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RESEARCH ARTICLE

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 sarcoplasmic reticulum (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 the 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 by ~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 that improvements in submaximal SERCA kinetics may contribute to the effects of nitrate on force production during fatigue.

NEW & 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 (soleus) but not type II fibers (EDL). These findings suggest that alterations in SERCA properties are a possible mechanism in which nitrate increases force during fatiguing contractions.

calcium homeostasis; contractile function; mitochondria; nitrate; SERCA

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, whereas 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-

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

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

Within skeletal muscle, SERCA activity accounts for ~25%–50% of cellular ATP use at rest (23) and during contraction (24), indicating the importance of energy transfer between SERCA and mitochondria. A link between the SR and mitochondria exists (25) that promotes the exchange of signaling molecules such as Ca^{2+} and reactive oxygen species (ROS) (26, 27), and 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 whether 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 wk 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 h light-dark cycle.

Experimental Design

Female C57Bl/6N mice (15–20 wk 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 of sodium pentobarbital. All tissue collection procedures were performed only after assurance of anesthesia depth checked by leg retraction after tail pinch and movement of whiskers. In one subset of mice (n = 7 in 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 four-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 ms trains at 100 Hz. Muscles were then equilibrated for 30 min before the stimulation protocol. All electrical pulses were 0.5 ms in duration with a train duration of 250 ms. The stimulation protocol consisted of a twitch stimulus followed by a sequential force-frequency stimulation protocol (1, 5, 10, 20, 30, 40, 50, 60, 70, 80, and 100 Hz stimulations). Following this, another twitch stimuli were performed. Immediately after the muscles were subjected to a fatigue protocol consisting of one contraction every 5 s 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 handheld 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 spectrofluorometric method previously adapted by our laboratory (28). Reaction buffer (1.425 mL) 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 μL muscle homogenate, and 0.2 mM NADH were added to the cuvette with a final volume of 1.5 mL. EDL muscle was diluted fivefold to account for increased activity. Assays were performed in duplicate at 37°C and 340 nm wavelength. SERCA activity was measured using successive 15 μL 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 the homogenate, 40 μM of cyclopiazonic acid (CPA) was added to the cuvette following the reaction to completely inhibit SERCA activity. SERCA activity was determined at each time point by subtracting the activity in the presence of CPA and used to construct a nonlinear regression analysis. Enzymatic kinetics were analyzed for maximal SERCA activity (V_{max}), the negative logarithm of [Ca^{2+}] needed to produce 50% of V_{max} (pCa_{50}), and the slope of the relationship between SERCA activity and Ca^{2+} between 10% and 90% V_{max} (Hill slope, n_H).

Mitochondrial Bioenergetics

Permeabilized muscle fibers (PmFbs) were prepared from white gastrocnemius (WG), red gastrocnemius (RG), and soleus for mitochondrial respiration experiments, as previously described (29). Muscle was placed in ice-cold biopsy preservation solution (BIOPS) (29) and fiber bundles were separated with fine-tipped forceps underneath a microscope (MX6 Stereoscope, Zeiss Microsystems, Wetzlar, Germany). Fibers were incubated in 40 μg/mL saponin for 30 min and washed in MiR05 respiration buffer (respiration experiments) (29) or Buffer Z (ROS experiments) (30) for 15 min.

Mitochondrial respiration experiments were performed in MiR05 respiration buffer in an Oxygraph high-resolution respirometer at 37°C (Oroboros Instruments, Innsbruck, Austria) with constant spinning at 750 rpm. Experiments were conducted at room air saturation with reoxygenation after the addition of each substrate (~180–195 μM O_2). All experiments were performed in the presence of 5 μM blebbistatin, 5 mM pyruvate, and 1 mM malate. For ADP experiments, ADP was titrated in various concentrations (25, 100, 250, 500, 1,000, 2,000, 4,000, 6,000, 8,000, and 10,000 μM ADP) followed by the addition of 10 mM glutamate, 10 mM succinate, and 10 μM cytochrome C. Respiratory control ratios (RCRs) were calculated by dividing 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 before titrations of $CaCl_2$ (25, 50, 100, 200, 250, 300, 350, 375, 400, 425, and 450 μM $CaCl_2$). When a plateau in $CaCl_2$-supported respiration was reached, 40 μM of 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, 1,000, 1,400, and 1,800 nM (corresponding to 25, 50, 100, 200, 250, 300, 350, 375, 400, 425, and 450 μM $CaCl_2$).

Mitochondrial ROS experiments were performed as previously described (30) by measuring the rate of hydrogen peroxide (H_2O_2) release using Amplex Red fluorescence quantification (Invitrogen, Carlsbad, CA) in Buffer Z at 37°C. 5 μM blebbistatin, 5 U/mL horseradish peroxidase (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 with 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, and 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), and incubated in blocking solutions and appropriate primary/secondary antibodies. Target proteins included SERCA1 (1:2,000, DSHB CaF2-5D2), SERCA2 (1:1,000, Abcam 2861), CSQ1 (1:10,000, Abcam 108289), CSQ2 (1:1,000, Abcam 191564), OXPHOS (1:1,000 Abcam 110413), ANT1 (1:1,000, Abcam 110322), SLN (1:500, Millipore ABT13), PLN (1:1,000, Abcam 2865), and pPLN (1:1,000, Cell Signaling 8496). α-Tubulin (1:1,000, Abcam 7291) or ponceau stains were used as loading controls. Ten micrograms of protein was loaded for EDL and soleus Western blots following dietary nitrate consumption (Fig. 2*F*), and for PLN and pPLN in RG homogenate (Fig. 7*A*). Five micrograms of protein was loaded for all other Westerns (Fig. 4, *D*, *E* and *F*; Fig. 7*A*). Western blots were quantified using FlourChem HD imaging chemiluminescence (Alpha Innotech, Santa Clara, CA). 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). 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, and SERCA activity). For comparisons between fiber types (WG, RG, and soleus) 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 are expressed as means ± 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 approximately fourfold in nitrate-consuming mice (37.3 ± 11.7 μM, n = 6, means ± SD) compared with control mice (9.2 ± 5.1 μM, n = 6, means ± 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. 1*A*), but not EDL (Fig. 1*B*). In addition, in soleus muscle, force production throughout a 25 min stimulation protocol (Fig. 1*C*), as well as the total force produced throughout the fatiguing protocol (Fig. 1*C*, *left inset*) was higher following nitrate consumption. At the end of the fatigue protocol, there was a nonsignificant trend (P = 0.08, two-tailed unpaired Student's *t* test; Fig. 1*C*, *right inset*) for the percentage of initial force production to be higher in the soleus of nitrate compared with control mice, suggesting a potential fatigue resistance. In contrast, nitrate did not attenuate fatigue within the EDL (Fig. 1*D* 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 for 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 nonsignificant trend (P = 0.053) toward a ~25% increase in the Hill slope (n_H) compared with controls (Fig. 2, *A* and *B*). Although 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, and 5.7) was higher in nitrate-consuming mice (Fig. 2, *A* and *B*). In contrast, EDL showed no differences in any of the SERCA kinetic properties examined (Fig. 2, *C* and *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 SERCA1/2, SLN, PLN, or CSQ1/2 protein content in soleus or EDL following 7 days of dietary nitrate consumption (Fig. 2, *E* and *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, means ± SD) and EDL (84.3 ± 25.1 units control, n = 5 vs. 79.5 ± 28.3 units nitrate, n = 6; relative to soleus control, means ± SD) muscles (representative blot shown in Fig. 2*F*). 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

The apparent increase in SERCA-mediated ATP hydrolysis appears at odds with the well-established finding that dietary nitrate decreases oxygen consumption during exercise. Therefore, we next aimed to examine the high-energy phosphate transfer between SERCA and mitochondria following nitrate consumption to determine whether this was improved, possibly explaining the discrepancy. To do so, we first established a methodology to measure the ability of SERCA-derived ADP to stimulate mitochondrial respiration in permeabilized muscle fibers. The theoretical premise of

Table 1. *Twitch characteristics following 7 days of nitrate supplementation in soleus and EDL muscle*

	Pre-Force Frequency		Post-Force Frequency	
	Control	Nitrate	Control	Nitrate
Soleus				
$+dp/dt_{max}$, $N \cdot s^{-1}$	896.2 ± 159.4	889.3 ± 323.3	888.5 ± 163.7	918.3 ± 319.2
$-dp/dt_{max}$, $N \cdot s^{-1}$	614.8 ± 109.4	554.1 ± 122.3	628.4 ± 144.5	600.3 ± 120.5
1/2 RT, s	0.54 ± 0.11	0.64 ± 0.16	0.51 ± 0.11	0.57 ± 0.11
EDL				
$+dp/dt_{max}$, $N \cdot s^{-1}$	1740.5 ± 262.2	1804.0 ± 233.4	1790.5 ± 125.9	1748.1 ± 196.5
$-dp/dt_{max}$, $N \cdot s^{-1}$	1580.2 ± 262.9	1610.5 ± 332.4	1512.9 ± 236.9	1555.3 ± 295.4
1/2 RT, s	0.36 ± 0.05	0.31 ± 0.02	0.30 ± 0.01	0.30 ± 0.01

Data were analyzed using two-way ANOVA. Data are expressed as means ± SD. n = 6 soleus, n = 6 EDL. EDL, extensor digitorum longus; 1/2 RT, half-relaxation time.

