Acute Inhibition of the S-Nitrosoglutathione Reductase Delays Recovery Post-Fatigue in Isolated Fast-Twitch Muscle

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Summary

During high-intensity exercise, nitric oxide (NO) production increases in fast-twitch myofibers due to the activation of NO synthases (NOS). Increased NO levels may lead to S-nitrosylation of intracellular glutathione (GSH), thereby increasing S-nitrosoglutathione (GSNO) in myofibers. Although myofibers express GSNO reductase (GSNOR), an enzyme that degrades GSNO, little is known about the role of GSNOR in regulating intracellular GSNO levels during exercise and whether endogenous GSNO production affects muscle contractile function. We hypothesized that GSNOR in myofibers protects against NO-dependent nitrosative stress during repetitive contractions, and blocking GSNOR activity exacerbates fatigue-induced contractile dysfunction. We confirmed that the isolated *extensor digitorum longus* (EDL) muscle from mice expresses GSNOR by immunoblotting and determined that GSNOR activity was ~75 % lower in the presence of SPL-334 (10 μM). Isometric force and fatigue resistance of EDL from mice were tested in the presence of SPL-334 (10 μM), or with L-NMMA, a NOS inhibitor (400 μM) to block NO production upstream of GSNOR. The contralateral EDL served as an untreated control for both treatments. SPL-334 or L-NMMA treatment did not alter force at any stimulation frequencies or fatigue resistance. However, force recovery from fatigue at both submaximal and maximal stimulation frequencies was significantly impaired in SPL-334-treated muscles, but not altered with SPL-334 + L-NMMA. We can conclude that fatiguing contractions increase NO-dependent GSNO production, and in the absence of GSNOR activity, it delays the recovery of force post-fatigue. Thus, GSNOR serves as a protective anti-nitrosative stress mechanism against prolonged fatigue-induced force depression.

Key words

Muscle fatigue • Nitric oxide • S-nitrosoglutathione reductase • S-nitrosothiols

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Introduction

Transient changes in cytosolic Ca^{2+} concentrations ($[Ca^{2+}]c$) during brief tetanic contractions are known to activate the nitric oxide (NO) synthases (NOS) in skeletal myofibers. This leads to sustained myofiber NO synthesis during repetitive contractions that mimic exercise [1]. NO production during contractions and exercise contributes to maintaining muscle force production through increases in muscle blood flow [2,3], cGMP-dependent signaling [4], and intracellular calcium handling [5]. Also, a lack of NOS activity significantly reduces fatigue resistance due to blood flow restriction to working muscles [6]. Conversely, excess NO leads to post-translational oxidative and nitrosative modifications of myofibrillar proteins. This, in turn, can decrease submaximal force production during repetitive contractions, thereby accelerating fatigue.

It has been demonstrated that exercise results in muscle protein S-nitrosylation (i.e., adding a nitroso (–NO) group to protein thiols) of several contractile and

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Ca^{2+}-handling proteins [7,8] leading to the inhibition of myosin ATPase [7] and leaky ryanodine receptors (RyR1) [9]. Treating isolated myofibers and purified contractile proteins with exogenous low-molecular-weight S-nitrosothiols (LMW-SNOs; e.g., S-nitrosoglutathione [GSNO] or S-nitrosocysteine [CysNO]) results in myofiber weakness [10,11]. However, it remains poorly understood whether the NO produced by myofibers during contractions is converted to form LMW-SNOs, particularly GSNO, the most abundant LMW-SNO [12], and whether LMW-SNOs interfere with myofiber contractile function and energy metabolism under fatiguing conditions mimicking intense exercise [13].

Intracellular protein S-nitrosylation in several cell types, including muscle fibers, is believed to be regulated by the enzyme GSNO reductase (GSNOR), which regulates intracellular GSNO levels [14]. When GSNOR is pharmacologically inhibited or genetically ablated (GSNOR knockout [KO] mice), protein S-nitrosylation in muscle fibers is enhanced [15-17]. These data suggest that at least part of the NO synthesized in muscle fibers is converted to GSNO, which then transfers its nitroso group to proteins [18]. Although it has been shown that adult GSNOR KO mice exhibit increases in nerve-stimulated muscle force production and greater fatigue resistance [25], it is unclear how much of these effects can be attributed to possible lifelong compensation for GSNOR absence. Therefore, whether GSNOR controls exercise-induced GSNO production and the consequences of acute GSNOR inhibition during repetitive contractile activity remain unknown.

