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Dietary nitrate increases submaximal SERCA activity and ADP-transfer to mitochondria in slow-twitch muscle of female mice

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Abstract

Rapid oscillations in cytosolic calcium (Ca^{2+}) coordinate muscle contraction, relaxation, and physical movement. Intriguingly, dietary nitrate decreases ATP cost of contraction, increases force production, and increases cytosolic Ca^{2+}; which would seemingly necessitate a greater demand for sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) to sequester Ca^{2+} within the SR during relaxation. As SERCA is highly regulated, we aimed to determine the effect of 7-day nitrate supplementation (1 mM via drinking water) on SERCA enzymatic properties and the functional interaction between SERCA and mitochondrial oxidative phosphorylation. In soleus, we report that dietary nitrate increased force production across all stimulation frequencies tested, and throughout a 25 min fatigue protocol. Mice supplemented with nitrate also displayed an ~25% increase in submaximal SERCA activity and SERCA efficiency (p=0.053) in soleus. To examine a possible link between ATP consumption and production, we established a methodology coupling SERCA and mitochondria in permeabilized muscle fibers. The premise of this experiment is that the addition of Ca^{2+} in the presence of ATP generates ADP from SERCA to support mitochondrial respiration. Similar to submaximal SERCA activity, mitochondrial respiration supported by SERCA-derived ADP was increased ~20% following nitrate in red gastrocnemius. This effect was fully attenuated by the SERCA inhibitor cyclopiazonic acid and was not attributed to differences in mitochondrial oxidative capacity, ADP sensitivity, protein content, or reactive oxygen species emission. Overall, these findings suggest improvements in submaximal SERCA kinetics may contribute to the effects of nitrate on force production during fatigue.

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Key words: Calcium homeostasis, contractile function, mitochondria, nitrate, SERCA

Abbreviations: ADP, adenosine diphosphate; ANT, adenine nucleotide translocase; ATP, adenosine triphosphate; BRJ, beet root juice; C, cytochrome C; Ca^{2+}, calcium; CPA, cyclopiazonic acid; CSQ, calsequestrin; EDL, extensor digitorum longus; G, glutamate; JO_2, oxygen consumption; K_m, Michaelis-Menten constant; mH_2O_2, mitochondrial hydrogen peroxide emission; NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; NOx, nitrate+nitrite; OXPHOS, oxidative phosphorylation system; pCa_{50}, negative logarithm of [Ca^{2+}] required to elicit half maximal SERCA activity; PLN, phospholamban; PM, pyruvate+malate; PmFb, permeabilized muscle fibers; pPLN, phosphorylated phospholamban; RCR, respiratory control ratio; RG, red gastrocnemius; ROS, reactive oxygen species; RyR, ryanodine receptor; S, succinate; SERCA, sarcoplasmic reticulum calcium ATPase; SLN, sarcolipin; Sol, soleus; SR, sarcoplasmic reticulum; VDAC, voltage-dependent anion channel; V_{max}, maximal enzymatic activity; WG, white gastrocnemius.

New and Noteworthy: We show that nitrate supplementation increased force production during fatigue and increased submaximal SERCA activity. This was also evident regarding the high-energy phosphate transfer from SERCA to mitochondria, as nitrate increased mitochondrial respiration supported by SERCA-derived ADP. Surprisingly, these observations were only apparent in muscle primarily expressing type I fibers (soleus) but not type II fibers (EDL). These findings suggest alterations in SERCA properties are a possible mechanism in which nitrate increases force during fatigue.

Wordcount: 75

Introduction

Muscle contraction and relaxation cycles are dependent on rapid fluctuations in cytosolic calcium (Ca^{2+}) and the activity of numerous ATP consuming and producing enzymes. Cytosolic free Ca^{2+} concentrations in skeletal muscle are tightly regulated by uptake and release from the lumen of the sarcoplasmic reticulum (SR) (1). The transient increase in cytosolic Ca^{2+} upon excitation results in contractile unit recruitment and force production by the actin-myosin ATPase, while SR Ca^{2+} ATPase (SERCA) is responsible for Ca^{2+} reuptake back into the SR for relaxation. Ca^{2+} homeostasis is essential for maintaining contractile function, as reduced Ca^{2+} release from the SR (2) and reduced SERCA activity (3) in part contribute to muscle fatigue. As both SERCA and actin-myosin ATPase consume ATP, mitochondria are essential for maintaining ATP production and allowing continuous physical movement to occur. Coordination of ATP turnover is highly modifiable, and as a result considerable interest has been placed on studying nutritional approaches that may alter contractile properties and enhance exercise performance through regulation of ATP consumption or ATP production (4, 5).

One such compound, nitric oxide (NO) is a small hydrophobic signaling molecule that influences a number of physiological processes such as vasodilation (6), mitochondrial biogenesis (7, 8) and skeletal muscle excitation-contraction coupling (9, 10). NO can be synthesized from the nitrate-nitrite-NO pathway through serial reduction of nitrate (NO_3^-) (11), providing a dietary means for obtaining the bioactive effects of NO. Indeed, exogenous nitrate supplementation has been shown to decrease the oxygen cost of exercise (12–15) in humans, delay time to fatigue (15), and increase low-frequency force production in both human (16) and mouse (10) skeletal muscle. The original theory to explain these observations suggested nitrate-mediated improvements in mitochondrial coupling efficiency (P/O ratio) (13). However, changes

95 in mitochondrial protein content (UCP3, ANT) and mitochondrial respiratory bioenergetics have
96 not been observed following 7 days of beet root juice (BRJ) supplementation in humans (17) or
97 nitrate supplementation in mice (18), suggesting improvements in mitochondrial bioenergetics
98 are not mediating the beneficial ergogenic effects of nitrate consumption.

99 Alternatively, nitrate consumption has been linked to a reduction in PCr degradation and
100 ADP and P_i accumulation during muscle contraction (15), indicative of a reduced ATP turnover
101 rate. As a result, it is now believed that alterations in Ca^{2+} handling, ion pumping, or actin-
102 myosin cross-bridge sensitivity are mechanisms central to the beneficial effects of nitrate.
103 Indeed, nitrate consumption has been reported to increase cytosolic Ca^{2+} concentrations in both
104 cardiac (19) and skeletal (10) muscles. However, increased cytosolic Ca^{2+} would seemingly
105 necessitate a greater ATP demand for SERCA to sequester Ca^{2+} within the SR during relaxation,
106 indicating an energetically demanding process that would contrastingly increase ATP cost.
107 Alternatively, it is possible that dietary nitrate reduces the ATP cost of force production through
108 improvements in SERCA enzymatic efficiency within skeletal muscle, in the absence of
109 increasing ATP hydrolysis. In support, there is evidence that NO can alter the function of Ca^{2+}
110 handling proteins (20, 21), and acute incubation with nitrite (NO_2^-) improves SR Ca^{2+} pumping
111 in single muscle fibers (22). Therefore, the possibility that nitrate improves intracellular Ca^{2+}
112 handling through SERCA efficiency is a plausible hypothesis that warrants further investigation.

113 Within skeletal muscle, SERCA activity accounts for ~40-50% of cellular ATP use at rest
114 (23) and during contraction (24), indicating the importance of energy transfer between SERCA
115 and mitochondria. A link between the SR and mitochondria exists (25) which promotes the
116 exchange of signaling molecules such as Ca^{2+} and reactive oxygen species (ROS) (26, 27), and
117 allows for a structural proximity for ATP turnover between organelles. As a result, an increase in

SERCA efficiency could be linked to an increase in the high-energy phosphate transfer between the SR and mitochondria. Therefore, we aimed to determine if the increase in force production following dietary nitrate supplementation could be attributed to improvements in SERCA enzymatic efficiency. We hypothesized that SERCA-mediated rates of ATP hydrolysis would be decreased following nitrate consumption, contributing to the well-established reduction in whole-body oxygen consumption. Furthermore, using a readout of mitochondrial respiration, we examined the high-energy phosphate transfer from SERCA to mitochondria to assess the functional interaction between these organelles as a secondary determination of the energetic cost of controlling cytosolic Ca^{2+}.

Methods

Ethical approval

All experimental procedures were approved by the Animal Care Committee at the University of Guelph (4241). Female C57Bl/6N mice (15-20 weeks old) used for experiments were bred-in house at the University of Guelph Animal Facility. Animals were given access to food and water *ad libitum* and were kept in a temperature-controlled (22˚C) environment with a 12-12 hour light-dark cycle.

Experimental design

Female C57Bl/6N mice (15-20 weeks old) were randomized to consume either standard drinking water or water supplemented with 1 mM sodium nitrate ($NaNO_3$) for 7 days (10). Following supplementation, mice were anesthetized with 60 mg/kg sodium pentobarbital. All tissue collection procedures were performed only after assurance of anaesthesia depth checked

by leg retraction after tail pinch and movement of whiskers. In one subset of mice (n = 7 each group), soleus and extensor digitorum longus (EDL) muscles were removed for *in vitro* stimulation protocols to measure contractile function. Following this, soleus and EDL were immediately snap-frozen for western blot analysis or homogenized for SERCA activity. Blood was collected via cardiac puncture, centrifuged at 3,000 g for 10 min at 4˚C, and serum was aliquoted for later analysis of total and nitrate+nitrate (NOx) levels using a commercially available kit (Cayman Chemicals, Ann Arbor, MI). In a second subset of mice (n = 13 control, n = 15 nitrate), mitochondrial bioenergetic experiments were performed and fibers were recovered for western blot analysis.

In vitro *stimulation protocol*

Soleus (slow-twitch) and EDL (fast-twitch) muscles were isolated from the hindlimb. Surgical thread was used to tie off tendons to mount muscles in the stimulation chamber, connected to S-hooks on a 4-channel linear force transducer (Glass Telefactor S88 Stimulator). Each chamber contained ~150 mL of Krebs solution (118 mM NaCl, 4.69 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_47H_2O$, 24.76 mM $NaHCO_3$, 11.10 mM glucose, 2.52 mM $CaCl_2$, 10 U/L insulin, 3 mg/L tubarine) that was continually bubbled with 95% O_2 and 5% CO_2 (pH 7.35-7.45) and maintained at a constant temperature of 27°C. The same muscle from each animal was used for the entirety of the force-frequency and fatigue protocols. Optimal muscle length (L_o) was set when force plateaued following 250 msec trains at 100 Hz. Muscles were then equilibrated for 30 min prior to the stimulation protocol. All electrical pulses were 0.5 msec in duration with a train duration of 250 msec. The stimulation protocol consisted of a twitch stimulus followed by sequential force frequency stimulation protocol (1, 5, 10, 20, 30, 40, 50, 60,

70, 80, and 100 Hz stimulations). Following this, another twitch stimuli was performed. Immediately after the muscles were subjected to a fatigue protocol consisting of 1 contraction every 5 sec for 25 min at 40 Hz (soleus) or 60 Hz (EDL) frequencies. After measuring length and weight, one soleus and EDL were immediately frozen in liquid nitrogen and stored at -80°C for western blot analysis. The second soleus and EDL were diluted 1:10 (wt/vol) in ice-cold SERCA homogenizing buffer (pH 7.5) containing 0.2 mM PMSF, 250 mM sucrose, 5 mM HEPES, and 0.2% sodium azide (NaN_3), and homogenized on ice in a hand-held glass homogenizer. Homogenates were then frozen in liquid N_2 and stored at -80°C for later analysis.