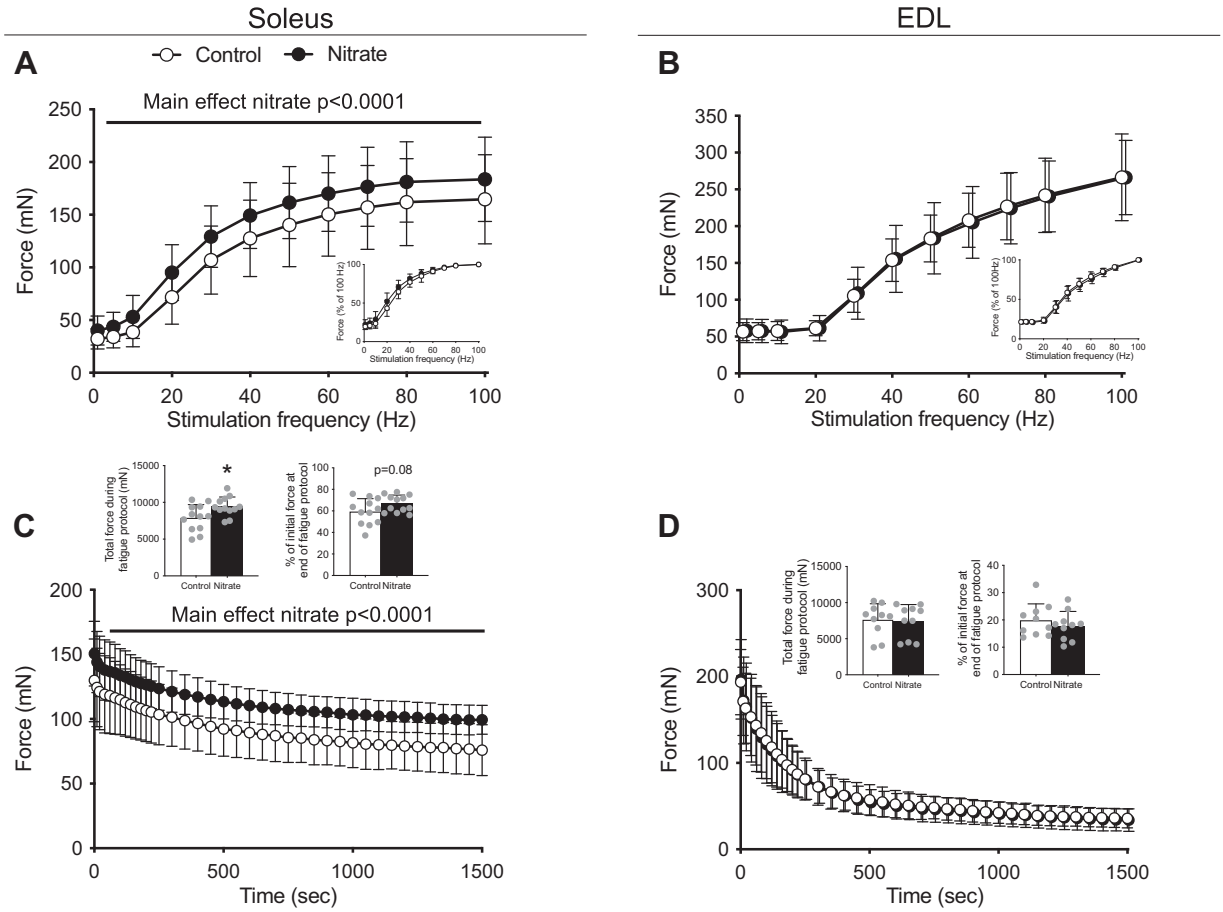

Figure 1. Dietary nitrate increases force production in soleus, but not EDL, of female mice. Force production over various stimulation frequencies in soleus (*A*) and EDL (*B*) muscle. Force production over a 25 min fatigue protocol in soleus (*C*) and EDL (*D*). *Insets* depict force production as a percentage of 100 Hz (maximum tested) (*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* and *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 were analyzed using two-way ANOVA (*A–D*) or two-tailed unpaired Student's *t* tests (*C*, *D*, *insets*). $*P < 0.05$ vs. control. Data are expressed as means ± SD. $n = 12$ soleus, $n = 10$ EDL. EDL, extensor digitorum longus.

this experimental approach is that the addition of Ca^{2+} in the presence of ATP will generate ADP from SERCA, and this ADP will act as a substrate to support oxidative phosphorylation (Fig. 3*A*). In this experiment, our functional readout of ADP-supported mitochondrial respiration (oxygen consumption, JO_2) provides an indication of ATP hydrolysis from SERCA.

To develop this approach, mitochondrial respiration (JO_2) was monitored in permeabilized fibers from red gastrocnemius muscle (Fig. 3*B*). Respiration was supported by pyruvate + malate, followed by the addition of ATP (Fig. 3*B*). We then titrated sequentially increasing concentrations of $CaCl_2$ in the presence of ATP to stimulate SERCA-mediated ATP hydrolysis and create ADP to support mitochondrial respiration. This revealed that maximal SERCA-supported respiration was ~25% of oxidative capacity in the presence of saturating ADP (Fig. 3, *B* and *C*). The addition of cyclopiazonic acid (CPA), a SERCA-specific inhibitor, fully attenuated mitochondrial respiration to that of pyruvate + malate (Fig. 3*B*), suggesting SERCA-derived ADP is capable of supporting mitochondrial respiration in our experimental design. Importantly, Ca^{2+} did not increase mitochondrial respiration following the prior addition of CPA (Fig. 3*D*), and titrations of Ca^{2+} after 500 μM ADP (~ADP K_m) did not increase submaximal ADP-supported respiration or attenuate maximal respiratory capacity (Fig. 3*E*). 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 used white gastrocnemius (WG), red gastrocnemius (RG), and soleus given the dramatic differences in SERCA activity (Fig. 4, *A–C*: *WG > RG>SOL)*, and content of SERCA1/2 (Fig. 4*D*), CSQ1/2 (Fig. 4*E*), and mitochondrial proteins (Fig. 4*F*: *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. 5*A*). In addition, the apparent mitochondrial ADP K_m was higher in soleus and RG compared with WG (Fig. 5*B*).