This study tested the hypothesis that NO production in muscle during fatiguing contractions increases intracellular GSNO, modifying myofibrillar function and ultimately force development during fatigue and recovery from fatigue, and GSNOR regulates this system by preventing excessive protein S-nitrosylation during fatiguing conditions. In the present investigation, we aimed to examine the effects of acute GSNOR inhibition on *ex vivo* isolated muscle contractility during fatiguing contractions and during recovery from fatigue in both normal conditions and with NOS pharmacologically inhibited.

Materials and Methods

Ethical approval

All experimental procedures performed in animals were approved by the San Diego State University Institutional Animal Care and Use Committee (protocol No. IACUC-25-071, respectively) and comply with the American Physiological Society's Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training. Male C57BL/6J mice (12-16 weeks old; The Jackson Laboratory, Bar Harbor, ME, USA Cat. No. 00664) were housed in a vivarium with a 12-h:12-h light-dark cycle, and regular chow and water were provided *ad libitum*. On the day of the experiment, the animals were euthanized by an overdose of sodium pentobarbital (Fatal Plus, 150 mg/kg body weight, i.p.), administered by a single intraperitoneal injection followed by rapid cervical dislocation after loss of consciousness. Immediately after death, the extensor digitorum longus (EDL) muscle was harvested from both hindlimbs.

Ex vivo EDL force recordings and contractile protocol

EDL muscles from both hind limbs were carefully dissected, mounted to organ bath chambers connected to an isolated muscle test system for rodents (Model #1200A, Aurora Scientific, Ontario, Canada), with Tyrode's solution (121 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 24 mM $NaHCO_3$, 5.5 mM glucose, 0.1 mM EGTA, 25 µM d-tubocurarine) bubbled with 95 % O_2-5 % CO_2 (pH 7.4) at 22 °C as previously reported [19]. Muscles were electrically stimulated using square-waved biphasic pulses (0.6 A), and force was acquired. At the end of each experiment, the optimal length (L_0) was measured, and each muscle was blotted dry and weighed to determine cross-sectional area (CSA; in mm^2), by dividing muscle mass (in mg) by the product of muscle length (in mm) and muscle density (1.06 mg/mm^3). Force was normalized to muscle CSA for each muscle and reported in kPa. Relative force-frequency curves were fit by a sigmoidal nonlinear regression using the equation $P=P_{min} + (P_0 \cdot F^N/F_{50}{}^N + F^N)$, where P is the tension developed at different frequencies of stimulation (F), P_{min} is the minimum tension developed, F_{50} is the midpoint of the curve, and *N* is the Hill coefficient (nH), which is correlated to the steepness of the curve.

Mice were randomly assigned to two groups (n=6 mice each), in which EDL muscles from the left leg were treated *ex vivo* with either 10 µM SPL-334 (solubilized in DMSO; Sigma Aldrich) (Group #1) or 10 µM SPL-334 + 400 µM N^G-Monomethyl-L-arginine acetate salt (L-NMMA solubilized in Tyrode's solution; Cayman Chemicals) (Group #2). The right leg from the same mouse served as an untreated control. An overview of the experimental scheme of the mechanical and fatigue

testing and drug treatment protocols is shown in Figure 1. After mounting EDL muscles in testing chambers, the length of each EDL was adjusted to achieve maximal isometric twitch force (L_0) evoked by single 0.5 ms pulses (16 V), after which the muscles were allowed to rest for 10 min. Then, muscles were stimulated at different frequencies (350 ms trains, 0.5 ms pulses, 1-150 Hz; i.e., force-frequency protocol) with 100-second intervals.