SERCA activity assay

Ca^{2+}-induced SERCA activity was measured in muscle homogenates using a spectrophotometric method previously adapted by our laboratory (28). 1.425 mL of reaction buffer containing 200 mM KCl, 20 mM HEPES, 10 mM NaN_3, 1 mM EGTA, 15 mM $MgCl_2$, 10 mM PEP, and 5 mM ATP (pH 7) was added to a glass cuvette. Immediately before the reaction, 18 U/mL lactate dehydrogenase, 18 U/mL pyruvate kinase, 25 µM blebbistatin, 5 uL muscle homogenate and 0.2 mM NADH were added to the cuvette with a final volume of 1.5 mL. EDL muscle was diluted 5-fold to account for increased activity. Assays were performed in duplicate at 37°C and 340 nm wavelength. SERCA activity was measured using successive 15 uL additions of 10 mM $CaCl_2$ every 2 min until a plateau was observed (V_{max}). Free Ca^{2+} concentrations were calculated using an online calculator (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/CaMgATPEGTA-TS.htm) given buffer conditions of pH 7.0, ionic strength 0.28, temperature 37˚C, 1 mM EGTA, 5 mM ATP, and 15 mM Mg^{2+}, as previously described (28). Due to the presence of other ATPases in

187 the homogenate, 40 μM of cyclopiazonic acid (CPA) was added to the cuvette following the
188 reaction in order to completely inhibit SERCA activity. SERCA activity was determined at each
189 time point by subtracting the activity in the presence of CPA and used to construct a non-linear
190 regression analysis. Enzymatic kinetics were analyzed for maximal SERCA activity (V_{max}), the
191 negative logarithm of [Ca^{2+}] needed to produce 50% of V_{max} (pCa_{50}), and the slope of the
192 relationship between SERCA activity and Ca^{2+} between 10% and 90% V_{max} (Hill slope, n_H).
193
194 *Mitochondrial bioenergetics*
195 Permeabilized muscle fibers (PmFb) were prepared from white gastrocnemius (WG), red
196 gastrocnemius (RG), and soleus for mitochondrial respiration experiments as previously
197 described (29). Muscle was placed in ice-cold BIOPS (29) and fiber bundles were separated with
198 fine-tipped forceps underneath a microscope (MX6 Stereoscope, Zeiss Microsystems, Wetzlar,
199 Germany). Fibers were incubated in 40 μg/mL saponin for 30 min and washed in MiR05
200 respiration buffer (respiration experiments) (29) or Buffer Z (ROS experiments) (30) for 15 min.
201 Mitochondrial respiration experiments were performed in MiR05 respiration buffer in an
202 Oxygraph high-resolution respirometer at 37˚C (Oroboros Instruments, Innsbruck, Austria) with
203 constant spinning at 750 rpm. Experiments were conducted at room air saturation with
204 reoxygenation after the addition of each substrate (~180-195 μM O_2). All experiments were
205 performed in the presence of 5 μM blebbistatin, 5 mM pyruvate, and 1 mM malate. For ADP
206 experiments, ADP was titrated in various concentrations (25, 100, 250, 500, 1000, 2000, 4000,
207 6000, 8000, 10000 μM ADP) followed by the addition of 10 mM glutamate, 10 mM succinate,
208 and 10 μM cytochrome C. Respiratory control ratios (RCR) were calculated by dividing
209 maximal state 3 respiration (presence of ADP) by state 2 respiration (pyruvate+malate, absence

of ADP). Ca^{2+} experiments were performed with the addition of 5 mM ATP prior to titrations of $CaCl_2$ (25, 50, 100, 200, 250, 300, 350, 375, 400, 425, 450 µM $CaCl_2$). When a plateau in $CaCl_2$-supported respiration was reached, 40 µM CPA was added to inhibit SERCA activity. Free Ca^{2+} concentrations were calculated using an online system (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/CaMgATPEGTA-TS.htm) given the buffer conditions of: pH 7.1, ionic strength 0.095 mM, temperature 37˚C, 0.5 mM EGTA, 5 mM ATP, and 3 mM Mg^{2+}. Estimated free Ca^{2+} concentrations were 17.2, 36.2, 81.2, 214.1, 317.9, 467.9, 701.1, 870.9, 1000, 1400, 1800 nM (corresponding to 25, 50, 100, 200, 250, 300, 350, 375, 400, 425, 450 µM $CaCl_2$).

Mitochondrial ROS experiments were performed as previously described (30) by measuring the rate of H_2O_2 release using Amplex Red fluorescence quantification (Invitrogen, Carlsbad, CA, USA) in Buffer Z at 37˚C. 5 µM blebbistatin, 5 U/mL HRP, and 40 U/mL SOD were added to the cuvette. Maximal ROS emission rates were examined in the presence of 20 mM succinate and submaximal ROS emission rates were examined following the addition of 100 µM ADP. H_2O_2 emission rates were calculated compared to a standard curve generated with known H_2O_2 concentrations. After mitochondrial bioenergetic experiments, fiber bundles were recovered and freeze-dried to normalize data to fiber bundle weight and to perform western blot analysis.

Western blotting

Whole muscle (soleus, EDL, WG, RG) was homogenized as previously described (29) and diluted to 0.5 µg/µL protein concentration. Permeabilized muscle fibers from WG, RG, and soleus muscle were digested in fiber lysis buffer for 60 min at 65˚C (31) for western blot

analysis. All samples were loaded equally onto a standard SDS-PAGE gel and separated for 1 h at 150 V. Proteins were transferred to PVDF membranes (1 h at 100 V), incubated in blocking solutions and appropriate primary/secondary antibodies. Target proteins included SERCA1 (1:2000, DSHB CaF2-5D2), SERCA2 (1:1000, Abcam 2861), CSQ1 (1:10,000, Abcam 108289), CSQ2 (1:1000, Abcam 191564), OXPHOS (1:1000 Abcam 110413), ANT1 (1:1000, Abcam 110322), SLN (1:500, Millipore ABT13), PLN (1:1000, Abcam 2865), and pPLN (1:1000, Cell Signaling 8496). α-tubulin (1:1000, Abcam 7291) or ponceau stains were used as loading controls. 10 μg of protein was loaded for EDL and soleus western blots following dietary nitrate consumption *(Fig. 2F)*, and for PLN and pPLN in RG homogenate *(Fig. 7A)*. 5 μg of protein was loaded for all other westerns *(Fig. 4D-F; Fig. 7A)*. Western blots were quantified using FlourChem HD imaging chemiluminescence (Alpha Innotech, Santa Clara, US). To limit variability, all samples for each protein were loaded and detected on the same membrane.

Statistics

Statistical analyses were completed using GraphPad Prism 9 software (GraphPad Software Inc., La Jolla, CA, US). Data comparing control and nitrate animals were analyzed using two-tailed unpaired Student's t-tests or two-way ANOVA with LSD post-hoc test when an interaction was determined (force-frequency, fatigue, SERCA activity). For comparisons between fiber types (WG, RG, Sol) in control animals establishing the SERCA-supported respiration methodology, one-way ANOVA was used with LSD post-hoc test where appropriate. ADP titrations were analyzed using constrained Michaelis-Menten kinetic curves and Ca^{2+} titrations were analyzed using constrained one-phase associations, whereby maximal respiration was defined (constraint set to 100). Statistical significance was determined as $p<0.05$. Data

expressed as mean ± SD and depicted as bar-and-scatter plots of individual values. Appropriate n sizes and statistical analysis details are listed in respective figure and table legends.

Results

Dietary nitrate increases force production in soleus muscle

Following 7 days of dietary nitrate consumption, serum NOx was increased ~4-fold in nitrate-consuming mice (37.3 ± 11.7 μM, n = 6, mean ± SD) compared to control mice (9.2 ± 5.1 μM, n = 6, mean ± SD, p=0.0006, two-tailed unpaired Student's t-test), confirming the effectiveness of nitrate supplementation and allowing us to examine the influence of nitrate on contractile properties. In both soleus and EDL muscles, there were no differences in the rate of force development ($+dp/dt_{max}$), rate of relaxation ($-dp/dt_{max}$), or half relaxation time (1/2 RT) between control and nitrate mice *(Table 1).* However, supplementation with nitrate increased force production at all stimulation frequencies tested (main effect of nitrate) in soleus *(Fig. 1A),* but not EDL *(Fig. 1B)*. In addition, in soleus muscle, force production throughout a 25 min stimulation protocol *(Fig. 1C)*, as well as the total force produced throughout the fatiguing protocol (*Fig. 1C, left inset*) was higher following nitrate consumption. At the end of the fatigue protocol, there was a non-significant trend (p=0.08, two-tailed unpaired Student's t-test; *Fig. 1C right inset*) for the percentage of initial force production to be higher in soleus of nitrate compared to control mice, suggesting a potential fatigue resistance. In contrast, nitrate did not attenuate fatigue within the EDL *(Fig. 1D and insets).*

Dietary nitrate improves submaximal SERCA activity in soleus muscle

Ca^{2+} handling is essential for maintaining contractile function at low frequencies of stimulation. Dietary nitrate has previously been shown to increase cytosolic Ca^{2+} concentrations in both skeletal (10) and cardiac (19) muscle, indicating a possible mechanism improving summation of force at low frequencies of stimulation. However, since this would necessitate a greater excursion of Ca^{2+} through SERCA we aimed to determine possible changes in SERCA enzymatic properties following nitrate consumption. Soleus muscle of nitrate animals displayed a non-significant trend ($p=0.053$) towards an ~25% increase in the Hill slope (n_H) compared to controls *(Fig. 2A, B).* While maximal SERCA activity (V_{max}) and the negative logarithm of $[Ca^{2+}]$ required to elicit half maximal SERCA activity (pCa_{50}) were unchanged in soleus, absolute ATPase activity at three pCa values (4.9, 5.43, 5.7) was higher in nitrate-consuming mice *(Fig. 2A, B).* In contrast, EDL showed no differences in any of the SERCA kinetic properties examined *(Fig 2C, D).* Dietary nitrate has previously been shown to increase the content of Ca^{2+} handling proteins in EDL muscle (10), however we did not observe any differences in SERCA, SLN, PLN, or CSQ protein content in soleus or EDL following 7 days of dietary nitrate consumption *(Fig. 2E, F).* The absence of a change in α-tubulin confirmed consistent loading in both soleus (100 ± 35 units Control, n = 6 vs. 86.8 ± 30.7 units Nitrate, n = 6, mean ± SD) and EDL (84.3 ± 25.1 units Control, n = 5 vs. 79.5 ± 28.3 units Nitrate, n =6; relative to soleus Control, mean ± SD) muscles (representative blot shown in *Fig. 2F).* Altogether, these data suggest that dietary nitrate increased indexes of submaximal SERCA activity in the soleus muscle.