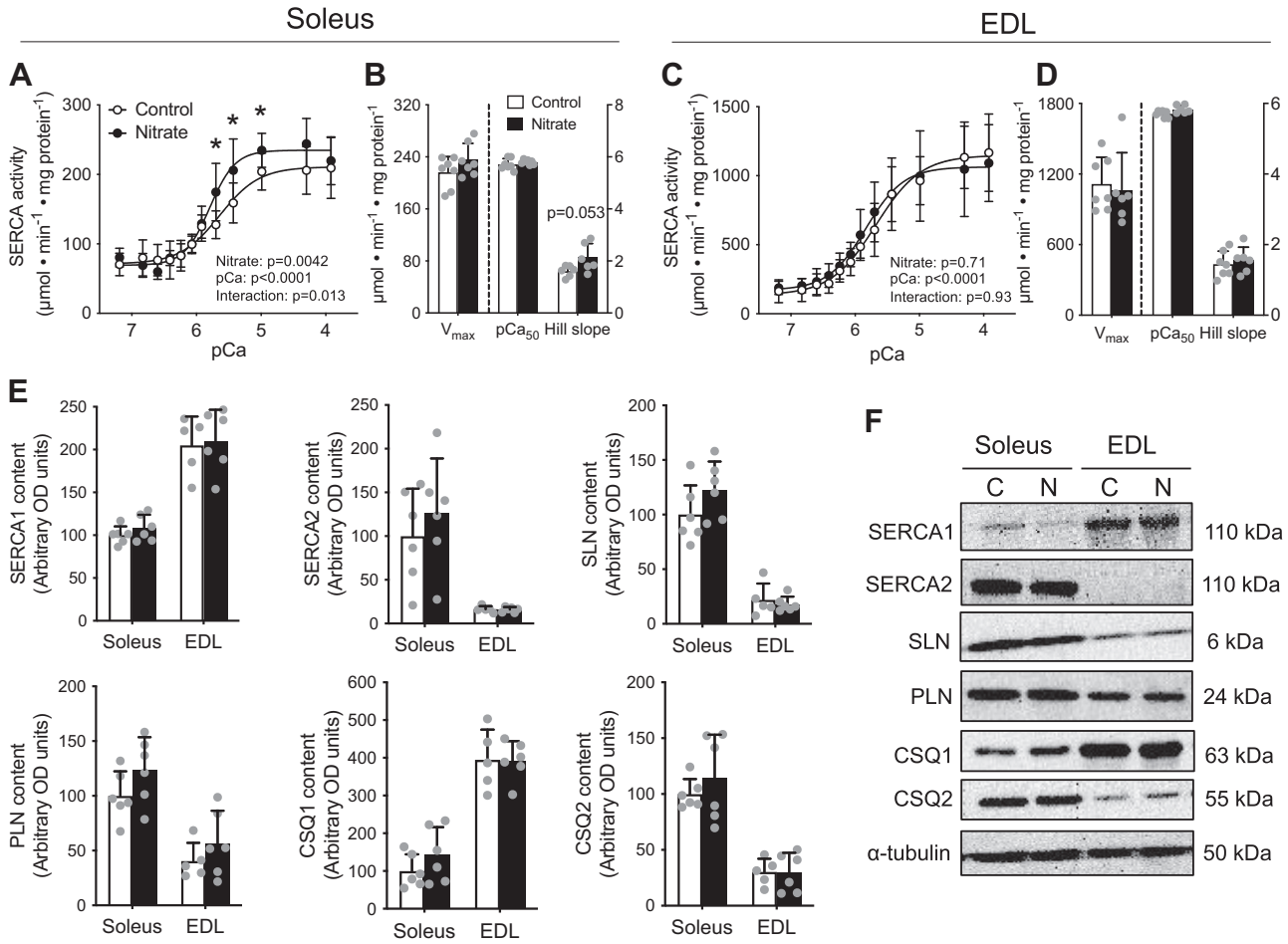

Figure 2. Dietary nitrate increases submaximal SERCA activity in soleus muscle, but does not alter content of Ca^{2+}-handling proteins. SERCA activity (*A*) and enzymatic properties (*B*) within soleus muscle and EDL muscle (*C*, *D*). Protein content of Ca^{2+}-handling proteins (SERCA1, SERCA2, CSQ1, 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 were 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 are expressed as means ± SD. $n = 5$–7. CSQ, calsequestrin; EDL, extensor digitorum longus; pCa_{50}, negative logarithm of [Ca^{2+}] required to elicit half-maximal SERCA activity; PLN, phosholamban; SERCA, sarcoplasmic reticulum calcium ATPase; SLN, sarcolipin; V_{max}, maximal enzymatic activity.

Although SERCA-supported respiration (presence of ATP and Ca^{2+}) was greatest in soleus (Fig. 5*C*), this largely reflected the global differences in mitochondrial respiratory capacity between tissues. When expressed as a percentage of maximal ADP-supported respiration (Fig. 5*D*), the ability of SERCA-derived ADP to stimulate respiration was higher in WG compared with RG and soleus, in line with the differences in SERCA activity between these tissues (Fig. 4, *A* and *B*). This occurred in the absence of changes in the sensitivity of mitochondria to SERCA-derived ADP (Fig. 5*E*), similar to that of the SERCA pCa_{50} (Fig. 4*C*). Combined, these data supported 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.

Dietary Nitrate Supplementation Increases SERCA-Derived Mitochondrial Respiration

We next aimed to examine global mitochondrial respiratory function, as well as the high-energy phosphate interaction between SERCA and mitochondria, following dietary nitrate consumption. Maximal respiratory capacity and the sensitivity of mitochondria to submaximal concentrations of ADP were not affected by nitrate consumption (Fig. 6*A*), confirming the absence of changes in mitochondrial respiratory function. With respect to the SERCA-mitochondria energy transfer, dietary nitrate did not alter the sensitivity of mitochondria to Ca^{2+}-mediated ADP supply (Fig. 6*B*). However, the maximal ability of Ca^{2+} to drive respiration was increased by ~20% in nitrate-consuming mice compared with controls (Fig. 6*C*). The difference in SERCA-supported respiration between control and nitrate animals that was fully attenuated by the addition of CPA (Fig. 6*C*), was not due to global changes in mitochondrial ADP-supported respiration (Fig. 6, *A* and *D*), and therefore was likely linked to SERCA activity. In support, although absolute mitochondrial respiration in the presence of 250 μM and 1000 μM ADP was comparable between control and nitrate mice (Fig. 6*D*), respiration supported by SERCA-derived ADP at the same absolute respiration rate of ~300 pmol·s·mg^{-1} dry wt (max Ca^{2+}, Fig. 6*C*) was greater following

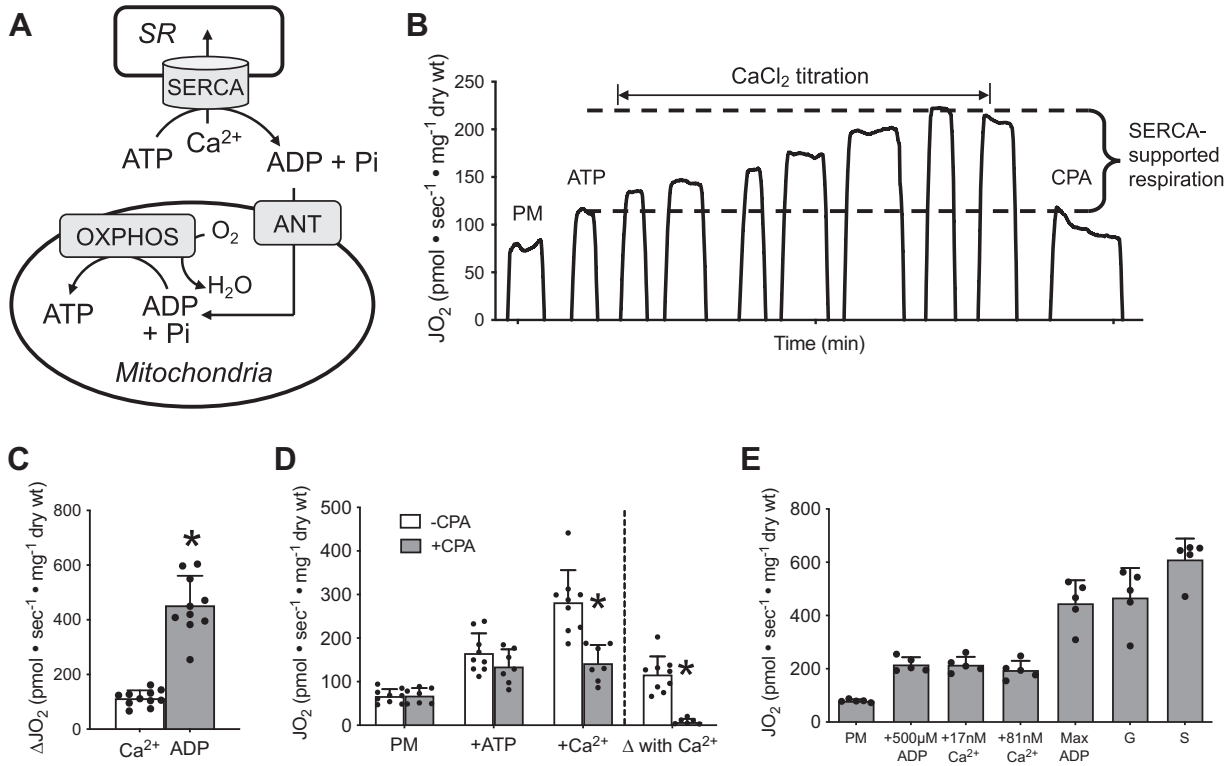

Figure 3. Establishing a methodology to examine the high-energy phosphate transfer between SERCA and mitochondria. ADP released from SERCA, in the presence of ATP and Ca^{2+}, is capable of supporting mitochondria oxidative phosphorylation in permeabilized muscle fibers (*A*). $CaCl_2$ was titrated in 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 used did not increase mitochondrial respiration or alter the ability of subsequent substrates to drive respiration (*E*). Data were analyzed using two-tailed unpaired Student's *t* tests. *$P < 0.05$ vs. Ca^{2+} (*C*) or -CPA (*D*). Data are expressed as means ± 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; Pi, inorganic phosphate; PM, pyruvate + malate; S; succinate; SERCA, sarcoplasmic reticulum calcium ATPase; SR, sarcoplasmic reticulum.

