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A mitochondrial-targeted antioxidant improves myofilament Ca^{2+} sensitivity during prolonged low frequency force depression at low P_{O_2}

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Key points

- Skeletal muscle contractile activity is associated with an enhanced reactive oxygen species (ROS) generation.
- At very low P_{O_2}, ROS generation by mitochondria can be elevated in intact cells.
- An elevated intracellular oxidant activity may affect muscle force development and recovery from fatigue.
- We treated intact single muscle fibres with a mitochondrial antioxidant and stimulated the fibres to contract at a low extracellular P_{O_2} that is similar to the intracellular P_{O_2} that is observed during moderate to intense exercise *in vivo*.
- The mitochondrial antioxidant prevented a sustained decrease in the myofibrillar Ca^{2+} sensitivity and improved muscle submaximal force development after fatigue at low extracellular P_{O_2}.

Abstract Skeletal muscle can develop a prolonged low frequency-stimulation force depression (PLFFD) following fatigue-inducing contractions. Increased levels of reactive oxygen species (ROS) have been implicated in the development of PLFFD. During exercise the skeletal muscle intracellular P_{O_2} decreases to relatively low levels, and can be further decreased when there is an impairment in O_2 diffusion or availability, such as in certain chronic diseases and during exercise at high altitude. Since ROS generation by mitochondria is elevated at very low P_{O_2} in cells, we tested the hypothesis that treatment of muscle fibres with a mitochondrial-targeted antioxidant at a very low, near hypoxic, P_{O_2} can attenuate PLFFD. We treated intact single fibres from mice with the mitochondrial-specific antioxidant SS31, and measured force development and intracellular $[Ca^{2+}]$ 30 min after fatigue at an extracellular P_{O_2} of ∼5 Torr. After 30 min following the

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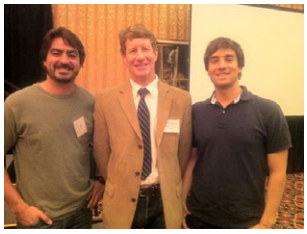

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end of the fatiguing contractions, fibres treated with SS31 showed significantly less impairment in force development compared to untreated fibres at submaximal frequencies of stimulation. The cytosolic peak $[Ca^{2+}]$ transients (peak $[Ca^{2+}]_c$) were equally decreased in both groups compared to pre-fatigue values. The combined force and peak $[Ca^{2+}]_c$ data demonstrated that myofibrillar Ca^{2+} sensitivity was diminished in the untreated fibres 30 min after fatigue compared to pre-fatigue values, but Ca^{2+} sensitivity was unaltered in the SS31 treated fibres. These results demonstrate that at a very low P_{O_2}, treatment of skeletal muscle fibres with a mitochondrial antioxidant prevents a decrease in the myofibrillar Ca^{2+} sensitivity, which alleviates the fatigue induced PLFFD.

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Introduction

Following a period of fatigue inducing contractions, skeletal muscle can demonstrate a long lasting impairment in submaximal force development that has been named "prolonged low frequency force depression" (PLFFD) (Edwards *et al.* 1977; Bruton *et al.* 2008; Balog, 2010; Cheng *et al.* 2015). PLFFD can last for up to 24 h or more in humans (Edwards *et al.* 1977; Place *et al.* 2015). Therefore, understanding the alterations that occur in skeletal muscle following fatigue may be of great relevance for the development of strategies to avoid a reduction in muscle function during and after strenuous physical activity. This is particularly important under conditions in which fatigue resistance is impaired by tissue hypoxia, such as during exercise at high altitude or for different chronic diseases states as in chronic obstructive pulmonary disease (COPD) (Gea *et al.* 2013) and chronic heart failure (CHF) (Rehn *et al.* 2012).