After the first force-frequency protocol, SPL-334 was added to the perfusion solution of one EDL in the testing chamber, while the contralateral EDL muscle remained untreated. After 20 min of incubation, a second force-frequency protocol was performed on both muscles, followed by a 10-min rest. Then, both muscles were simultaneously repetitively stimulated to contract at a 120 Hz pulse frequency (350 ms trains, 0.5 ms pulses) every 4 s for the first minute, and the train rate was increased by 20 % every minute thereafter. The contraction was interrupted when the muscle fatigued, which we defined as the point at which peak force decreased by 30 % relative to the initial contraction (peak force). Force recovery was measured by simultaneously stimulating both muscles to contract after 10, 30, 60, and 120 min post-fatigue at 30, 50, and 120 Hz pulse-stimulation (350 ms trains, 0.5 ms pulses, 100 s intervals). In Group #2, the experimental protocol was repeated as previously described for Group #1, but one EDL muscle was co-treated with SPL-334 and L-NMMA, and the contralateral EDL served as an untreated internal control. Muscles were fatigued and force recovery was measured over 120 min as described.

In vitro GSNOR activity and immunoblotting

In a separate group of mice (n=5 mice), the EDL muscles from both hind limbs were dissected, immediately homogenized in 100 µl for each 10 mg tissue in 20 mM Tris-HCl pH 8, 0.5 mM K_2EDTA, 0.1 % NP-40 supplemented with 1 % (v/v) mammalian protease inhibitor cocktail (Sigma-Aldrich, Cat #P-8340) and centrifuged at 1,000× g for 3 min at 4 °C. Protein concentration was determined using the BCA Protein Assay Kit (Pierce; Cat #23227; Rockford, IL). GSNO was prepared immediately before the experiment, as described [15] and neutralized in 50 mM HEPES buffer, pH 7.0. The supernatant (0.3 mg/ml) was incubated with 300 µM NADH and 300 µM GSNO in homogenization buffer in the presence of 10 µM SPL-334 (solubilized in DMSO) or with an equal volume of DMSO (control) at room temperature. Absorbance was continuously measured at 340 nm for 20 min and converted to NADH concentration (Molar coefficient at 340 nm = 6,300 $M^{-1} \cdot cm^{-1}$). GSNOR activity was determined from the initial velocity of NADH fluorescence decay and normalized to protein concentration.