nitrate supplementation. Similar to findings in soleus, this was not due to changes in protein content, as there were no differences in Ca^{2+}-handling proteins (SERCA1/2, CSQ1) or mitochondrial proteins (OXPHOS complexes, ANT1) in permeabilized muscle fibers used to perform SERCA-derived ADP experiments (Fig. 7, *A* and *B*). In addition, phospholamban (PLN) phosphorylation in RG muscle homogenate was not different between control and nitrate animals (Fig. 7, *A* and *B*). As BRJ consumption in humans has been shown to increase mitochondrial ROS 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. 7*C*) or submaximal rates in the presence of 100 μM ADP (Fig. 7*D*).

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, the soleus muscle of nitrate-consuming animal 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 a trend toward 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). Although functional changes with dietary nitrate have been reported exclusively in fast-twitch EDL of mice (10, 34), work in the human muscle of mixed fiber type composition (16,

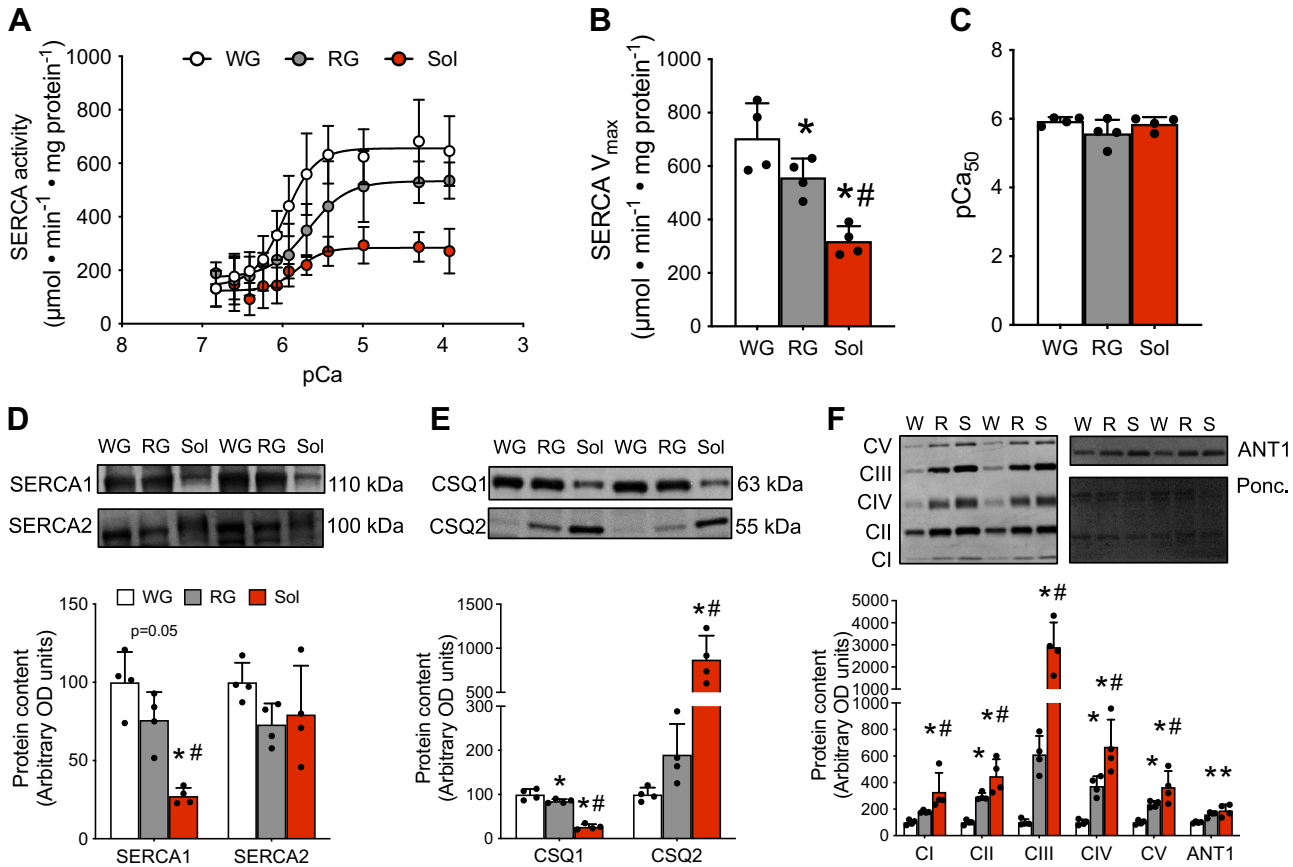

Figure 4. SERCA activity and protein content display fiber-type differences. SERCA activity (*A*, *B*) and the negative logarithm of [Ca^{2+}] required to elicit half-maximal SERCA activity (pCa_{50}, *C*) in WG, RG, and soleus. SERCA1 and SERCA2 (*D*), CSQ1 and CSQ2 (*E*), and mitochondrial (*F*) protein content in WG, RG, and soleus. Molecular weights for representative Western blots in *F* are CV (55 kDa), CIII (45 kDa), CIV (37 kDa), CII (25 kDa), CI (18 kDa), and ANT1 (32 kDa). Data were analyzed using one-way ANOVA with LSD post hoc multiple comparisons. *$P < 0.05$ vs. WG and #$P < 0.05$ vs. RG. Data are expressed as means ± SD. $n = 4$. ANT1, adenine nucleotide translocase 1; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V; CSQ, calsequestrin; OD, optical density; pCa_{50}, negative logarithm of [Ca^{2+}] required to elicit half-maximal SERCA activity; Ponc, ponceau stain; R and RG, red gastrocnemius; S and Sol, soleus; SERCA, sarcoplasmic reticulum calcium ATPase; V_{max}, maximal enzymatic activity; W and WG, white gastrocnemius.

35) and in cardiac muscle (19) would suggest that 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, the fiber type composition of the mouse soleus muscle is more similar than EDL to the human muscle (36–38), and therefore the increase in force production that we observed in the 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 $\dot{V}O_2$ 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 with faster twitch muscle of rats (41). Alternatively, our study was performed in female mice, whereas 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. Although previous work has also shown that acute (2.5 h) and chronic (8 days) 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, whereas some previous studies examining the influence of nitrate and nitrite on contractile properties used 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 used 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 report that