The mechanisms causing PLFFD remain unclear (Bruton *et al.* 2008; Balog, 2010; Place *et al.* 2015; Watanabe *et al.* 2015). One of the proposed mechanisms is that an increased generation of reactive oxygen species (ROS) resulting from a fatiguing contractile bout leads to oxidative modifications of proteins that will decrease the sarcoplasmic reticulum (SR) Ca^{2+} release and myofibrillar Ca^{2+} sensitivity during PLFFD (Bruton *et al.* 2008; Cheng *et al.* 2015; Watanabe *et al.* 2015). However, most of the studies examining PLFFD in isolated muscle models have been performed at supraphysiological extracellular ($\geq$150 Torr) oxygen tensions (P_{O_2}) (Bruton *et al.* 2008; Cheng *et al.* 2015). Since ROS generation in various cellular compartments can be significantly affected by the P_{O_2} (Clanton, 2007; Clanton *et al.* 2013), it is important to determine the role of ROS on the development of PLFFD under physiological, low P_{O_2} conditions. During moderate to high intensity exercise the intracellular P_{O_2} in human skeletal muscle fibres *in vivo* is quite low, decreasing from ~30 Torr at rest to ~3–5 Torr or less (Richardson *et al.* 2001, 2015; Wagner, 2012). Such physiological range of intracellular P_{O_2} (i.e. 3–30 Torr) should not be restrictive to the rate of mitochondrial oxidative phosphorylation during exercise in healthy subjects (Wagner, 2012) due to the high affinity of cytochrome *c* oxidase for oxygen (Wilson *et al.* 1988). Conversely, the O_2 availability can limit oxidative phosphorylation (i.e. hypoxia) (Connett *et al.* 1990) in highly trained subjects during maximal exercise (i.e. exercise induced hypoxia), or when exercise is performed at high altitude or in certain chronic disease patients (e.g. COPD and CHF) (Esposito *et al.* 2010; Poole & Jones, 2012; Cano *et al.* 2015; Hirai *et al.* 2015). In accordance with the idea that relatively low extracellular P_{O_2} values are necessary to limit oxidative phosphorylation in muscle fibres, the critical extracellular P_{O_2} estimated for rat skeletal myofibres working at their maximal oxygen consumption rate was estimated to be ~4–6 Torr (Wüst *et al.* 2009). While controversial, it has been suggested that at very low P_{O_2}, mitochondria may have an important role in ROS generation in intact contracting skeletal muscle as it has been demonstrated for other cell types (Guzy *et al.* 2005; Guzy & Schumacker, 2006; Clanton, 2007; Waypa *et al.* 2010; Clanton *et al.* 2013). Yet, the exact molecular mechanisms underlying ROS generation in skeletal muscle fibres, and potentially in skeletal muscle mitochondria, during contractions under near hypoxic P_{O_2} are not clear.

The purpose of the present study was to test the hypothesis that under a very low extracellular P_{O_2}, an exogenous mitochondrial-targeted antioxidant can blunt or attenuate the development of PLFFD. We measured whether intact single muscle fibres treated with the mitochondrial-targeted antioxidant SS31 preserves force development, free intracellular Ca^{2+} levels, and myofibrillar Ca^{2+} sensitivity 30 min after fatigue at an extracellular P_{O_2} of 5 Torr. The results of the present study demonstrated that at near hypoxic conditions, treatment of skeletal muscle fibres with SS31 avoided a decrease in myofibrillar Ca^{2+} sensitivity and resulted in higher submaximal force development after fatigue.

Methods

Ethical approval

All experiments were approved by the University of California San Diego institutional animal care and use committee (IACUC) and complied with the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals in Research*, all the regulations issued by the United States Department of Agriculture (USDA), and all regulations issued by the USDA implementing the Animal Welfare act. Male C57BL/6J mice (total of 14 mice) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were fed a standard diet and had access to food and water *ad libitum*. The animals (12–13 weeks old) were killed by an overdose of sodium pentobarbital administered by intraperitoneal injection, followed by cervical dislocation. Immediately after the animals were killed flexor digitorum brevis (FDB) muscles from both hindlimb feet were dissected.

Single fibre preparation, force and $[Ca^{2+}]_c$ measurement

Isolated single muscle fibres with tendons intact from FDB muscles were mechanically dissected under dark field illumination at room temperature with dissecting solution (~22°C; 136 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$ and 5.5 mM glucose). After dissection, each fibre was pressure injected with the Ca^{2+} sensitive fluorescent probe FURA-2 (Invitrogen, Carlsbad, CA, USA) as previously described (Nogueira *et al.* 2013), and allowed to rest for 60 min. Platinum clips were attached to each tendon of the fibre. The fibre was mounted on a Small Intact Muscle Apparatus (model no. 801C, Aurora Scientific Inc., Aurora, ON, Canada) and placed on the stage of an inverted microscope. Tetanic contractions were evoked by parallel platinum electrodes (250 ms train duration, 0.5 ms biphasic pulses, 8 V) using a Grass S88X stimulator (Quincy, MA, USA). Force development was measured by a force transducer (model no. 403A, Aurora Scientific Inc.). The fibre length was adjusted to achieve the maximal isometric tetanic tension (L_0; set with 100 Hz stimulations), allowed to rest for 10 min, and perfused with Tyrode 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 and 0.1 mM K_2EGTA) which was continuously bubbled with 5% O_2, 5% CO_2 and N_2 balance.