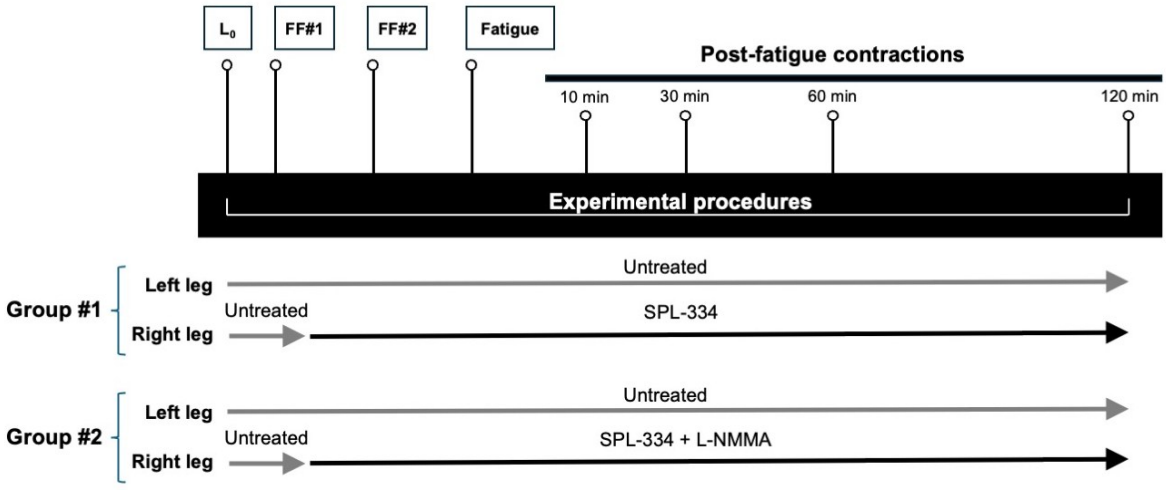


Fig. 1. Experimental scheme of extensor digitorum longus (EDL) drug treatment, mechanical testing, and fatigue protocols. Muscles from the left and right legs underwent a stimulation protocol, and isometric force was recorded. All stimulations were performed using 350 ms trains and 0.5 ms pulses. First, the optimal length (L_0) of each EDL was determined using single-twitch pulses at 16 V and by adjusting the muscle length in the testing apparatus (60 s intervals between stimulations). Muscles were allowed to rest (10 min), followed by force-frequency (FF) protocol #1 (1-150 Hz; 100 s intervals between stimulations). Immediately after FF#1, the drugs were added directly into the muscle perfusion solution for the remainder of the experiment (Group #1: GSNOR inhibitor [SPL-334], Group #2: GSNOR inhibitor and NOS inhibitor co-incubation [SPL-334 + L-NMMA]). FF#2, performed using the same protocol as FF #1, began 20 min after the drug incubation. After FF#2, the muscles were allowed to rest (10 min), and a fatigue protocol was performed (120 Hz pulse frequency) with a train rate of contractions every 4 s. After the first minute, the train rate was increased by 20 % each minute until the muscle was fatigued (measured as 30 % of the initial force). Post-fatigue force recovery was tracked at 10, 30, 60, and 120 min time points, with contractile force measured at 30, 50, and 120 Hz pulse frequencies.

After measuring GSNOR activity, the supernatant was frozen, diluted to 1 mg/ml protein in 4 % SDS, 20 % glycerol, 0.2 M beta-mercaptoethanol, 125 mM Tris-HCl pH 6.8, and equal amounts of protein were loaded onto 4-15 % mini-protean TGX gels (Bio-Rad). Following electrophoresis (SDS-PAGE), proteins were transferred to polyvinylidene difluoride membrane, blocked for 1 h at room temperature (RT) in Odyssey blocking buffer, and incubated for 12 h at 4 °C with primary antibodies (1:1,000 dilution) for either GSNOR (Polyclonal Antibody, Proteintech, Cat #11051-1-AP), SERCA1 (Monoclonal antibody, Thermo Fisher Scientific, Cat #MA3-912), or GAPDH (Polyclonal antibody, Thermo Fisher Scientific, Cat #PA1-987) in Odyssey blocking buffer supplemented with 0.1 % (v/v) Tween-20 (TBST). Membranes were washed with phosphate-buffered solution supplemented with 0.1 % (v/v) Tween-20, incubated for 1 h at RT with infrared conjugated secondary antibodies (Li-COR, Goat anti-mouse 680, Cat #926-68070 and goat anti-rabbit 800, Cat. #926-32211, 1:5,000 dilution) in Odyssey blocking buffer, and washed again. The bands were visualized using an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE) and analyzed using ImageJ.

Statistical analysis

The experimental results are presented as mean ± standard deviation (s.d.). A p-value less than 0.05 was considered significantly different. For comparison between two groups, a paired Student's *t*-test was used. For multiple comparisons, a one-way ANOVA followed by the Tukey post-test, or a two-way ANOVA followed by a Bonferroni post-test were performed. All analyses were conducted using GraphPad Prism version 10 (San Diego, California).

Results

GSNOR activity in EDL muscle homogenates significantly decreased (75±11 %, P<0.0001, Fig. 2A) with 10 µM SPL-334. GSNOR and SERCA1 (as a marker of muscle protein content) were detected by immunoblotting (Fig. 2B).