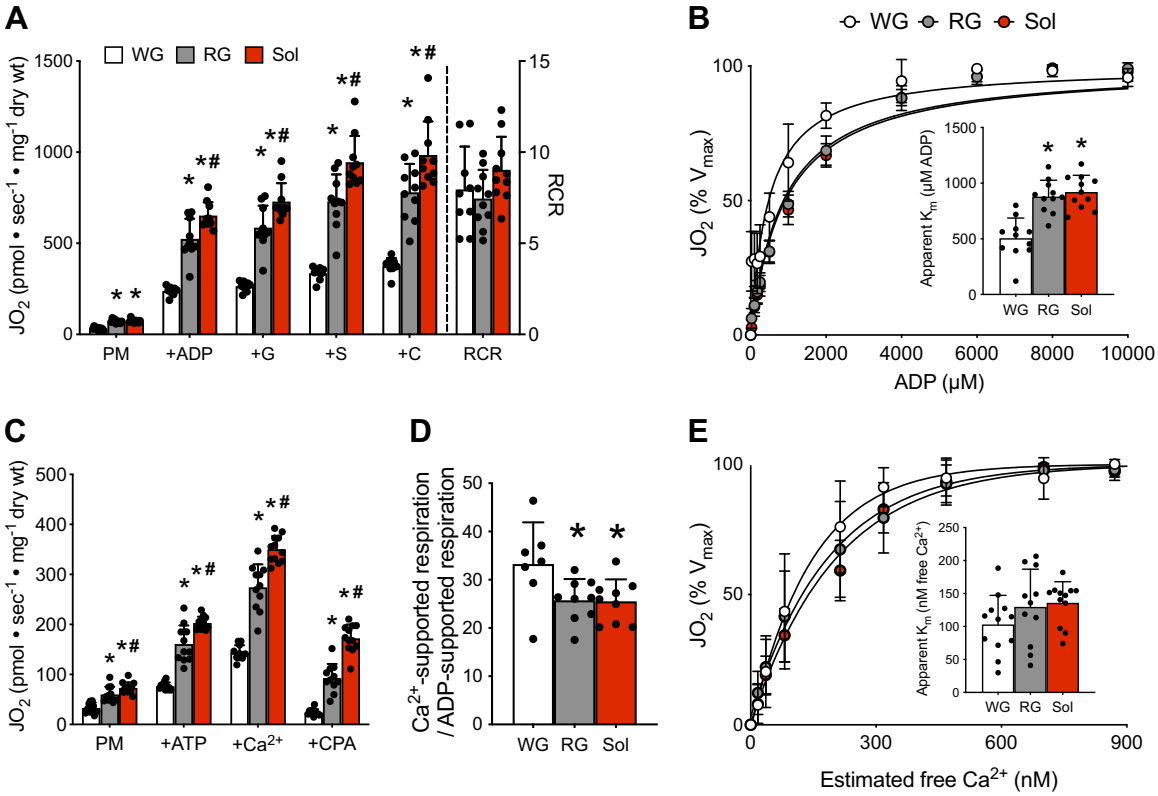

Figure 5. Mitochondrial respiration supported by global ADP supply and SERCA-derived ADP display fiber-type differences. Maximal mitochondrial respiration (*A*) and ADP sensitivity (*B*) in WG, RG, and soleus. Mitochondrial respiration supported by SERCA-derived ADP (*C*) and the ratio of Ca^{2+}-supported respiration/ADP-supported respiration (*D*) in different muscle fiber types. Sensitivity of mitochondria to SERCA-derived ADP from Ca^{2+} titrations (*E*). Data were analyzed using one-way ANOVA with LSD post hoc multiple comparisons. *$P < 0.05$ vs. WG and #$P < 0.05$ vs. RG. Data are expressed as means ± 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.

submaximal SERCA activity was increased ~25% at three pCa values in the soleus of nitrate-consuming animals, supporting the previous finding that in vitro incubations with nitrite increase 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, although 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 whether SERCA efficiency was improved; however, the trend (P = 0.053) toward 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 suggest 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 although this cannot contribute to an increase in submaximal force, it could provide a mechanism for the improvement in fatigue with nitrate supplementation.

Although mechanisms underlying the change in submaximal SERCA activity and trend toward 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 ~22 free cysteine residues,

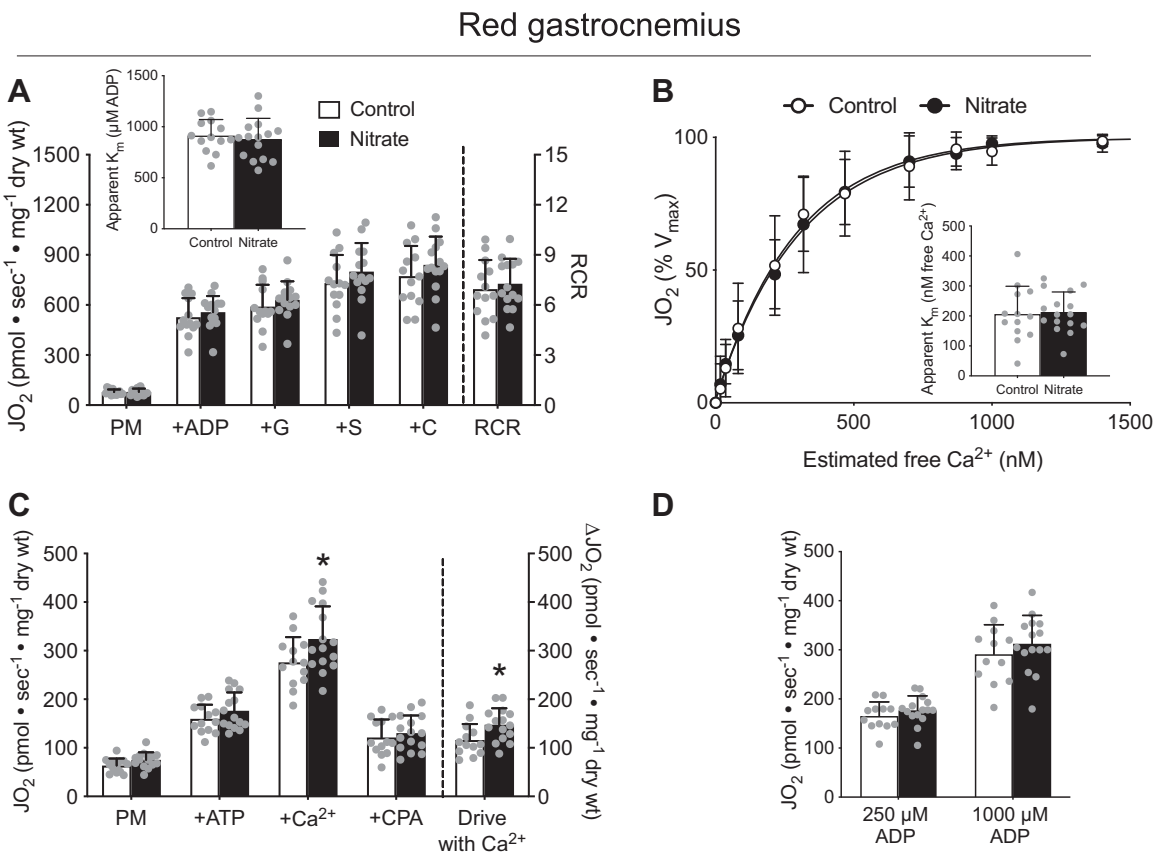

Figure 6. Dietary nitrate increases mitochondrial respiration supported by SERCA-derived ADP, but not global ADP supply. Maximal mitochondrial respiratory capacity and the sensitivity of mitochondria to ADP within RG following nitrate consumption (*A*). Mitochondrial sensitivity to SERCA-mediated ADP supply (*B*) and maximal respiration supported by Ca^{2+} (*C*). Submaximal ADP-supported respiration at a similar absolute respiration rate as SERCA-derived ADP (Ca^{2+}) titrations (*D*). Data were analyzed using two-tailed unpaired Student's *t* tests. *$P < 0.05$ vs. control. Data are expressed as means ± SD. $n = 13$ control, $n = 15$ nitrate. 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; V_{max}, maximal enzymatic activity.