Establishing a methodology to determine SERCA-mitochondria high-energy phosphate transfer

300 The apparent increase in SERCA-mediated ATP hydrolysis appears at odds with the
301 well-established finding that dietary nitrate deceases oxygen consumption during exercise.
302 Therefore, we next aimed to examine the high-energy phosphate transfer between SERCA and
303 mitochondria following nitrate consumption to determine if this was improved, possibly
304 explaining the discrepancy. To do so, we first established a methodology to measure the ability
305 of SERCA-derived ADP to stimulate mitochondrial respiration in permeabilized muscle fibers.
306 The theoretical premise of this experimental approach is that the addition of Ca^{2+} in the presence
307 ATP will generate ADP from SERCA, and this ADP will act as a substrate to support oxidative
308 phosphorylation *(Fig. 3A).* In this experiment, our functional readout of ADP-supported
309 mitochondrial respiration (oxygen consumption, JO_2) provides an indication of ATP hydrolysis
310 from SERCA.

311 To develop this approach, mitochondrial respiration (JO_2) was monitored in
312 permeabilized fibers from red gastrocnemius muscle *(Fig. 3B).* Respiration was supported by
313 pyruvate+malate, followed the addition of ATP *(Fig. 3B*). We then titrated sequentially
314 increasing concentrations of Ca^{2+} in the presence of ATP to stimulate SERCA-mediated ATP
315 hydrolysis and create ADP to support mitochondrial respiration. This revealed that maximal
316 SERCA-supported respiration was ~25% of oxidative capacity in the presence of saturating ADP
317 *(Fig. 3B, C)*. The addition of cyclopiazonic acid (CPA), a SERCA-specific inhibitor, fully
318 attenuated mitochondrial respiration to that of pyruvate+malate *(Fig. 3B),* suggesting SERCA-
319 derived ADP is capable of supporting mitochondrial respiration in our experimental design.
320 Importantly, Ca^{2+} did not increase mitochondrial respiration following the prior addition of CPA
321 *(Fig. 3D)*, and titrations of Ca^{2+} after 500 μM ADP (~ADP K_m) did not increase submaximal
322 ADP-supported respiration or attenuate maximal respiratory capacity *(Fig. 3E)*. Combined, this

methodology enables the examination of a high-energy phosphate cycling microdomain between ATP hydrolysis from SERCA and mitochondrial ADP provision.

The SERCA-mitochondria high-energy phosphate transfer displays fiber-type differences

To further establish the validity of the high-energy phosphate transfer methodology, we examined this interaction across various muscle fiber types with known differences in SERCA and mitochondrial content. We utilized white gastrocnemius (WG), red gastrocnemius (RG), and soleus given the dramatic differences in SERCA activity *(Fig. 4A, B, C: WG>RG>SOL)*, and content of SERCA1/2 *(Fig. 4D)*, CSQ1/2 *(Fig. 4E)*, and mitochondrial proteins *(Fig. 4F: SOL>RG>WG)* between fiber types. In line with the hierarchy of mitochondrial content, maximal mitochondrial respiration in the presence of ADP and various complex I- and II-linked substrates was greatest in soleus muscle, followed by RG and WG *(Fig. 5A)*. In addition, the apparent mitochondrial ADP K_m was lower in soleus and RG compared to WG *(Fig. 5B)*. While SERCA-supported respiration (presence of ATP and Ca^{2+}) was greatest in soleus *(Fig. 5C)*, this largely reflected the global differences in mitochondrial respiratory capacity between tissues. When expressed as a percentage of maximal ADP-supported respiration *(Fig. 5D)*, the ability of SERCA-derived ADP to stimulate respiration was higher in WG compared to RG and soleus, in line with the differences in SERCA activity between these tissues *(Fig. 4A, B)*. This occurred in the absence of changes in the sensitivity of mitochondria to SERCA-derived ADP *(Fig. 5E)*, similar to that of the SERCA pCa_{50} (*Fig. 4C*). Combined, these data support the methodology measuring the ability of SERCA-derived ADP to drive mitochondrial respiration and allowed us to examine the influence of dietary nitrate on the high-energy phosphate interaction between SERCA and mitochondria.

346

347 *Dietary nitrate supplementation increases SERCA-derived mitochondrial respiration*

348 We next aimed to examine global mitochondrial respiratory function, as well as the high-
349 energy phosphate interaction between SERCA and mitochondria, following dietary nitrate
350 consumption. Maximal respiratory capacity and the sensitivity of mitochondria to submaximal
351 concentrations of ADP were not affected by nitrate consumption *(Fig. 6A),* confirming the
352 absence of changes in mitochondrial respiratory function. With respect to the SERCA-
353 mitochondria energy transfer, dietary nitrate did not alter the sensitivity of mitochondria to Ca^{2+}-
354 mediated ADP supply *(Fig. 6B)*. However, the maximal ability of Ca^{2+} to drive respiration was
355 increased ~20% in nitrate-consuming mice compared to controls *(Fig. 6C)*. The difference in
356 SERCA-supported respiration between control and nitrate animals was fully attenuated by the
357 addition of CPA *(Fig. 6C),* was not due to global changes in mitochondrial ADP-supported
358 respiration *(Fig. 6D)*, and therefore is likely linked to SERCA activity. In support, while absolute
359 mitochondrial respiration in the presence of 250 μM and 1000 μM ADP was comparable
360 between control and nitrate mice *(Fig. 6D)*, respiration supported by SERCA-derived ADP at the
361 same absolute respiration rate of ~300 $pmol \cdot sec \cdot mg^{-1}$ dry wt (max Ca^{2+}, *Fig. 6C*) was greater
362 following nitrate supplementation. Similar to findings in soleus, this was not due to changes in
363 protein content, as there were no differences in Ca^{2+} handling proteins (SERCA1/2, CSQ1) or
364 mitochondrial proteins (OXPHOS, ANT1) in permeabilized muscle fibers used to perform
365 SERCA-derived ADP experiments *(Fig. 7A,B)*. In addition, phospholamban (PLN)
366 phosphorylation in RG muscle homogenate was not different between control and nitrate animals
367 *(Fig. 7A,B)*. As BRJ consumption in humans has been shown to increase mitochondrial ROS
368 emission rates (17), and H_2O_2 directly increases force production (32) it is possible that

mitochondrial-derived ROS are influencing nitrate-induced changes in SERCA activity and contractile properties. However, we did not detect any differences in mitochondrial ROS emission rates in red gastrocnemius permeabilized muscle fibers following nitrate supplementation, with respect to both maximal rates in the presence of succinate *(Fig. 7C)* or submaximal rates in the presence of 100 μM ADP *(Fig. 7D)*.

Discussion

In the current study, we provide mechanistic insight into the ability of dietary nitrate to alter skeletal muscle contractile properties. Following 7 days of 1 mM nitrate supplementation, soleus muscle of nitrate-consuming animals mice displayed greater force production over various stimulation frequencies and during a 25 min fatiguing contraction protocol. This was mirrored by an increase in submaximal SERCA activity and trend towards an increase in binding efficiency (Hill slope, p=0.053), which would suggest an improvement in Ca^{2+}/ATP coupling ratios in the absence of changes in V_{max}. To examine the interaction between ATP hydrolysis from SERCA and mitochondrial function, we established a methodology to measure the high-energy phosphate transfer between organelles. However, in contrast to our hypothesis, this approach revealed that mitochondrial respiration supported by SERCA-derived ADP was increased following dietary nitrate consumption, suggestive of increased rates of ATP hydrolysis.

Contractile function and SERCA activity

Nitrate has previously been shown to alter contractile function, and we aimed to examine this in the soleus (slow-twitch) and EDL (fast-twitch) muscles of mice. Considerable interest has been placed on the effects of nitrate in type II fibers (fast-twitch), as microvascular oxygen

tension is lower than in type I muscle fibers, which could promote the conversion of nitrite to NO for subsequent biological effects (33). While functional changes with dietary nitrate have been reported exclusively in fast-twitch EDL of mice (10, 34), work in human muscle of mixed fiber type composition (16, 35) and in cardiac muscle (19) would suggest there is no preferential target of NO. In our study, only soleus muscle (predominantly type I fibers) displayed improvements in contractile function following dietary nitrate supplementation, encompassing an increase in force production at various stimulation frequencies and throughout a 25 min fatiguing contraction protocol. Importantly, fiber type composition of mouse soleus muscle is more similar than EDL to human muscle (36–38), and therefore the increase in force production that we observed in soleus would also be in line with findings in mixed fiber type human muscle (16, 35). In addition, as nitrate is postulated to raise cytosolic Ca^{2+} concentrations by increasing the probability of ryanodine receptor 1 (RyR1) opening (39), and the RyR1 isoform is present in all muscle fiber types (40), it would be expected that the effects of nitrate are not fiber-type specific. Functionally, it also seems likely that nitrate influences type I fibers as these are predominantly recruited during submaximal exercise when reductions in VO_2 following nitrate have been observed (12, 14); and NO_3^- and NO_2^- concentrations are reported to be higher in slow-twitch soleus muscle compared to faster twitch muscle of rats (41). Alternatively, our study was performed in female mice, while most previous research was conducted in male mice and humans (10, 16). It is therefore possible that nitrate preferentially influences type I muscle fibers in females, in contrast to type II muscle fibers in males. While previous work has also shown that acute (2.5 hours) and chronic (8 day) BRJ supplementation increased low-frequency force production at 10 Hz in females (42), research examining the influence of nitrate on performance in females is limited and warrants further investigation. We also performed our experiments in

C57Bl/6N mice, while some previous studies examining the influence of nitrate and nitrite on contractile properties utilized C57Bl/6J mice (22) which possess a mutation in the nicotinamide nucleotide transhydrogenase (Nnt) gene involved in antioxidant defense (43). It is therefore possible that the presence of the Nnt gene in the N strain utilized in our study could influence the ability of nitrate to alter skeletal muscle properties in slow-twitch muscles.