FURA-2 fluorescence was measured combined with force measurements. FURA-2 injected fibres were illuminated with two rapidly alternating (200 Hz) excitation wavelengths of 340 nm and 380 nm, and the ratio of fluorescence excitation (340 nm/380 nm; R) at an emission length of 510 nm was obtained. Fluorescence was converted to $[Ca^{2+}]_c$ according to the following equation (Nogueira *et al.* 2013):

$$[Ca^{2+}]_c = K_D\beta[(R - R_{min})/(R_{max} - R)] \quad (1)$$

In this equation K_D is the dissociation constant for Ca^{2+}-FURA-2 and was set to 224 nM (Westerblad & Allen, 1991). β is the fluorescence ratio between high and no $[Ca^{2+}]_c$ at 380 nm. R_{min} and R_{max} are the fluorescence ratios when Ca^{2+}-FURA-2 binding is absent and saturated, respectively. R_{min} was determined using an internal *in vivo* calibration described by Kabbara and Allen (Kabbara & Allen, 1999) with modifications (Nogueira *et al.* 2013). To determine R_{min}, fibres were incubated for 30 min with a no-calcium Ringer–EGTA solution (116.5 mM NaCl, 2 mM KCl, 2 mM NaH_2PO_4, 10 mM EGTA, at pH 7.3) supplemented with 30 mM caffeine. Fibres were then incubated with 100 μM BAPTA-AM (the acetoxymethyl ester form of BAPTA), solubilized in DMSO (final [DMSO] = 1%), and incubated in Ringer–EGTA solution for 30 min, followed by washout with Ringer–EGTA solution for an additional 20 min. R_{min} was determined as the smallest R value observed. To determine R_{max}, another group of fibres were treated with 10 mM caffeine and 500 μM 2,5-di-tert-butylhydroquinone (TBQ), a sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor, and stimulated at 120 Hz with 2 s train duration. To obtain the peak $[Ca^{2+}]_c$ during contractions, the average FURA-2 fluorescence ratio in the final 100 ms of stimulation was used.

The force–$[Ca^{2+}]_c$ relationship was accessed in each fibre before and 30 min after fatigue by fitting the force relative to the maximum against the estimated peak tetanic $[Ca^{2+}]_c$ for tetanic contractions evoked at 10–120 Hz with the following Hill equation (for details see Westerblad & Allen, 1993; Nogueira & Hogan, 2010).

$$P = P_{max}[Ca^{2+}]_c{}^N \Big/ \left(Ca^{2+}{}_{50}{}^N + [Ca^{2+}]_c{}^N\right) \quad (2)$$

where P is the relative force, P_{max} is the force at saturating $[Ca^{2+}]_c$, $Ca^{2+}{}_{50}$ is the mid-point of the force–$[Ca^{2+}]_c$ relationship and N is a constant which describes the steepness of the function.

Since temperature can affect ROS generation, the fatigue protocol was performed at 28°C because it mimics the temperature of the FDB muscle *in vivo* (Bruton *et al.* 1998). In preliminary experiments, we observed a rapid loss of the FURA-2 emission response to Ca^{2+} when fibres were kept at temperatures above 28°C and a very slow loss at 22°C. Due to the long-lasting nature of the present experiments, the temperature was kept at 28°C only during the time period of the fatigue protocol and at 22°C in the remaining time of the experiment to avoid loss of the FURA-2 fluorescent response to Ca^{2+}.

SS31 treatment

Fibres were perfused with Tyrode solution in the absence (control) or presence of 1 μM SS31, a water soluble peptide that has antioxidant properties, but localizes primarily in the mitochondrial inner membrane ($n = 7$ fibres per group; each fibre was obtained from a different animal) (Zhao *et al.* 2004). The amount of SS31 used was based on an established dose–response curve for SS31 (Anderson *et al.* 2009). The average fibre diameter was not different between groups (35 $\pm$ 6 μm for control group and 36.8 $\pm$ 10 μm for the SS31 group).