In two groups of mice (Groups #1 [SPL-334] and #2 [SPL-334 + L-NMMA], see methods), EDL muscles *ex vivo* were subjected to two consecutive

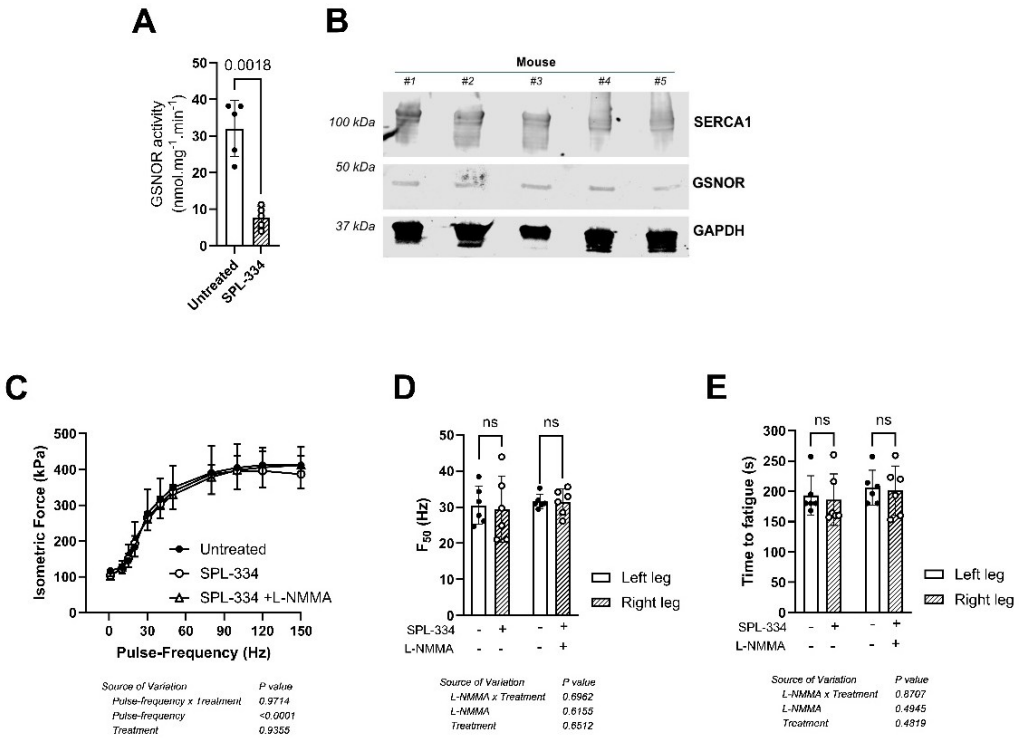


Fig. 2. Effects of SPL-334 incubation or SPL-334 + L-NMMA co-incubation on force development during a single bout of repetitive contractions until task failure. (**A**) GSNOR activity of isolated EDL muscle homogenates in the absence and presence of 10 µM of SPL-334; (**B**) GSNOR, SERCA1, and GAPDH detection in EDL muscle homogenates by immunoblotting; (**C-D**) Force developed by EDL muscles at increasing frequencies of pulse-stimulation (C), and the midpoint of the force-frequency relationship (F_{50}) (D), 20 min after beginning of treatments (SPL-334 or SPL-334 + L-NMMA or untreated); (**E**) Time to fatigue was measured in untreated muscle and muscles treated with either SPL-334 or L-NMMA, or SPL-334 + L-NMMA. Two-way ANOVA, repeated measures, statistics are shown below each panel. Mean ± SD, n=5–6 muscles per group.

force-frequency protocols, and twenty minutes before the second force-frequency protocol, one muscle was treated with SPL-334 or with SPL-334 + L-NMMA, while the contralateral muscle served as an internal untreated control. SPL-334 or SPL-334 + L-NMMA treatments did not affect submaximal or maximal force development (Fig. 2C), or the midpoint of the force-frequency relationship (F_{50}) (Fig. 2D) compared to untreated muscles. After the second force-frequency protocol, the muscles underwent a fatiguing bout of contractions, which was interrupted when peak force reached 30 % of the initial force. Time to fatigue was not different between groups (193±13 s vs. 186±17 s for untreated vs SPL-334, respectively; and 206±12 s vs. 201±17 s for untreated vs SPL-334 + L-NMMA, respectively, P>0.05 for treatment and for interaction, two-way ANOVA, repeated measures; Fig. 2E). Therefore, neither SPL-334 nor SPL-334 + L-NMMA treatments affected force development and time to fatigue during a single bout of repetitive contractions.