The ergogenic effects of dietary nitrate have been linked to Ca^{2+} handling, as previous work has indicated that dietary nitrate supplementation increases cytosolic Ca^{2+} concentrations (10, 19). However, this would necessitate a greater activity of SERCA to sequester Ca^{2+} within the SR for relaxation to occur. In the present study we observed submaximal SERCA activity was increased ~25% at three pCa values in soleus of nitrate-consuming animals, supporting the previous finding that *in vitro* incubations with nitrite increases SR Ca^{2+} pumping in single muscle fibers of mice (22). These data would suggest an increased ATP cost of controlling cytosolic Ca^{2+} following nitrate exposure, seemingly in contrast to the previous findings indicating a reduction in ATP turnover following nitrate supplementation (15). However, while SERCA generally transports Ca^{2+} ions across the SR membrane at the cost of ATP in a 2:1 ratio, SERCA function can be altered under various situations of external regulation, resulting in changes to the coupling efficiency of SERCA (i.e. Ca^{2+} transport/ATP) (44). Without direct measurements of Ca^{2+} uptake in the present study it is difficult to determine if SERCA efficiency was improved; however, the trend ($p=0.053$) towards an increase in Hill slope (n_H) following nitrate consumption indicates a greater Ca^{2+}affinity/cooperativity, and therefore possibly improved Ca^{2+}/ATP coupling ratios. Regardless, the present data suggests that dietary nitrate increases SERCA ATP hydrolysis. This may contribute to fatigue resistance, however, cannot explain an improvement in low-frequency force or well-established reductions in whole-body

oxygen consumption. In this respect, dietary nitrate likely has several mechanisms-of-action and assessments of whole-body oxygen consumption reflects a sum/amalgamation of these responses. Possible improvements in the efficiency of actin-myosin ATPase, which represents the vast majority of ATP hydrolysis during exercise, may mask increased SERCA-mediated ATP hydrolysis. In support, NO has been shown to alter actin-myosin cross-bridge sensitivity (9, 45), which could contribute to a reduction in net ATP turnover despite greater Ca^{2+} pumping. Regardless, an increase in SERCA activity will reduce cytosolic Ca^{2+} concentrations, and while this cannot contribute to an increase in submaximal force, it could provide a mechanism for the improvement in fatigue with nitrate supplementation.

While mechanisms underlying the change in submaximal SERCA activity and trend towards a change in the Hill slope observed in the current study remain unknown, this could involve NO-dependent modification of the SERCA enzyme. NO has been shown to activate RyR Ca^{2+} release channels at high concentrations, and inactivate channels at low concentrations (21), supporting the ability of NO to modulate Ca^{2+} handling proteins on the SR. In addition, with approximately 22 free cysteine residues, SERCA is highly susceptible to post-translational redox modifications (46). NO itself can directly modify SERCA at these protein thiols (47), but can also react with superoxide anions to produce peroxynitrite (48), a potent oxidizing agent that can modify important cysteine residues of SERCA (47). Following BRJ supplementation, Whitfield *et al.* (17) found an increased propensity towards mitochondrial H_2O_2 emission in human skeletal muscle, thus presenting a possible mechanism connecting the improvements in excitation-contraction coupling reported in humans. However, this occurred in the absence of changes in cellular redox markers (4HNE, Oxyblot, nitrotyrosine) (17), and in red gastrocnemius muscle of mice, we did not observe any increase in maximal or submaximal mitochondrial ROS emission

rates following nitrate supplementation. While it remains possible that cytosolic and non-mitochondrial ROS are influencing SERCA binding domains, it is more likely that NO-mediated modifications could explain the changes in function we observed following nitrate supplementation.

Alternatively, the small membrane bound proteins PLN and sarcolipin (SLN) can regulate SERCA activity through physical interactions. While we could not detect changes in PLN phosphorylation, commercially available antibodies that detect SLN phosphorylation do not exist. SLN has been shown to reduce the apparent Ca^{2+} binding affinity and maximal activity of SERCA (49), represented by a rightward shift in the SERCA kinetic curve. Therefore, it is possible that post-translational modifications on SLN could cause a dissociation of SLN from SERCA following nitrate consumption, and could explain the altered SERCA kinetic profile; however, this remains to be directly determined. SLN appears particularly important for SERCA regulation in slow-twitch muscle, as the SERCA pCa was increased in soleus and RG (indicating a leftward kinetic shift), but not EDL or WG, of mice lacking SLN (50). However, it does not seem that the changes in submaximal SERCA activity and efficiency we observed are mediated by protein content, as we did not detect any differences in content of SERCA, CSQ, PLN, or SLN following nitrate supplementation. This is in contrast to previous work in rodents determining an increase in Ca^{2+} handling proteins (10, 34) but in line with findings in human skeletal muscle reporting no differences (16) following nitrate supplementation. The absence of changes in protein content would further suggest post-translational modifications are important for the observed outcomes with nitrate consumption. In support, a reduction in the oxygen cost of exercise with dietary nitrate has been produced as acutely as 2.5 hours following ingestion (51) suggesting the effects of nitrate on skeletal muscle are due to more transient modifications

than protein synthesis. Regardless of the mechanisms mediating a change in SERCA activity, the greater submaximal enzymatic kinetics and greater Ca^{2+} availability (10) would suggest *in vivo* Ca^{2+} flux through SERCA is increased following nitrate and could indicate a greater reliance on the SERCA-mitochondria interaction for ATP provision.

Mitochondria-SERCA interaction

Within skeletal muscle, SERCA accounts for nearly 40-50% of energy consumption at rest (23), and ~50% during muscle contraction (24). Therefore, a link between SERCA and mitochondria is important for maintaining ATP turnover. Structurally, mitochondria and SR are highly integrated (25), and the outer mitochondrial protein voltage-dependent anion channel (VDAC) has been shown to be physically linked to Ca^{2+} release channel inositol 1,4,5-triphosphate receptor (IP_3R) on the endoplasmic reticulum (52). VDAC is a ubiquitous transport protein involved in the provision of numerous substrates to mitochondria, including the transport of ADP. This structural association would therefore suggest that mitochondrial ATP/ADP transport is highly concentrated in the proximity of the SR. As permeabilized fibers represent an *in vitro* preparation of intact cellular network (53), it is possible to measure the high-energy phosphate transfer between organelles. Indeed, we have established a method to measure the ability of SERCA-derived ADP to support mitochondrial respiration by titrating Ca^{2+} in the presence of ATP. While Ca^{2+} has been shown to increase ADP-supported respiration in isolated mitochondria (54), we did not detect an ability of Ca^{2+} alone to alter oxygen consumption in permeabilized muscle fibers. This may be due to the presence of the SR in permeabilized fiber preparations, in which Ca^{2+} uptake into the SR alters the ability of free Ca^{2+} to accumulate within the mitochondrial matrix and influence O_2 consumption.

507 The structural and functional interactions between SR and mitochondria appear to be
508 influenced by cellular energetic state. For instance, impairments in ER-mitochondrial coupling
509 are evident in skeletal muscle of insulin resistant individuals (55), a situation known to influence
510 mitochondrial function (56) and Ca^{2+} homeostasis (57, 58). Given that dietary nitrate also
511 appears capable of influencing these processes (10), the SR-mitochondria high-energy phosphate
512 interaction is of particular interest. In line with our findings of an increase in submaximal
513 SERCA activity, dietary nitrate increased the ability of Ca^{2+} to drive mitochondrial respiration.
514 This would therefore suggest that greater rates of ATP hydrolysis from SERCA following nitrate
515 consumption provide an increased provision of ADP to mitochondria. Similar to previous work
516 (17, 18), we did not detect any ability of dietary nitrate to improve global mitochondrial
517 respiratory capacity or mitochondrial ADP sensitivity. This would therefore indicate that the
518 increase in mitochondrial respiration we observed in response to SERCA-derived ADP largely
519 reflects the change in SERCA activity and ATP hydrolysis, as opposed to an influence of dietary
520 nitrate on mitochondrial function. Nevertheless, while we observed an increase in submaximal,
521 but not maximal SERCA activity; in stark contrast, we observed an increase in maximal, but not
522 submaximal, SERCA-supported mitochondrial respiration. One possible explanation for this
523 discrepancy is that all respiratory experiments reflect submaximal SERCA activity, as greater
524 additions of Ca^{2+} in our *in vitro* preparation appeared to elicit a detrimental effect on
525 mitochondrial respiration (see *Fig. 3B*). Altogether, our data does suggest that SERCA-derived
526 mitochondrial respiration was increased following nitrate consumption, and while this may
527 contribute to fatigue resistance, cannot explain the reduction in submaximal VO_2 observed with
528 dietary nitrate.
529

Limitations

While our SERCA-supported mitochondrial respiration experiment is a functional readout of the link between two organelles, we are not able to determine any structural interactions between mitochondria and the SR. It remains unknown if physical changes occurred following nitrate supplementation, such as the extent of VDAC-IP_3R interactions which can be altered by insulin resistance (55). In addition, it is possible that the rise in cytosolic Ca^{2+} with dietary nitrate (10) increases Ca^{2+} provision to mitochondria and influences other *in vivo* mitochondrial signaling pathways. While this remains to be determined, our methodology nonetheless establishes a link between mitochondria and SERCA and can demonstrate changes in mitochondrial respiration in response to altered SERCA kinetics. However, while the increase in submaximal SERCA activity following nitrate supplementation can contribute to fatigue resistance and compensate for the increase in cytosolic Ca^{2+} concentrations, it cannot explain the nitrate-mediated reduction in ATP turnover during exercise or an improvement in low-frequency force production. It is therefore likely that these responses involve a mechanism affecting actin-myosin ATPase to improve ATP turnover efficiency. In support, NO has been shown to alter actin-myosin cross-bridge sensitivity (9, 45); however, this remains a subject of future research.

Perspectives and Conclusion

We provide evidence that 7 days of dietary nitrate consumption increases submaximal SERCA activity and tends ($p=0.053$) to increase Ca^{2+} binding efficiency. This finding was also evident in a methodology examining the high-energy phosphate transfer from SERCA to mitochondria, where we report a greater ability of SERCA-derived ADP to increase mitochondrial respiration. However, while the increase in submaximal SERCA activity likely

553 represents a compensatory mechanism to counter the rise in cytosolic Ca^{2+} following nitrate
554 supplementation, our findings do not appear to explain the nitrate-mediated decrease in ATP
555 turnover or increase in low-frequency force production, suggesting other cellular targets of
556 nitrate such as actin-myosin ATPase. Overall, dietary nitrate improves submaximal SERCA
557 activity, efficiency, and SERCA-supported mitochondrial respiration while functionally
558 increasing force production throughout a fatiguing contraction protocol. These findings suggest
559 that alterations in SERCA enzymatic properties are a mechanism in which dietary nitrate
560 enhances fatigue resistance, providing insight into the ability of nitrate to influence exercise
561 performance.
562

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

HLP, SB, BV, HB, LJCvL, CLM, and GPH designed the study. HLP, SB, BV, HSB, RMH, and GPH organized and performed experiments. All authors analyzed and interpreted the data. HLP, SB, and GPH drafted the manuscript, and all authors approved the final version.

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Figure Legends

Figure 1: Dietary nitrate increases force production in soleus, but not EDL, of female mice. Force production over various stimulation frequencies in soleus (A) and EDL (B) muscle. Force production over a 25 min fatigue protocol in soleus (C) and EDL (D). Insets depict force production as a percentage of maximum (A, B), total force produced during the fatigue protocol (C, D left inset), and percentage of initial force production at the end of the fatigue protocol (C, D right inset). Control and nitrate data points in (B, D) were obtained at the same stimulation frequency (B) or time (D), however the x-axes are off-set to better distinguish the two groups. Data analyzed using two-way ANOVA (A-D) or two-tailed unpaired Student's t-tests (C, D insets). * $p<0.05$ vs. Control. Data expressed as mean ± SD. n = 12 soleus, n = 10 EDL.

