeleton is

important for skeletal muscle glucose uptake in response to both contraction and insulin (Su *et al.*, 2005; Wang, 2011; Sylow *et al.*, 2013a) and given that skeletal muscle nNOS is associated with the cytoskeleton (Percival *et al.*, 2010), it is possible that this could be playing a role. Depolymerization of the actin cytoskeleton decreases glucose uptake (Sylow *et al.*, 2013b) and rearrangement of the actin cytoskeleton by Rac1 (Ras-related C3 botulinum toxin substrate 1), a small Rho family GTPase, is necessary for insulin-stimulated GLUT4 translocation in L6 myotubes (Ueda *et al.*, 2008). In addition, Rac1 and its downstream target, PAK1, are activated by contraction/exercise in human and mouse skeletal muscle and insulin-stimulated GLUT4 translocation is impaired in skeletal muscle from Rac1 knockout mice (Sylow *et al.*, 2013a; Sylow *et al.*, 2013b). Inhibition of Rac1 or Rac1 knockout reduces both contraction-stimulated and insulin-stimulated glucose uptake in mouse muscle (Sylow *et al.*, 2013a; Sylow *et al.*, 2013b). There is also some evidence of interactions between Rac1 and NO, including in C_2C_{12} muscle cells (Su *et al.*, 2005; Cheng *et al.*, 2006; Godfrey & Schwarte, 2010). Follow up studies should examine whether NOS inhibition during insulin exposure attenuates increases in pPAK1 after prior *ex vivo* skeletal muscle contraction.

The cGMP/PKG pathway, which is present in skeletal muscle, is generally considered to be the major downstream signaling pathway of NO (Stamler & Meissner, 2001). However, modification of cGMP/PKG signalling with the soluble guanylate cyclase inhibitor ODQ (guanylate cyclase produces cGMP in response to NO), the PDE 5 inhibitor T1032 (PDE5 breaks down cGMP) and the cGMP-dependent protein kinase (PKG) inhibitor Rp-8-Br-PET-cGMPS, had no significant effect on the insulin-sensitizing effects of prior contraction in mouse muscle *ex vivo* (Fig. 3A-B). These results suggest that NO increases skeletal muscle insulin sensitivity post-contraction via cGMP/PKG independent mechanism(s). This is similar to what we have found previously during *ex vivo* contractions where L-NMMA attenuates the increase in skeletal muscle glucose uptake during *ex vivo* contractions but there is no effect of inhibition of sGC or PKG (Merry *et al.*, 2010a). Moreover, Wang et al. (Wang *et al.*, 2013) found in endothelial cells and Kaddai et al (Kaddai *et al.*, 2008) found

in adipocytes that the stimulatory effect of NO donors on insulin transport was not through cGMP/PKG but through S-nitrosylation.

The alternatively-spliced isoform of nNOS, nNOSμ, is the primary source of skeletal muscle NO during contraction in mouse muscle (Silvagno *et al.*, 1996) and in contracting muscle cells (Hirschfield *et al.*, 2000). Indeed, it has been shown contraction increases cGMP during *ex vivo* skeletal muscle contraction in normal mice and eNOS KO mice but not in nNOSμ KO mice (Lau *et al.*, 2000). Therefore, it is possible that in the current study skeletal muscle NO production was from nNOSμ. Follow up studies should examine whether the increase in insulin sensitivity after *ex vivo* contraction is attenuated in nNOSμ mouse muscle. In addition, studies with NOS inhibition in humans could be conducted to determine if NO production plays a role in the insulin sensitizing effects of exercise in humans. We have infused local NOS inhibitors into the femoral artery of humans during exercise in studies examining the role of NO in glucose uptake during exercise (Bradley *et al.*, 1999). Similar methods could be used with infusion of a NOS inhibitor during insulin several hours after acute exercise. It has been shown that 4 hours after single leg exercise there is 50% or greater increases in insulin-stimulated glucose uptake into the exercised leg compared with the rested leg (Richter *et al.*, 1989).

Due to technical difficulties and the small muscle mass we were unable to measure soluble guanylate cyclase activity to confirm the efficacy of ODQ or to measure PKG activity to confirm the efficacy of Rp-8-Br-PET-cGMP in our study. It should be considered, however, that we have found that the same concentration of ODQ used in the current study prevents NO donor stimulated increases in glucose uptake in EDL muscle (Merry *et al.*, 2010a).

In conclusion, we have shown that NO is required for normal increases in insulin sensitivity several hours after *ex vivo* contraction of mouse muscle. NOS inhibition during contraction had no effect on insulin sensitivity 3.5 hrs later but, remarkably, NOS inhibition during 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.
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Additional information section

Competing interests

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

Author contributions

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

Funding

This study was funded by the National Health and Medical Research Council (NHMRC, Project grant number 1012181 to GKM) and Biomedical & Lifestyle Diseases (BioLED) to XZ in Australia.

Acknowledgements

The authors thank Associate Professor Itamar Levinger for fruitful discussion and helpful suggestions.

Figures and Legends

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

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

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

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

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