After the end of the fatigue protocol, pulse-stimulation frequencies that evoked unfused submaximal tetanic contractions (30 and 50 Hz) and maximal tetanic contractions (120 Hz) were used to investigate the time course of force recovery post-fatigue. In the absence of the drug (untreated muscles from Groups #1 and #2), the force evoked by 30 Hz stimulation remained lower than pre-fatigue for the first 2 h of recovery. At the 2-h recovery time point, force was 75±3 % [P=0.0006] and 78±5 % [P=0.0192] of the pre-fatigue force, for groups #1 and #2, respectively (Fig. 3A and 3D). At 50 Hz, force was lower than pre-fatigue only in the first 15 min of recovery, but at 30 min recovery, it either trended lower (85±5 % [P=0.0562]) or did not statistically differ (88±4 % [P=0.0786]) from the pre-fatigue force, for groups #1 and #2, respectively (Fig. 3B and 3E). At 120 Hz, force was not different than pre-fatigue at 30 min recovery timepoint (99±7 % [P=0.9999] and 101±2 % [P=0.9935] of the pre-fatigue force, for groups #1 and #2, respectively [Fig. 3C and 3F]).

In SPL-334-treated muscles from Group #1, force recovery was statistically lower compared to untreated muscles over the time course of the recovery from fatigue in all three frequencies of pulse-stimulation (Fig. 3A-C, * P<0.05, Bonferroni post-test). Conversely, in treated muscles from Group #2, in which L-NMMA was used to inhibit NO synthesis during contractions (i.e., SPL-334 + L-NMMA), force recovery was not statistically different from untreated muscles (Fig. 3D-F, P>0.05, Bonferroni post-test). Therefore, force recovery