Figure 2: Dietary nitrate increases submaximal SERCA activity in soleus muscle, but does not alter content of Ca^{2+} handling proteins. SERCA activity (A) and enzymatic properties (B) within soleus muscle and EDL muscle (C, D). Protein content of Ca^{2+} handling proteins (SERCA1, SERCA2, CSQ1, CSQ2, PLN, SLN) in soleus and EDL (E, F). α-tubulin was used as a loading control (F), and arbitrary protein content did not differ between control and nitrate animals in soleus (100 ± 34.7 units Control, n=6 vs. 86.8 ± 30.7 units Nitrate, n=6) or EDL (84.3 ± 25.1 units Control, n=5 vs. 79.5 ± 28.3 units Nitrate, n=6; relative to soleus Control). Data analyzed using two-way ANOVA (A, C) with LSD post-hoc test when an interaction was determined (A) or two-tailed unpaired Student's t-tests (B, D, E). * $p<0.05$ vs. Control. Data expressed as mean ± SD. n = 5-7. CSQ, calsequestrin; pCa_{50}, negative logarithm of $[Ca^{2+}]$ required to elicit half maximal SERCA activity; PLN, phosholamban; SERCA, sarcoplasmic reticulum calcium ATPase; SLN, sarcolipin.

Figure 3: Establishing a methodology to examine the high-energy phosphate transfer between SERCA and mitochondria. ADP released from SERCA, in the presence of ATP and Ca^{2+}, is capable of supporting mitochondria oxidative phosphorylation in permeabilized muscle fibers (A). $CaCl_2$ was titrated in the presence of PM and ATP while measuring oxygen consumption (JO_2), and the addition of SERCA-specific inhibitor CPA fully attenuated respiration (B). The ability of SERCA-derived ADP (Ca^{2+}) to increase mitochondrial respiration was ~25% of maximal ADP-supported respiratory capacity (C) and the prior addition of CPA prevented the increase in mitochondria respiration supported by Ca^{2+} (D). Ca^{2+} in the concentrations utilized did not increase mitochondrial respiration or alter the ability of subsequent substrates to drive respiration (E). Data analyzed using two-tailed unpaired Student's t-tests. * $p<0.05$ vs. Ca^{2+} (C) or -CPA (D). Data expressed as mean ± SD. n = 5-11. ADP, adenosine diphosphate; ANT, adenine nucleotide translocase; ATP, adenosine triphosphate; Ca^{2+}, calcium; CPA, cyclopiazonic acid; G, glutamate; JO_2, oxygen consumption; OXPHOS, oxidative phosphorylation system; PM, pyruvate+malate; S; succinate; SERCA, sarcoplasmic reticulum calcium ATPase; SR, sarcoplasmic reticulum.

Figure 4: SERCA activity and protein content display fiber-type differences. SERCA activity (A,B) and the negative logarithm of [Ca^{2+}] required to elicit half maximal SERCA activity (pCa_{50}, C) in soleus, RG, and WG. SERCA1 and SERCA2 (D), CSQ1 and CSQ2 (E), and mitochondrial (F) protein content in WG, RG, and soleus. Molecular weights for representative western blots in panel (F) are CV (55 kDa), CIII (45 kDa), CIV (37 kDa), CII (25 kDa), CI (18 kDa), and ANT1 (32 kDa). Data analyzed using one-way ANOVA with LSD post-

hoc multiple comparisons. * $p<0.05$ vs. WG and # $p<0.05$ vs. RG. Data expressed as mean ± SD. n = 4. ANT, adenine nucleotide translocase; CSQ, calsequestrin, pCa_{50}, negative logarithm of $[Ca^{2+}]$ required to elicit half maximal SERCA activity; Ponc, ponceau stain; R, RG; RG, red gastrocnemius; S, Sol; SERCA, sarcoplasmic reticulum calcium ATPase; Sol, soleus; V_{max}, maximal enzymatic activity; W, WG; WG, white gastrocnemius.

Figure 5: Mitochondrial respiration supported by global ADP supply and SERCA-derived ADP display fiber-type differences. Maximal mitochondrial respiration (A) and ADP sensitivity (B) in soleus, RG, and WG. Mitochondrial respiration supported by SERCA-derived ADP (C) and the ratio of Ca^{2+}-supported respiration / ADP-supported respiration (D) in different muscle fiber types. Sensitivity of mitochondria to SERCA-derived ADP from Ca^{2+} titrations (E). Data analyzed using one-way ANOVA with LSD post-hoc multiple comparisons. * $p<0.05$ vs. WG and # $p<0.05$ vs. RG. Data expressed as mean ± SD. n=9-12 (A-C, E). n=7-9 (D) because some animals were used for just ADP or just Ca^{2+} titration experiments, therefore only animals used for both experiments were included in calculating the ratio of Ca^{2+}-supported respiration/ADP-supported respiration. ADP, adenosine diphosphate; ATP, adenosine triphosphate; C, cytochrome C; Ca^{2+}, calcium; CPA, cyclopiazonic acid; G, glutamate; JO_2, oxygen consumption; K_m, Michaelis-Menten constant; PM, pyruvate+malate; RCR, respiratory control ratio; RG, red gastrocnemius; S, succinate; Sol, soleus; $V_{max,}$ maximal enzymatic activity; WG, white gastrocnemius.

840

841 **Figure 6: Dietary nitrate increases mitochondrial respiration supported by SERCA-**
842 **derived ADP, but not global ADP supply.** Maximal mitochondrial respiratory capacity and the
843 sensitivity of mitochondria to ADP within RG following nitrate consumption (A). Mitochondrial
844 sensitivity to SERCA-mediated ADP supply (B) and maximal respiration supported by Ca^{2+} (C).
845 Submaximal ADP-supported respiration at a similar absolute respiration rate as SERCA-derived
846 ADP (Ca^{2+}) titrations. (D). Data analyzed using two-tailed unpaired Student's t-tests. * $p<0.05$
847 vs. Control. Data expressed as mean ± SD. n = 13 control, n = 15 nitrate. ADP, adenosine
848 diphosphate; ATP, adenosine triphosphate; C, cytochrome C; Ca^{2+}, calcium; CPA, cyclopiazonic
849 acid; G, glutamate; JO_2, oxygen consumption; K_m, Michaelis-Menten constant; PM,
850 pyruvate+malate; RCR, respiratory control ratio; RG, red gastrocnemius; S, succinate; $V_{max,}$
851 maximal enzymatic activity.

852

853 **Figure 7: Dietary nitrate does not alter content of Ca^{2+} handling proteins, mitochondrial**
854 **proteins, or mitochondrial ROS emission rates in red gastrocnemius.** SERCA-related protein
855 content and mitochondrial protein content in RG. Permeabilized muscle fibers were used for
856 SERCA1/2, CSQ1/1, ANT, and OXPHOS; while whole RG homogenate was used for PLN and
857 pPLN to detect protein phosphorylation (A, B). Maximal (succinate; C) and submaximal (+100
858 μM ADP, D) mitochondrial ROS emission rates in RG permeabilized muscle fibers. Data
859 analyzed using two-tailed unpaired Student's t-tests. Data expressed as mean ± SD. n = 8-12.
860 ADP, adenosine diphosphate; ANT1, adenine nucleotide translocase; C, control; CSQ,
861 calsequestrin; mH_2O_2, mitochondrial hydrogen peroxide emission; PLN, phospholamban; pPLN,
862 phosphorylated phospholamban; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; N, nitrate.