Experimental protocol

The P_{O_2} in the working chamber was measured by using a fibre optic oxygen sensor (Oxymicro, World precision Instruments, Sarasota, FL, USA), placed near the muscle fibre, throughout the experimental procedure, except when $[Ca^{2+}]_c$ was measured to avoid interference from the light emitted by the O_2 probe. Untreated and SS31 treated fibres were initially perfused with Tyrode solution continuously bubbled with a mixture of N_2 and O_2, so the final extracellular P_{O_2} in the working chamber was either 40 Torr or 5 Torr. All gas mixtures contained 5% CO_2 to maintain the solution's pH at ~7.4. Each fibre was initially perfused with an extracellular P_{O_2} of 40 Torr for 30 min, and stimulated to contract at different frequencies of pulses (1–150 Hz, 250 ms train duration, 0.5 ms biphasic pulses, 8 V) with 1 min intervals between contractions (to obtain a force–frequency curve) at 22°C. To minimize the contractile differences usually detected between the tested fibres, this work only used fibres in which the contraction force evoked at 30 Hz developed ~40–60% of maximum tetanic force (52 $\pm$ 4% and 54 $\pm$ 5% of maximum force for untreated control and SS31 treated groups, respectively). After the first force–frequency curve at 40 Torr, the P_{O_2} of the perfused solution was decreased to 5 Torr, which was kept until the end of the experimental procedure. In one group of fibres, 1 μM SS31 was added into the solution to scavenge mitochondrial ROS after the first force–frequency curve (22°C). Each fibre was equilibrated for 30 min with SS31, followed by a second force–frequency curve (pre-fatigue force–frequency curve; 22°C). After a 10 min resting period, the temperature was raised to 28°C and each fibre performed a fatigue protocol. The fatigue protocol consisted of a series of repetitive contractions (stimulation trains) evoked by 100 Hz stimulations at a train frequency of 0.25 trains s^{-1} that was increased each minute to 0.33 and 0.5 trains s^{-1} as previously described (Gandra *et al.* 2012). The protocol was terminated when force was decreased to 30% of the initial, then the temperature was lowered to 22°C during recovery. To examine the fatigue-induced PLFFD, a third force–frequency curve (22°C) was performed 30 min after the end of the fatigue protocol.

We have consistently observed for single fibres in our laboratory that the time to fatigue is significantly shorter when the extracellular P_{O_2} is set to 5 Torr compared to 40 Torr (as exemplified in Fig. 1*A*), which is similar to the resting mean capillary and intracellular P_{O_2} *in vivo*

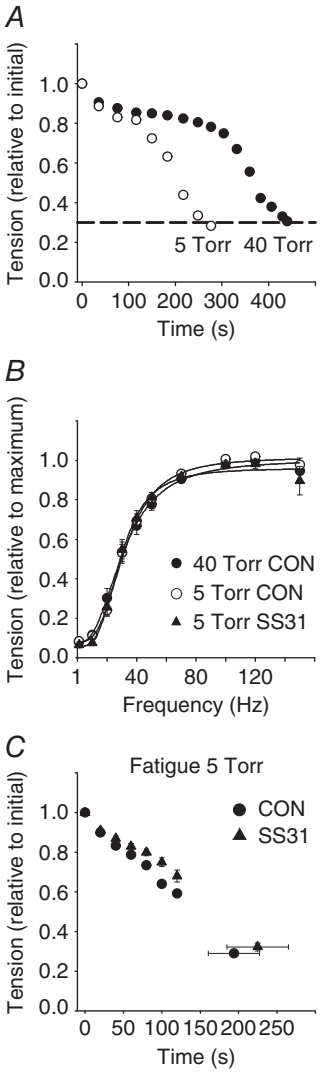

Figure 1. Single fibres treated with SS31 show no alterations in force generation and fatigue resistance at a low extracellular P_{O_2}
A, representative changes in tension generated by a single FDB fibre during fatiguing stimulations at an extracellular P_{O_2} of 40 Torr (filled circles) and 5 Torr (open circles) under control conditions. *B*, force *vs.* stimulation frequency data from single fibres repeated after 60 min of constant perfusion at an extracellular P_{O_2} of 40 Torr and 5 Torr, and 5 Torr in the presence of SS31 (40 Torr, filled circles; 5 Torr, open circles; and at 5 Torr plus SS31, filled triangles; $n = 7$). *C*, changes in tension generation during a fatiguing stimulation protocol at an extracellular P_{O_2} of 5 Torr for control and SS31 treated fibres (control, filled circles; SS31, filled triangles; $n = 7$ fibres per group).