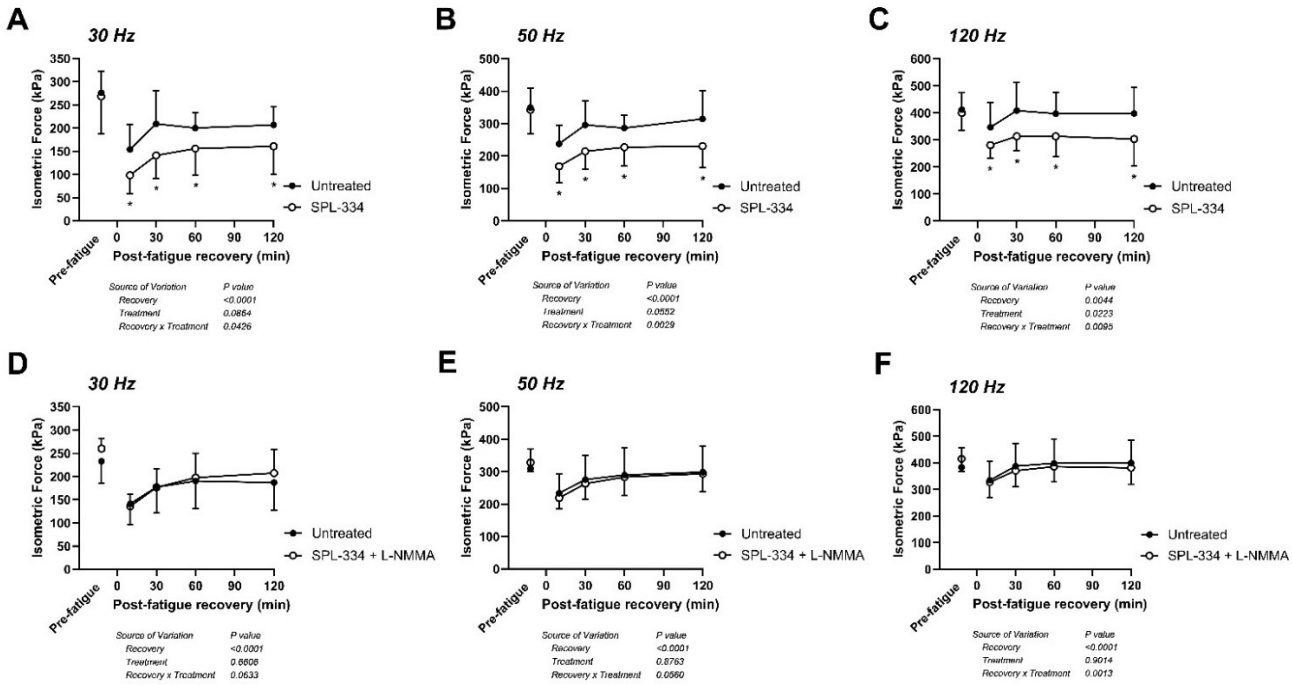


Fig. 3. Post-fatigue force depression with SPL-334 incubation is prevented with SPL-334 + L-NMMA co-incubation. Time-course of force recovery after fatigue (0-120 min) measured by submaximal (30 and 50 Hz) and maximal (120 Hz) frequencies of pulse-stimulation. Pre-fatigue force was obtained using the force-frequency protocol. Muscles were incubated with SPL-334 (**A-C**) or with or SPL-334 + L-NMMA (**D-F**) before the second force-frequency protocol and were present in the incubation media during the fatiguing contractions and during recovery from fatigue. Two-way ANOVA, repeated measures, statistics are shown below each panel. * P<0.05 vs. contralateral untreated muscles, Bonferroni post-test. Mean ± SD, n=6 muscles per group.

post-fatigue was impaired in SPL-334-treated muscles, and this effect was prevented by the presence of L-NMMA in the incubation medium.

Discussion

We demonstrated that muscle force and fatigue resistance in isolated EDL muscle from C57BL/6J male mice are not impaired by inhibiting GSNOR with SPL-334 or NOS activity with L-NMMA. However, after fatigue, muscles treated with SPL-334 exhibit sustained post-fatigue force depression at both low and high stimulation frequencies, and this effect is abolished when NOS activity is concurrently inhibited. This data suggests that increased NO synthesis during fatiguing contractions enhances intracellular GSNO in muscle fibers, but this effect is counteracted by GSNOR. When GSNOR is inhibited, repetitive contraction-dependent accumulation of GSNO impairs post-fatigue recovery of muscle force.

We confirmed the presence of GSNOR by immunoblotting and that SPL-334 significantly inhibited GSNOR activity (~75 %) in EDL muscle homogenates. GSNOR has been detected in other muscles with a high proportion of fast-twitch muscle fibers (e.g., gastrocnemius, quadriceps, and tibialis anterior) [17,20]. In mice, EDL muscles are primarily composed (~98 %) of type 2b and 2x muscle fibers [21], thereby reducing potential interference from slow-twitch muscle fibers. Fast-twitch fibers have greater nNOS expression compared to slow-twitch muscle fibers [22]. It is well known that nNOS activity depends on Ca^{2+}-calmodulin allosteric activation [23]. The intracellular calcium concentration required to bind calmodulin and activate nNOS (>500 nM) [23] matches the change in intracellular [Ca^{2+}] from rest (~100 nM) to tetanic contractions (~2 μM) [24]. It has also been shown that nNOS colocalizes with GSNOR in fast-twitch muscle [20], suggesting GSNOR can control any GSNO produced in the vicinity of nNOS in muscle fibers. However, GSNOR activity is highly dependent on substrate (i.e., GSNO) availability due to its relatively high reported K_M (~10-30 μM) [25,26]. Thus, GSNO production would occur only when nNOS activity is significantly increased, and in muscle fibers, this is likely to happen only during repetitive fatiguing contractions. Therefore, GSNOR activity has the potential to prevent the accumulation of GSNO in muscle fibers during sustained exercise.