Fig. 1

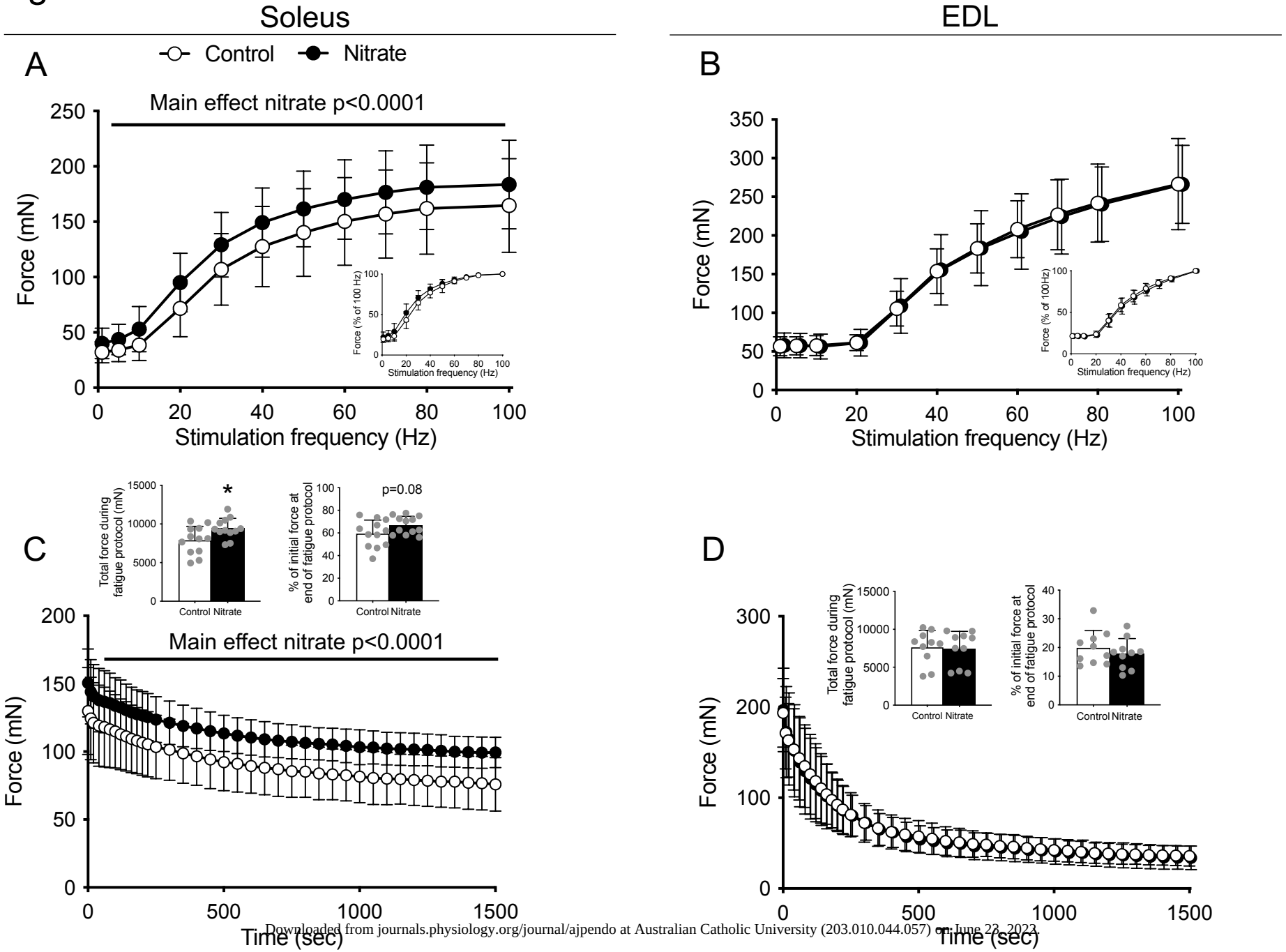
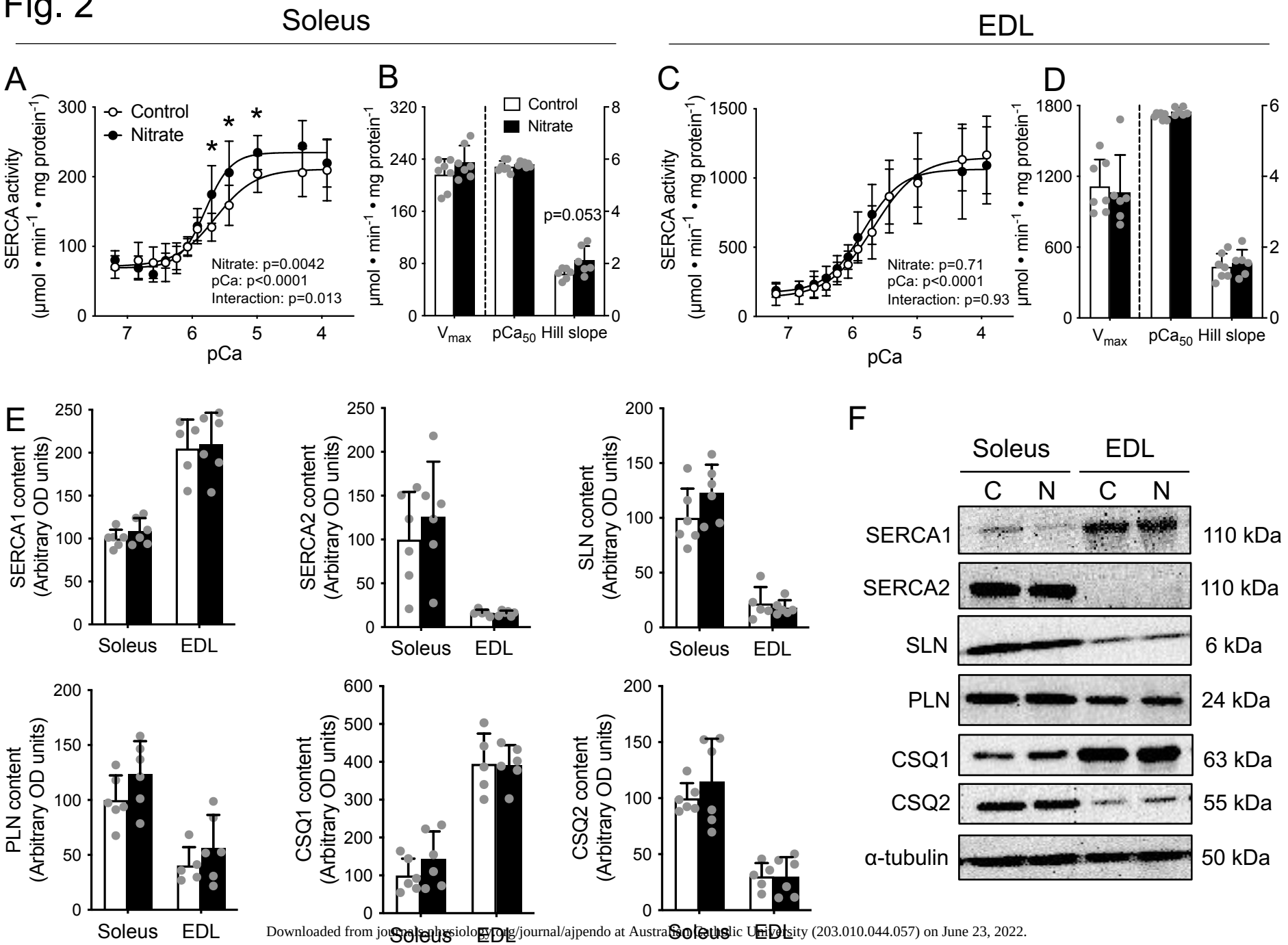


Fig. 2



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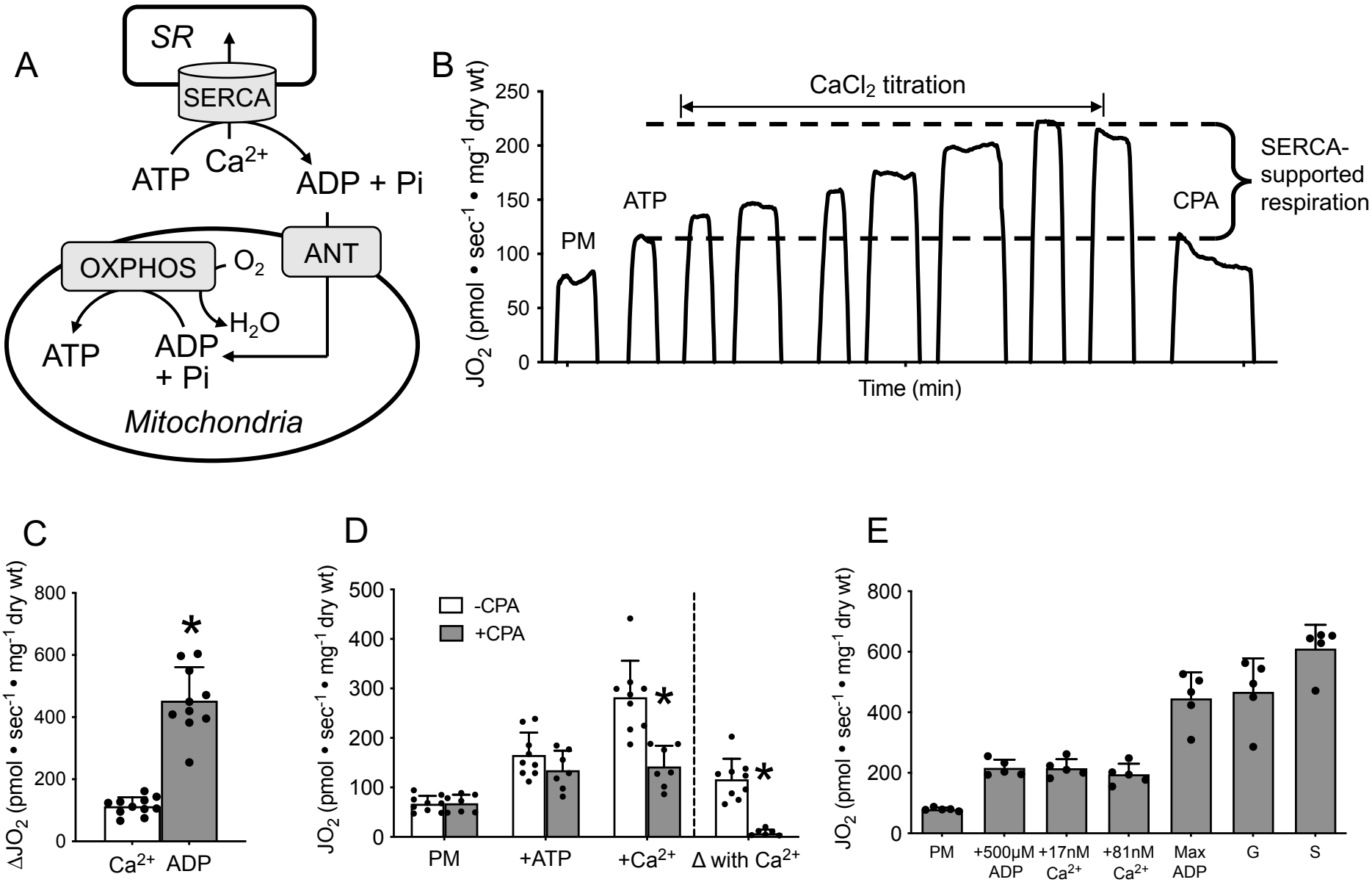

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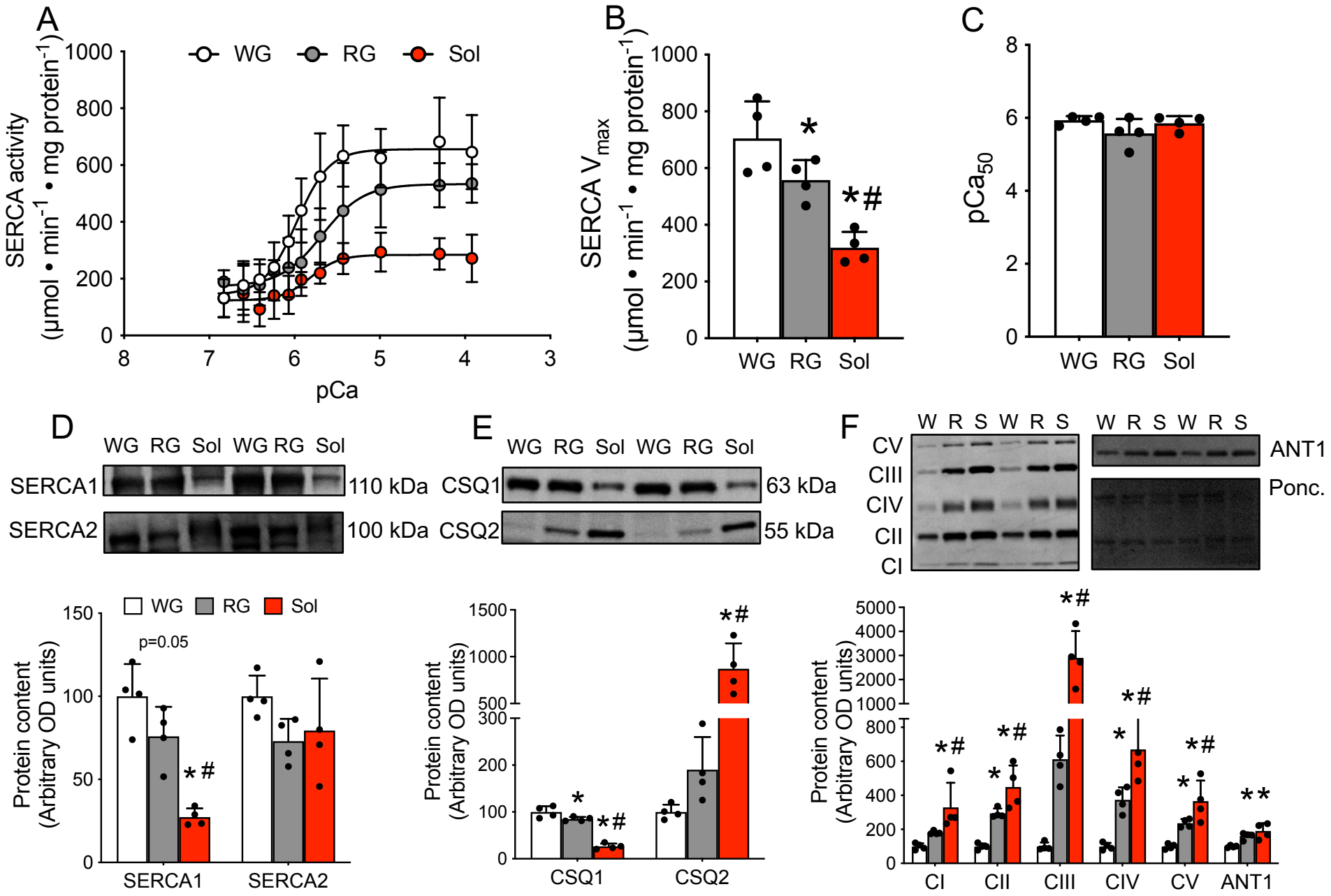