SERCA is highly susceptible to posttranslational 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 toward 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. Although it remains possible that cytosolic and nonmitochondrial 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. Although 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 posttranslational 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 the 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 posttranslational modifications are important for the observed outcomes with nitrate consumption. In support, a reduction in the oxygen

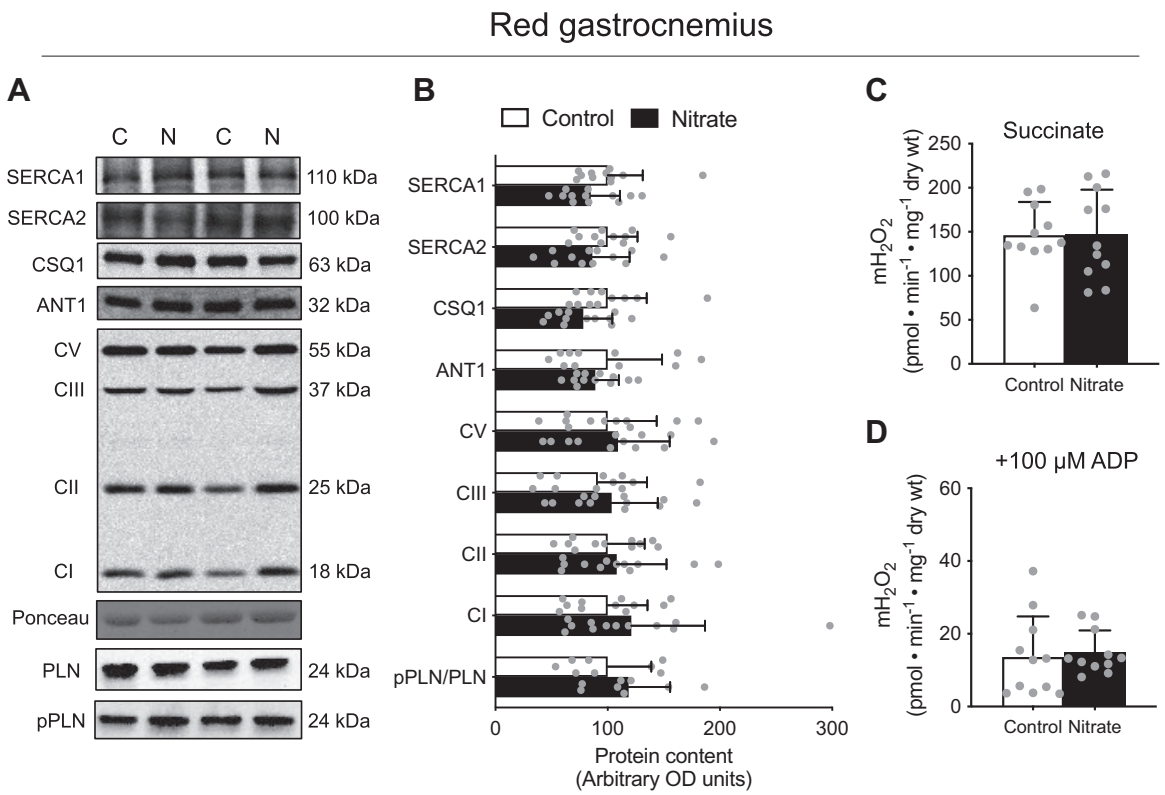

Figure 7. Dietary nitrate does not alter content of Ca^{2+}-handling proteins, mitochondrial proteins, or mitochondrial ROS emission rates in red gastrocnemius. SERCA-related protein content and mitochondrial protein content in RG (*A*, *B*). Permeabilized muscle fibers were used for SERCA1/2, CSQ1, ANT1, and OXPHOS complexes, whereas whole RG homogenate was used for PLN and pPLN to detect protein phosphorylation (*A*, *B*). Maximal (succinate; *C*) and submaximal (+100 μM ADP, *D*) mitochondrial ROS emission rates in RG permeabilized muscle fibers. Data were analyzed using two-tailed unpaired Student's *t* tests. Data are expressed as means ± SD. n = 8–12. ADP, adenosine diphosphate; ANT1, adenine nucleotide translocase; C, control; CI, complex I; CII, complex II; CIII, complex III; CV, complex V; CSQ, calsequestrin; mH_2O_2, mitochondrial hydrogen peroxide emission; PLN, phospholamban; pPLN, phosphorylated phospholamban; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; N, nitrate.

cost of exercise with dietary nitrate has been produced as acutely as 2.5 h 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 ~25%–50% of energy consumption at rest (23) and 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 an 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. Although 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.

The structural and functional interactions between SR and mitochondria appear to be influenced by cellular energetic state. For instance, impairments in ER-mitochondrial coupling are evident in skeletal muscle of insulin-resistant individuals (55), a situation known to influence mitochondrial function (56) and Ca^{2+} homeostasis (57, 58). Given that dietary nitrate also appears capable of influencing these processes (10), the SR-mitochondria high-energy phosphate interaction is of particular interest. In line with our findings of an increase in submaximal SERCA activity, dietary nitrate increased the ability of Ca^{2+} to drive mitochondrial respiration. This would therefore suggest that greater rates of ATP hydrolysis from SERCA following nitrate consumption provide an increased provision of ADP

to mitochondria. Similar to previous work (17, 18), we did not detect any ability of dietary nitrate to improve global mitochondrial respiratory capacity or mitochondrial ADP sensitivity. This would therefore indicate that the increase in mitochondrial respiration we observed in response to SERCA-derived ADP largely reflects the change in SERCA activity and ATP hydrolysis, as opposed to an influence of dietary nitrate on mitochondrial function. Nevertheless, although we observed an increase in submaximal, but not maximal SERCA activity; in contrast, we observed an increase in maximal, but not submaximal, SERCA-supported mitochondrial respiration. One possible explanation for this discrepancy is that all respiratory experiments reflect submaximal SERCA activity, as greater additions of Ca^{2+} in our in vitro preparation appeared to elicit a detrimental effect on mitochondrial respiration (see Fig. 3*B*). Altogether, our data does suggest that SERCA-derived mitochondrial respiration was increased following nitrate consumption, and while this may contribute to fatigue resistance, cannot explain the reduction in submaximal $\dot{V}O_2$ observed with dietary nitrate.

Limitations

Although 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 that 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. Although 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, although 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 CONCLUSIONS

We provide evidence that 7 days of dietary nitrate consumption increases submaximal SERCA activity and tends ($P = 0.053$) to increase Ca^{2+}-binding cooperativity. 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, although the increase in submaximal SERCA activity likely represents a compensatory mechanism to counter the rise in cytosolic Ca^{2+} following nitrate supplementation, our findings do not appear to explain the nitrate-mediated decrease in ATP turnover or increase in low-frequency force production, suggesting other cellular targets of nitrate such as actin-myosin ATPase. Overall, dietary nitrate improves submaximal SERCA activity, efficiency, and SERCA-supported mitochondrial respiration while functionally increasing force production throughout a fatiguing contraction protocol. These findings suggest that alterations in SERCA enzymatic properties are a mechanism in which dietary nitrate enhances fatigue resistance, providing insight into the ability of nitrate to influence exercise performance.

GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

H.L.P., S.B., L.J.C.v.L., C.L.M., and G.P.H. conceived and designed research; H.L.P., S.B., B.V., H.S.B., R.M.H., and G.P.H. performed experiments; H.L.P., S.B., B.V., H.S.B., R.M.H., C.L.M., and G.P.H. analyzed data; H.L.P., S.B., B.V., H.S.B., R.M.H., L.J.C.v.L., C.L.M., and G.P.H. interpreted results of experiments; H.L.P., S.B., R.M.H., and G.P.H. prepared figures; H.L.P., S.B., and G.P.H. drafted manuscript; H.L.P., B.V., H.S.B., L.J.C.v.L., C.L.M., and G.P.H. edited and revised manuscript; H.L.P., S.B., B.V., H.S.B., R.M.H., L.J.C.v.L., C.L.M., and G.P.H. approved final version of manuscript.

REFERENCES

1. **Berchtold MW**, **Brinkmeier H**, **Müntener M.** Calcium ion in skeletal muscle: Its crucial role for muscle function, plasticity, and disease. *Physiol Rev* 80: 1215–1265, 2000. doi:10.1152/physrev.2000.80.3.1215.
2. **Westerblad H**, **Allen DG.** Changes of myoplasmic calcium concentration during fatigue in single mouse muscle fibers. *J Gen Physiol* 98: 615–635, 1991. doi:10.1085/jgp.98.3.615.
3. **Tupling AR.** The sarcoplasmic reticulum in muscle fatigue and disease: role of the sarco(endo)plasmic reticulum Ca2 + -ATPase. *Can J Appl Physiol* 29: 308–329, 2004. doi:10.1139/h04-021.
4. **Holloway GP.** Nutrition and training influences on the regulation of mitochondrial adenosine diphosphate sensitivity and bioenergetics. *Sports Med* 47: 13–21, 2017. doi:10.1007/s40279-017-0693-3.
5. **Hawley JA**, **Burke LM**, **Phillips SM**, **Spriet LL.** Nutritional modulation of training-induced skeletal muscle adaptations. *J Appl Physiol* 110: 834–845, 2011. doi:10.1152/japplphysiol.00949.2010.
6. **Liu VWT**, **Huang PL.** Cardiovascular roles of nitric oxide: A review of insights from nitric oxide synthase gene disrupted mice. *Cardiovasc Res* 77: 19–29, 2008. doi:10.1016/j.cardiores.2007.06.024.
7. **Nisoli E**, **Clementi E**, **Paolucci C**, **Cozzi V**, **Tonello C**, **Sciorati C**, **Bracale R**, **Valerio A**, **Francolini M**, **Moncada S**, **Carruba MO.** Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science* 299: 896–899, 2003. doi:10.1126/science.1079368.
8. **Tengan CH**, **Rodrigues GS**, **Godinho RO.** Nitric oxide in skeletal muscle: role on mitochondrial biogenesis and function. *Int J Mol Sci* 13: 17160–17184, 2012. doi:10.3390/ijms131217160.
9. **Andrade FH**, **Reid MB**, **Allen DG**, **Westerblad H.** Effect of nitric oxide on single skeletal muscle fibres from the mouse. *J Physiol* 509: 577–586, 1998. doi:10.1111/j.1469-7793.1998.577bn.x.
10. **Hernández A**, **Schiffer TA**, **Ivarsson N**, **Cheng AJ**, **Bruton JD**, **Lundberg JO**, **Weitzberg E**, **Westerblad H.** Dietary nitrate increases tetanic [Ca 2 +]© and contractile force in mouse fast-twitch muscle. *J Physiol* 590: 3575–3583, 2012. doi:10.1113/jphysiol.2012.232777.