We found that incubating isolated EDL muscles with L-NMMA or SPL-334 did not affect unfused or fused tetanic force development. These results suggest that non-fatiguing tetanic contractions with a long recovery period (i.e., 2 min) likely do not lead to significant increases in NO or GSNO that could affect muscle contractile function. Furthermore, peak force decay induced by repetitive contractions was unaffected by SPL-334, whether or not L-NMMA was present. It has been previously shown that mice with long-life GSNOR gene ablation (GSNOR KO mice) have lower decay in peak force during nerve-stimulated tibialis anterior (TA) muscle *in situ* (i.e., with circulation intact) repetitive contractions [17]. However, it has not been evaluated whether compensatory mechanisms in response to the persistent absence of GSNOR alter motor neuron function or minimize the production of NO and GSNO in muscle fibers during repetitive contractile activity. Therefore, the use of pharmacological inhibitors for GSNOR is an alternative to investigate the acute effects of GSNO accumulation in cells, including muscle fibers [15,27] and to avoid possible compensations of GSNOR ablation that may occur in transgenic mice.

Although the force and fatigue resistance were not altered by SPL-334 treatment, the recovery of muscle force from fatigue at both low and high frequencies of stimulation was significantly impaired in SPL-334-treated muscles. This result contradicts fatigue recovery measured *in situ* in tibialis anterior (TA) muscles, which did not differ between GSNOR KO and wild-type mice [17]. However, the differences between muscles (TA vs. EDL), preparations (*in situ* vs. *ex vivo*), the magnitude of force decay during fatigue among genotypes [17], and possible compensations for the lifelong absence of GSNOR (discussed above) make the comparison difficult to interpret. The force depression during fatigue recovery in SPL-334-treated muscles can be due to a reduced contraction-induced Ca^{2+} release from the sarcoplasmic reticulum by a hypernitrosylation of RyR1 that produces a "SR Ca^{2+}-leak" and lower Ca^{2+} transients during contractions [28]. It has also been shown that GSNOR KO mice exhibit increased S-nitrosylation of RyR1 [17]. However, this was not evaluated in the present investigation. Interestingly, when the experiment was performed in the presence of SPL-334 and L-NMMA, force recovery did not differ from the respective control, untreated muscle. This suggests that the SPL-334 effect on recovery from fatigue depended on NO synthesis during the fatigue protocol, strengthening the hypothesis that contractions lead to a sustained NO synthesis followed by GSNO formation and reduction by GSNOR.

Overall, the data presented in this study suggest that repetitive contractions of fast-twitch muscle in male mice increase NO-dependent GSNO production. However, GSNO accumulation is prevented by constitutive GSNOR present in muscle fibers, thereby protecting against GSNO-induced protein S-nitrosylation that could potentially result in contractile dysfunction. Therefore, the present study suggests that GSNOR in muscle fibers serves as an intracellular mechanism to protect the contractile machinery of fast-twitch muscle against NO-dependent nitrosative stress during intense contractile activity and possibly during high-intensity exercise.

Limitations of the Study

It has been demonstrated that female mice show greater exercise tolerance and higher locomotor muscle nitric oxide synthase activity (isoform not specified) [29], and a greater lung GSNOR activity [30]. However, whether GSNOR activity in skeletal muscles and contraction-induced muscle GSNO production differ between sexes remains unknown. In the present study, muscles were isolated only from male mice, thereby preventing analysis of potential sex-dependent effects of NO-dependent GSNO production on force production and recovery, a possibility that warrants further investigation. In addition, it has recently been proposed that NO in muscle can alternatively be produced via the nitrate-nitrite-NO pathway under acidic and hypoxic conditions [31]. It has been systematically demonstrated that fast-twitch glycolytic myofibers during contractions show intracellular acidosis [32] and, during exercise, the intracellular oxygen pressure decays from 20-40 mmHg to 2-3 mmHg [33], which is considered to be under near-hypoxic conditions. In the present study, *ex vivo*-isolated EDL muscles were perfused with a solution containing high oxygen (i.e., 95 %) to preclude anoxic cores during repetitive contractions [34]. Therefore, the contribution of the nitrate-nitrite-NO pathway to GSNO production during fatiguing contractions under *in vivo* conditions still needs to be explored.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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