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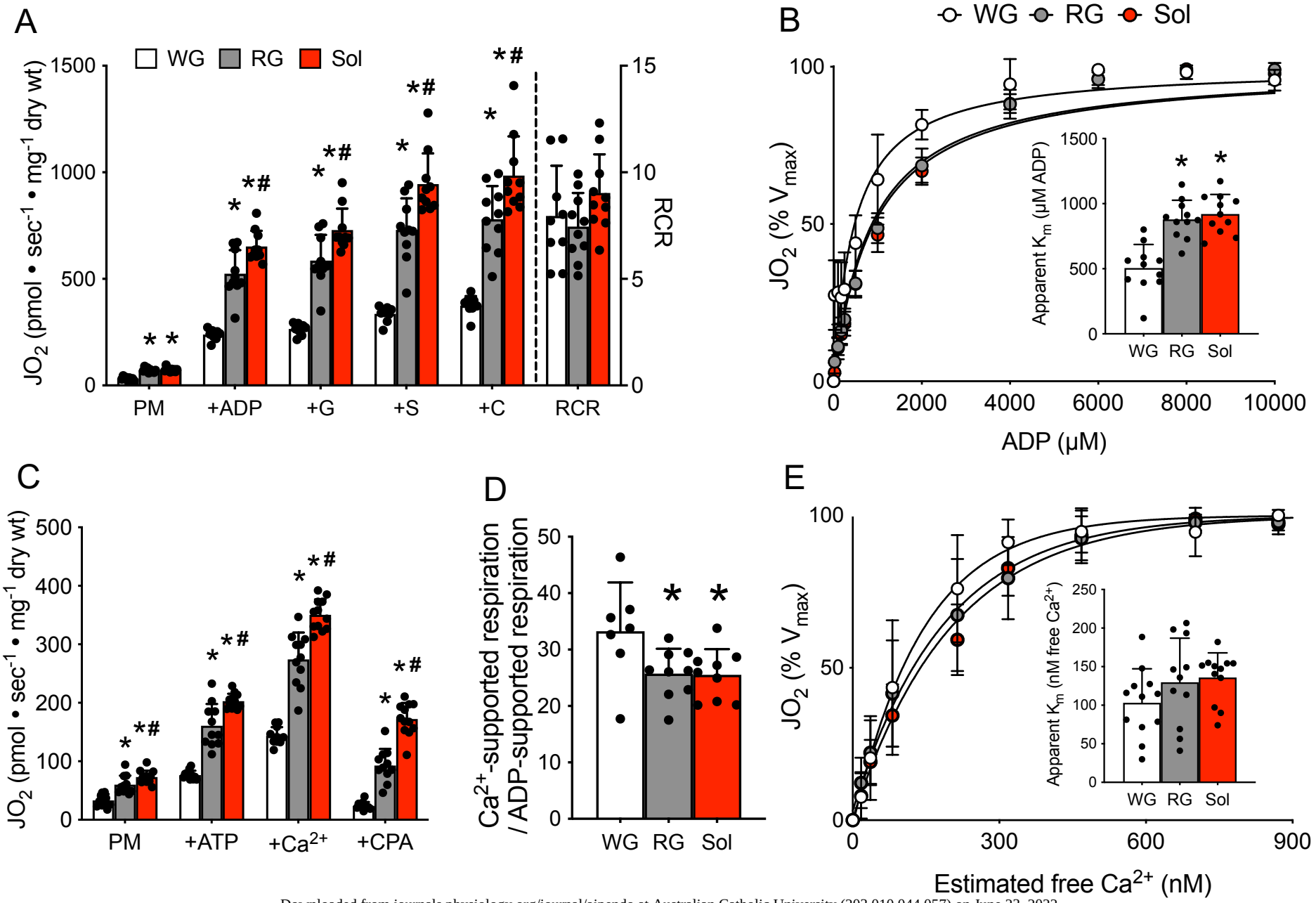


Fig. 6

Red gastrocnemius

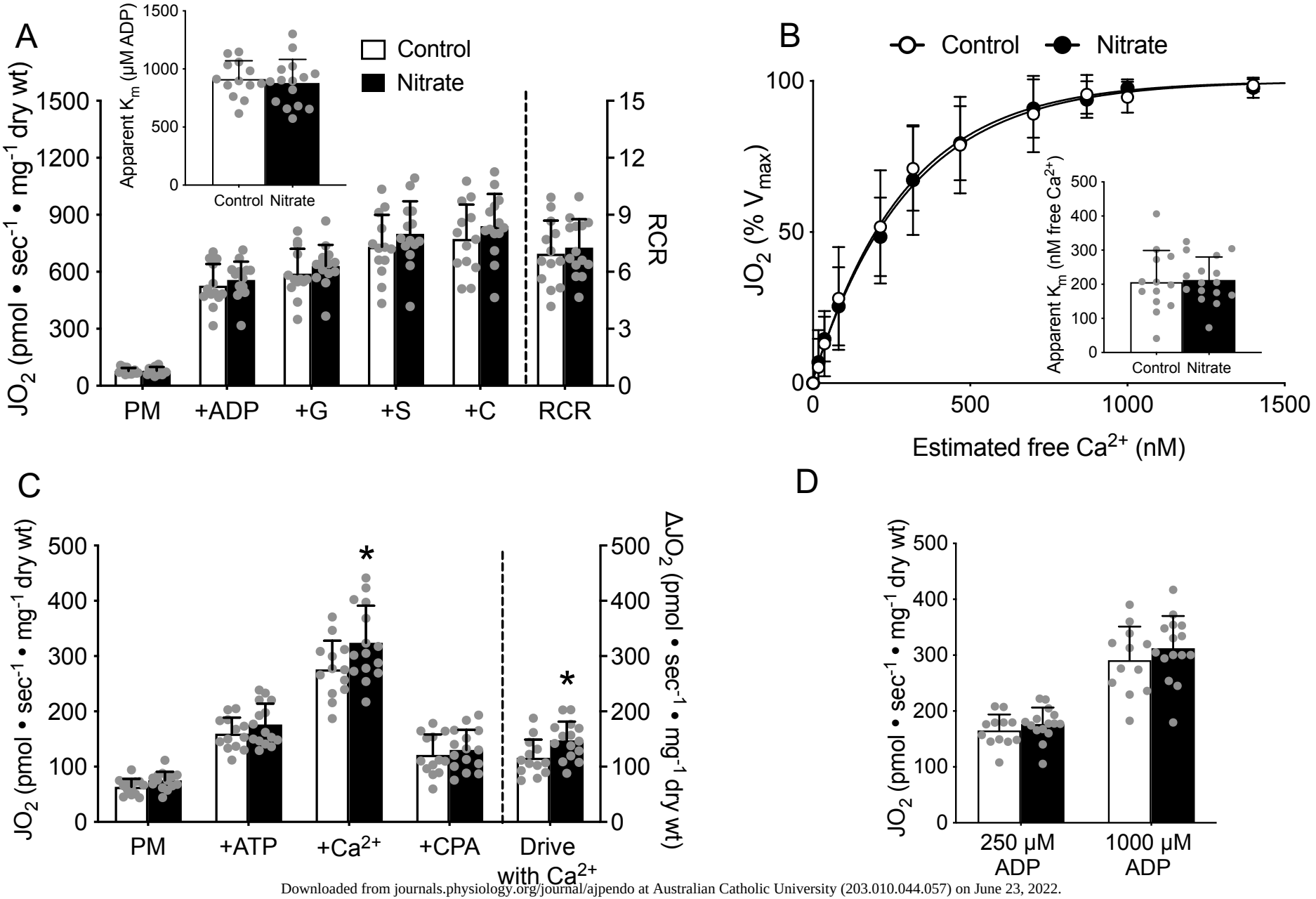
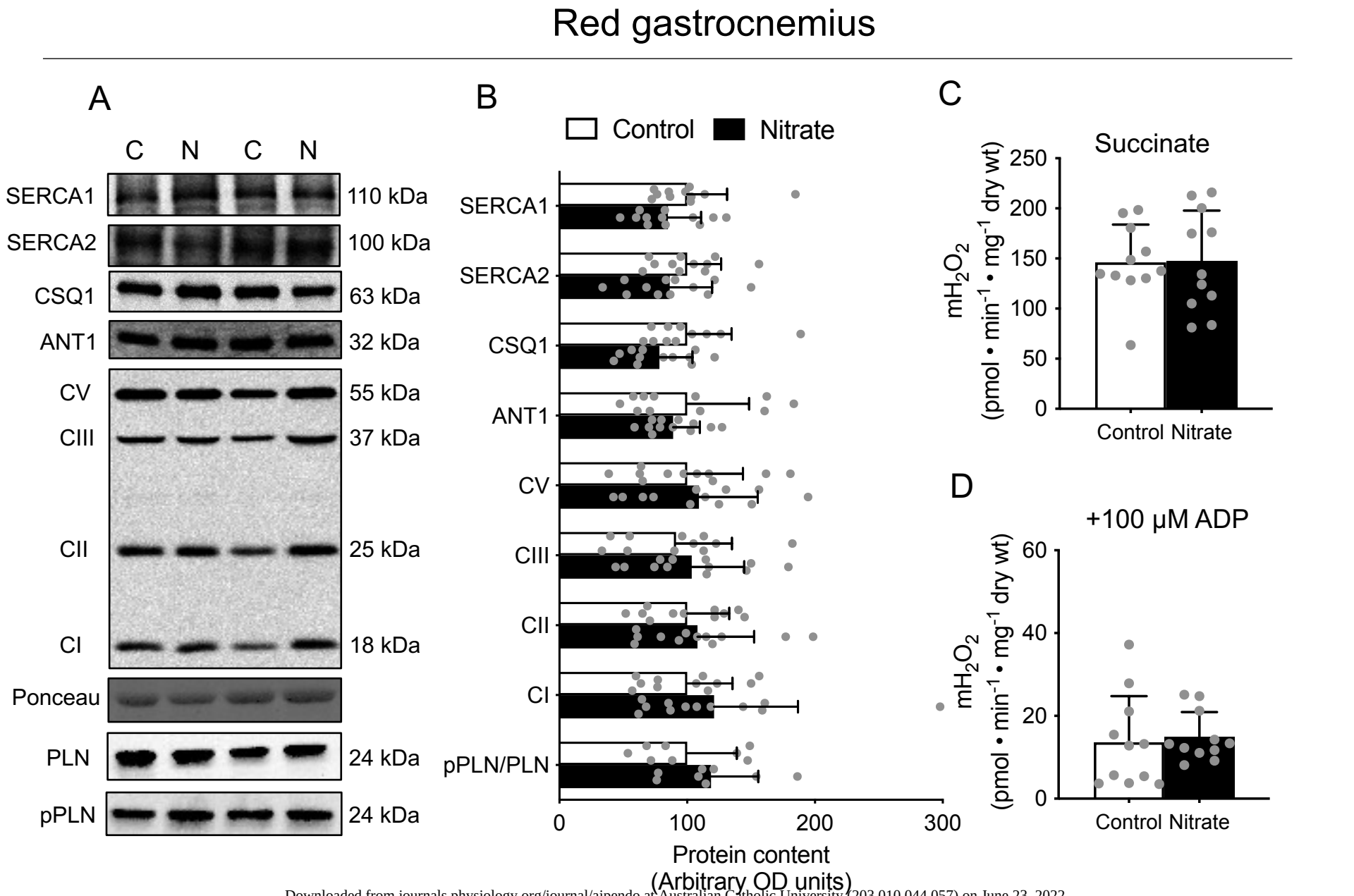