11. **Lundberg JO**, **Govoni M.** Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic Biol Med* 37: 395–400, 2004. doi:10.1016/j.freeradbiomed.2004.04.027.
12. **Larsen FJ**, **Weitzberg E**, **Lundberg JO**, **Ekblom B.** Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol* 191: 59–66, 2007. doi:10.1111/j.1748-1716.2007.01713.x.
13. **Larsen FJ**, **Schiffer TA**, **Borniquel S**, **Sahlin K**, **Ekblom B**, **Lundberg JO**, **Weitzberg E.** Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab* 13: 149–159, 2011. doi:10.1016/j.cmet.2011.01.004.
14. **Bailey SJ**, **Winyard P**, **Vanhatalo A**, **Blackwell JR**, **DiMenna FJ**, **Wilkerson DP**, **Tarr J**, **Benjamin N**, **Jones AM.** Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J Appl Physiol* 107: 1144–1155, 2009. doi:10.1152/japplphysiol.00722.2009.
15. **Bailey SJ**, **Fulford J**, **Vanhatalo A**, **Winyard PG**, **Blackwell JR**, **DiMenna FJ**, **Wilkerson DP**, **Benjamin N**, **Jones AM.** Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *J Appl Physiol* 109: 135–148, 2010. doi:10.1152/japplphysiol.00046.2010.
16. **Whitfield J**, **Gamu D**, **Heigenhauser GJF**, **van Loon LJC**, **Spriet LL**, **Tupling AR**, **Holloway GP.** Beetroot juice increases human muscle force without changing Ca2+-handling proteins. *Med Sci Sports Exerc* 49: 2016–2024, 2017. doi:10.1249/MSS.0000000000001321.
17. **Whitfield J**, **Ludzki A**, **Heigenhauser GJF**, **Senden JMG**, **Verdijk LB**, **van Loon LJC**, **Spriet LL**, **Holloway GP.** Beetroot juice supplementation reduces whole body oxygen consumption but does not improve indices of mitochondrial efficiency in human skeletal muscle. *J Physiol* 594: 421–435, 2016. doi:10.1113/JP270844.
18. **Ntessalen M**, **Procter NEK**, **Schwarz K**, **Loudon BL**, **Minnion M**, **Fernandez BO**, **Vassiliou VS**, **Vauzour D**, **Madhani M**, **Constantin-Teodosiu D**, **Horowitz JD**, **Feelisch M**, **Dawson D**, **Crichton PG**, **Frenneaux MP.** Inorganic nitrate and nitrite supplementation fails to improve skeletal muscle mitochondrial efficiency in mice and humans. *Am J Clin Nutr* 111: 79–89, 2020. doi:10.1093/ajcn/nqz245.
19. **Pironti G**, **Ivarsson N**, **Yang J**, **Farinotti AB**, **Jonsson W**, **Zhang SJ**, **Bas D**, **Svensson CI**, **Westerblad H**, **Weitzberg E**, **Lundberg JO**, **Pernow J**, **Lanner J**, **Andersson DC.** Dietary nitrate improves cardiac contractility via enhanced cellular Ca2+ signaling. *Basic Res Cardiol* 111: 34, 2016. doi:10.1007/s00395-016-0551-8.
20. **Ishii T**, **Sunami O**, **Saitoh N**, **Nishio H**, **Takeuchi T**, **Hata F.** Inhibition of skeletal muscle sarcoplasmic reticulum Ca2+-ATPase by nitric oxide. *FEBS Lett* 440: 218–222, 1998. doi:10.1016/S0014-5793(98)01460-4.
21. **Hart JDE**, **Dulhunty AF.** Nitric oxide activates or inhibits skeletal muscle ryanodine receptors depending on its concentration, membrane potential and ligand binding. *J Membr Biol* 173: 227–236, 2000. doi:10.1007/s002320001022.
22. **Bailey SJ**, **Gandra PG**, **Jones AM**, **Hogan MC**, **Nogueira L.** Incubation with sodium nitrite attenuates fatigue development in intact single mouse fibres at physiological PO2. *J Physiol* 597: 5429–5443, 2019. doi:10.1113/JP278494.
23. **Smith IC**, **Bombardier E**, **Vigna C**, **Tupling AR.** ATP consumption by sarcoplasmic reticulum Ca2+ pumps accounts for 40-50% of resting metabolic rate in mouse fast and slow twitch skeletal muscle. *PLoS ONE* 8: 1–11, 2013. doi:10.1371/journal.pone.0068924.
24. **Szentesi P**, **Zaremba R**, **van Mechelen W**, **Stienen GJM.** ATP utilization for calcium uptake and force production in different types of human skeletal muscle fibres. *J Physiol* 531: 393–403, 2001. doi:10.1111/j.1469-7793.2001.0393i.x.
25. **Csordás**, **GRGY**, **Renken**, **C**, **Várnai**, **P**, **Walter**, **L**, **Weaver**, **D**, **Buttle**, **KF**, **Balla**, **T**, **Mannella**, **CA**, **Hajnóczky**, **G.** Structural and functional features and significance of the physical linkage between ER and mitochondria. *J Cell Biol* 174: 915–921, 2006. doi:10.1083/jcb.200604016.
26. **Landolfi B**, **Curci S**, **Debellis L**, **Pozzan T**, **Hofer AM.** Ca2+ homeostasis in the agonist-sensitive internal store: Functional interactions between mitochondria and the ER measured in situ in intact cells. *J Cell Biol* 142: 1235–1243, 1998. doi:10.1083/jcb.142.5.1235.
27. **Csordas G**, **Hajnoczky G.** SR/ER-mitochondrial local communication: calcium and ROS. *Biochim Biophys Acta* 1787: 1352–1362, 2009. doi:10.1016/j.bbabio.2009.06.004.
28. **Jannas-Vela S**, **Brownell S**, **Petrick HL**, **Heigenhauser GJF**, **Spriet LL**, **Holloway GP.** Assessment of Na+/K+ ATPase activity in small rodent and human skeletal muscle samples. *Med Sci Sports Exerc* 51: 2403–2409, 2019. doi:10.1249/MSS.0000000000002063.
29. **Petrick HL**, **Holloway GP.** High intensity exercise inhibits carnitine palmitoyltransferase-I sensitivity to L-carnitine. *Biochem J* 476: 547–558, 2019. doi:10.1042/BCJ20180849.
30. **Miotto PM**, **Holloway GP.** Exercise-induced reductions in mitochondrial ADP sensitivity contribute to the induction of gene expression and mitochondrial biogenesis through enhanced mitochondrial H2O2 emission. *Mitochondrion* 46: 116–122, 2019. doi:10.1016/j.mito.2018.03.003.
31. **Barbeau P-A**, **Miotto PM**, **Holloway GP.** Mitochondrial derived reactive oxygen species influence ADP sensitivity, but not CPT-I substrate sensitivity. *Biochem J* 475: 2997–3008, 2018. doi:10.1042/BCJ20180419.
32. **Andrade FH**, **Reid MB**, **Westerblad H.** Contractile responses to low peroxide concentrations: myofibrillar calcium sensitivity as a likely target for redox-modulation of skeletal muscle function. *FASEB J* 15: 309–311, 2001. doi:10.1096/fj.00-0507fje.
33. **Jones AM**, **Ferguson SK**, **Bailey SJ**, **Vanhatalo A**, **Poole DC.** Fiber type-specific effects of dietary nitrate. *Exerc Sport Sci Rev* 44: 53–60, 2016. doi:10.1249/JES.0000000000000074.
34. **Ivarsson N**, **Schiffer TA**, **Hernández A**, **Lanner JT**, **Weitzberg E**, **Lundberg JO**, **Westerblad H.** Dietary nitrate markedly improves voluntary running in mice. *Physiol Behav* 1: 55–61, 2017. doi:10.1016/j.physbeh.2016.10.018.
35. **Haider G**, **Folland JP.** Nitrate supplementation enhances the contractile properties of human skeletal muscle. *Med Sci Sports Exerc* 46: 2234–2243, 2014. doi:10.1249/MSS.0000000000000351.
36. **Murgia M**, **Nogara L**, **Baraldo M**, **Reggiani C**, **Mann M**, **Schiaffino S.** Protein profile of fiber types in human skeletal muscle: a single-fiber proteomics study. *Skelet Muscle* 11: 24, 2001. doi:10.1186/s13395-021-00279-0.
37. **Gollnick PD**, **Armstrong RB**, **Saubert CW**, **Piehl K**, **Saltin B.** Enzyme activity and fiber composition in skeletal muscle of untrained and trained men. *J Appl Physiol* 33: 312–319, 1972. doi:10.1152/jappl.1972.33.3.312.
38. **Schiaffino S**, **Reggiani C.** Fiber types in mammalian skeletal muscles. *Physiol Rev* 91: 1447–1531, 2011. doi:10.1152/physrev.00031.2010.
39. **Eu JP**, **Sun J**, **Xu L**, **Stamler JS**, **Meissner G.** The skeletal muscle calcium release channel: Coupled O2 sensor and NO signaling functions. *Cell* 102: 499–509, 2000. doi:10.1016/S0092-8674(00)00054-4.
40. **Gehlert S**, **Bloch W**, **Suhr F.** Ca2+-dependent regulations and signaling in skeletal muscle: From electro-mechanical coupling to adaptation. *Int J Mol Sci* 16: 1066–1095, 2015. doi:10.3390/ijms16011066.
41. **Long GM**, **Gray DA**, **Troutman AD**, **Fisher A**, **Brown MB**, **Coggan AR.** Muscle fiber type differences in nitrate and nitrite storage and nitric oxide signaling in rats. bioRxiv. 2020. doi:10.1101/2020.06.01.128322.
42. **Wickham KA**, **McCarthy DG**, **Pereira JM**, **Cervone DT**, **Verdijk LB**, **van Loon LJC**, **Power GA**, **Spriet LL.** No effect of beetroot juice supplementation on exercise economy and performance in recreationally active females despite increased torque production. *Physiol Rep* 7: e13982, 2019. doi:10.14814/phy2.13982.
43. **Huang TT**, **Naeemuddin M**, **Elchuri S**, **Yamaguchi M**, **Kozy HM**, **Carlson EJ**, **Epstein CJ.** Genetic modifiers of the phenotype of mice deficient in mitochondrial superoxide dismutase. *Hum Mol Genet* 15: 1187–1194, 2006. doi:10.1093/hmg/ddl034.
44. **MacLennan DH**, **Asahi M**, **Tupling AR.** The regulation of SERCA-type pumps by phospholamban and sarcolipin. *Ann N Y Acad Sci* 986: 472–480, 2003. doi:10.1111/j.1749-6632.2003.tb07231.x.
45. **Heunks LMA**, **Cody MJ**, **Geiger PC**, **Dekhuijzen PNR**, **Sieck GC.** Nitric oxide impairs Ca2+ activation and slows cross-bridge cycling kinetics in skeletal muscle. *J Appl Physiol* 91: 2233–2239, 2001. doi:10.1152/jappl.2001.91.5.2233.
46. **Murphy AJ.** Sulfhydryl group modification of sarcoplasmic reticulum membranes. *Biochemistry* 15: 4492–4496, 1976. doi:10.1021/bi00665a025.
47. **Viner RI**, **Williams TD**, **Schöneich C.** Nitric oxide-dependent modification of the sarcoplasmic reticulum Ca-ATPase: localization of cysteine target sites. *Free Radic Biol Med* 29: 489–496, 2000. doi:10.1016/S0891-5849(00)00325-7.
48. **Pryor WA**, **Squadrito GL.** The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. *Am J Lung Cell Mol Physiol* 268: L699–L722, 1995. doi:10.1152/ajplung.1995.268.5.l699.