Fig. 7



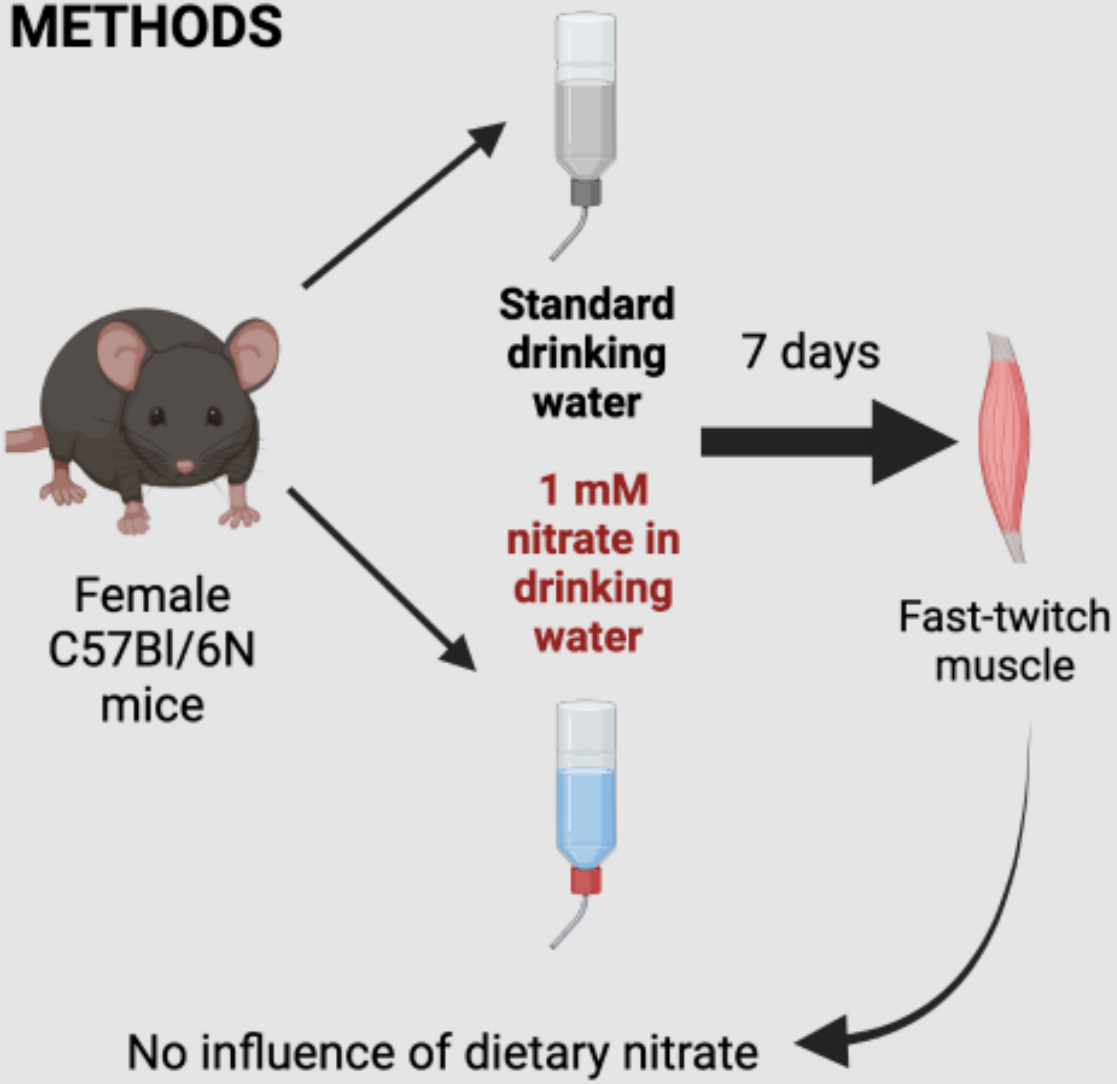
Tables

	Pre- Force Frequency		Post- Force Frequency	
	Soleus			
	Control	**Nitrate**	**Control**	**Nitrate**
+dp/dt$_{max}$ (N • sec^{-1})	896.2 ± 159.4	889.3 ± 323.3	888.5 ± 163.7	918.3 ± 319.2
-dp/dt$_{max}$ (N • sec^{-1})	614.8 ± 109.4	554.1 ± 122.3	628.4 ± 144.5	600.3 ± 120.5
½ RT (sec)	0.54 ± 0.11	0.64 ± 0.16	0.51 ± 0.11	0.57 ± 0.11
	EDL			
	Control	**Nitrate**	**Control**	**Nitrate**
+dp/dt$_{max}$ (N • sec^{-1})	1740.5 ± 262.2	1804.0 ± 233.4	1790.5 ± 125.9	1748.1 ± 196.5
-dp/dt$_{max}$ (N • sec^{-1})	1580.2 ± 262.9	1610.5 ± 332.4	1512.9 ± 236.9	1555.3 ± 295.4
½ RT (sec)	0.36 ± 0.05	0.31 ± 0.02	0.30 ± 0.01	0.30 ± 0.01

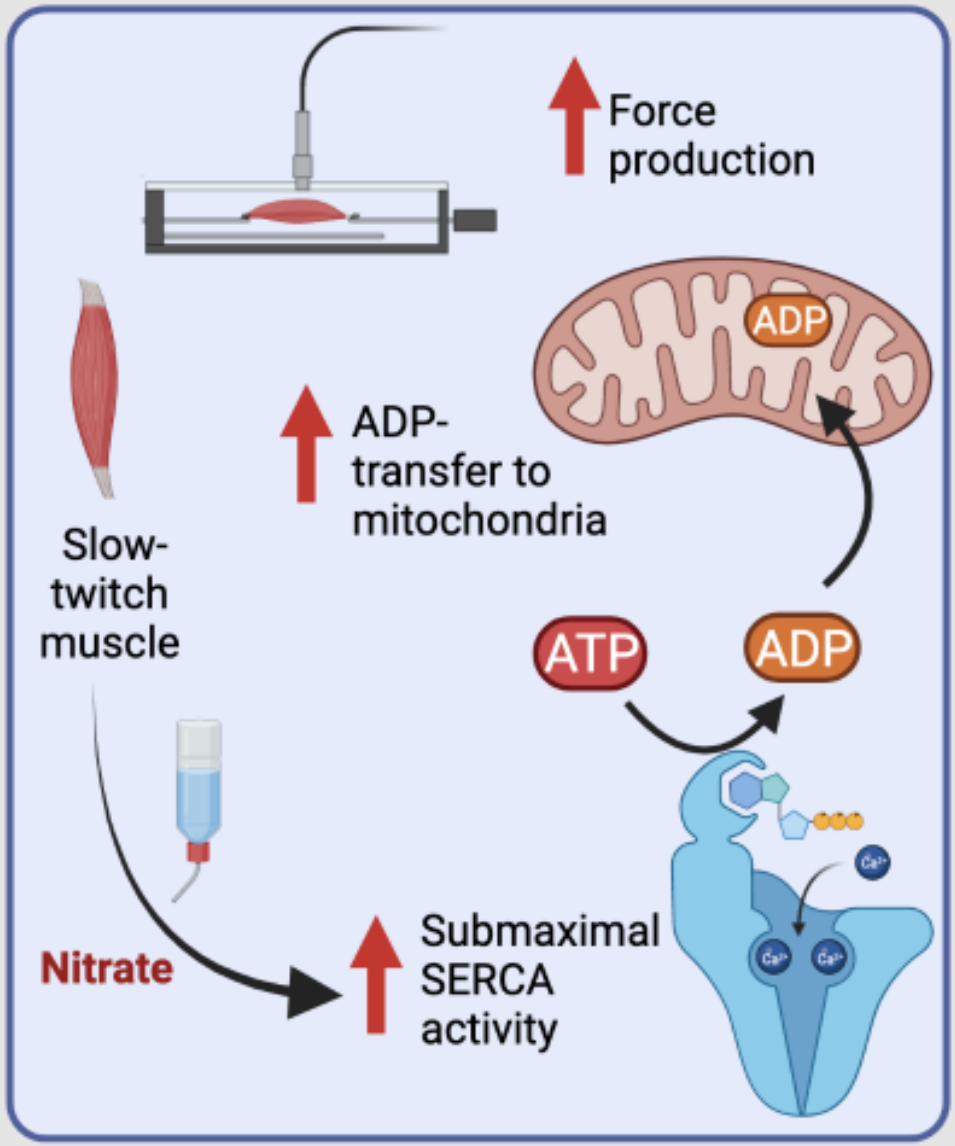
Table 1: Twitch characteristics following 7 days of nitrate supplementation in soleus and EDL muscle. Data analyzed using two-way ANOVA. Data expressed as mean ± SD. n = 6 soleus, n = 6 EDL.

Dietary nitrate increases submaximal SERCA activity and ADP-transfer to mitochondria in slow-twitch muscle of female mice

METHODS



RESULTS



CONCLUSIONS

Improvements in submaximal SERCA activity may represent a mechanism in which dietary nitrate increases force production during fatigue in slow-twitch muscle of female mice.