49. **Asahi M**, **Kurzydlowski K**, **Tada M**, **MacLennan DH.** Sarcolipin inhibits polymerization of phospholamban to induce superinhibition of sarco(endo)plasmic reticulum Ca2+-ATPases (SERCAs). *J Biol Chem* 277: 26725–26728, 2002. doi:10.1074/jbc.C200269200.
50. **Tupling A**, **Bombardier E**, **Gupta SC**, **Hussain D**, **Vigna C**, **Bloemberg D**, **Quadrilatero J**, **Trivieri MG**, **Babu GJ**, **Backx PH**, **Periasamy M**, **MacLennan DH**, **Gramolini AO.** Enhanced Ca2+ transport and muscle relaxation in skeletal muscle from sarcolipin-null mice. *Am J Physiol Cell Physiol* 301: C841–C849, 2011. doi:10.1152/ajpcell.00409.2010.
51. **Vanhatalo A**, **Bailey SJ**, **Blackwell JR**, **DiMenna FJ**, **Pavey TG**, **Wilkerson DP**, **Benjamin N**, **Winyard PG**, **Jones AM.** Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *Am J Physiol Regul Integr Comp Physiol* 299: R1121–R1131, 2010. doi:10.1152/ajpregu.00206.2010.
52. **Szabadkai G**, **Bianchi K**, **Várnai P**, **de Stefani D**, **Wieckowski MR**, **Cavagna D**, **Nagy AI**, **Balla T**, **Rizzuto R.** Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca2+ channels. *J Cell Biol* 175: 901–911, 2006. doi:10.1083/jcb.200608073.
53. **Kuznetsov A. V**, **Veksler V**, **Gellerich FN**, **Saks V**, **Margreiter R**, **Kunz WS.** Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nat Protoc* 3: 965–976, 2008. doi:10.1038/nprot.2008.61.
54. **Fink BD**, **Bai F**, **Yu L**, **Sivitz WI.** Regulation of ATP production: dependence on calcium concentration and respiratory state. *Am J Physiol Cell Physiol* 313: C146–C153, 2017. doi:10.1152/ajpcell.00086.2017.
55. **Tubbs E**, **Chanon S**, **Robert M**, **Bendridi N**, **Bidaux G**, **Chauvin MA**, **Ji-Cao J**, **Durand C**, **Gauvrit-Ramette D**, **Vidal H**, **Lefai E**, **Rieusset J.** Disruption of mitochondria-associated endoplasmic reticulum membrane (MAM) integrity contributes to muscle insulin resistance in mice and humans. *Diabetes* 67: 636–650, 2018. doi:10.2337/db17-0316.
56. **Miotto PM**, **LeBlanc PJ**, **Holloway GP.** High fat diet causes mitochondrial dysfunction as a result of impaired ADP sensitivity. *Diabetes* 67: 2199–2121, 2018. doi:10.2337/db18-0417.
57. **Jain SS**, **Paglialunga S**, **Vigna C**, **Ludzki A**, **Herbst EA**, **Lally JS**, **Schrauwen P**, **Hoeks J**, **Tupling AR**, **Bonen A**, **Holloway GP.** High-fat diet-induced mitochondrial biogenesis is regulated by mitochondrial-derived reactive oxygen species activation of CaMKII. *Diabetes* 63: 1907–1913, 2014. doi:10.2337/db13-0816.
58. **Bruton JD**, **Katz A**, **Lännergren J**, **Abbate F**, **Westerblad H.** Regulation of myoplasmic Ca2+ in genetically obese (ob/ob) mouse single skeletal muscle fibres. *Pflugers Arch Eur Arch* 444: 692–699, 2002. doi:10.1007/s00424-002-0882-1.