(Richardson *et al.* 2006; Golub & Pittman, 2012). During a repetitive contractile period the oxygen consumption rate increases resulting in a decrease in the intracellular P_{O_2} in a manner dependent on the extracellular P_{O_2} as previously demonstrated for isolated fibres (Howlett *et al.* 2007). Therefore, it is very likely that during a fatigue protocol at 5 Torr the intracellular P_{O_2} decreases to levels that limit oxidative phosphorylation in isolated fibres.

Statistics

Values are presented as mean ± SEM. Unpaired *t* test or two-way ANOVA with Bonferroni's post-test were used to determine statistically significant differences between untreated and SS31 treated groups as appropriate. Two-way ANOVA repeated measures was used to determine with Bonferroni's post-test intragroup differences. Analyses were performed using GraphPad PRISM version 4.00 software (GraphPad Software, La Jolla, CA, USA). The significance level was set at $P < 0.05$.

Results

Maximal and submaximal force development, as well as fatigue resistance, at very low extracellular P_{O_2} were not altered by SS31

Neither SS31 incubation nor exposure to an extracellular P_{O_2} of 5 Torr altered fibre maximal and submaximal force development evoked by different frequencies of stimulation under non-fatiguing contractions (one contraction each 100 s; Fig. 1*B*).

Treatment with SS31 did not affect fatigue resistance (determined by the time to fatigue) in single fibres stimulated at an extracellular P_{O_2} of 5 Torr (Fig. 1*C*). The time to observe a 70% fall in the initial tension during the fatigue protocol was 198 ± 29 s for control (untreated) fibres and 239 ± 38 s for SS31 treated fibres ($P = 0.2661$; $n = 7$; Fig. 1*C*).

The mitochondrial antioxidant SS31 improved submaximal force development 30 min after fatigue at low extracellular P_{O_2}

All fibres from the control and SS31 treated groups showed a complete recovery of the maximal tetanic force (evoked by high frequencies of pulse stimulation; i.e. 120 Hz) 30 min after the end of the fatigue protocol. Yet, fibres from both groups presented the expected PLFFD 30 min after fatigue (Fig. 2*A* and *B*). Compared to pre-fatigue values, the submaximal force development was significantly smaller 30 min after fatigue in control (between 20 and 70 Hz; Fig. 2*A*) and in SS31 treated fibres (between 20 and 50 Hz; Fig. 2*B*). However, fibres that were incubated with SS31 showed significantly greater force development during submaximal stimulation (between 30 and 50 Hz) 30 min after fatigue compared to control

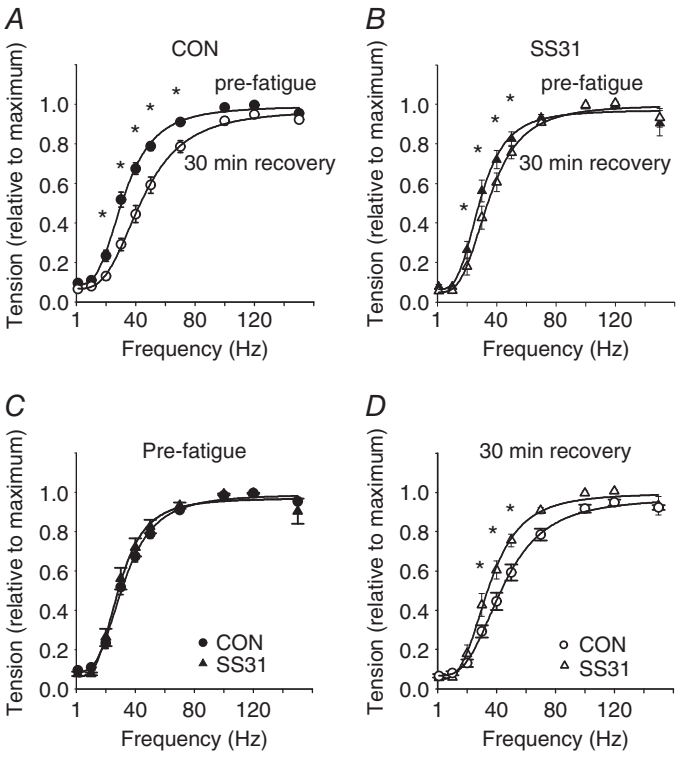

Figure 2. Submaximal force generation is higher in SS31 treated single fibres 30 min after fatigue at low extracellular P_{O_2}
A, force *vs.* stimulation frequency data from control fibres before and after fatigue at 5 Torr (pre-fatigue, filled circles; 30 min after fatigue, open circles; $n = 7$). *B*, force *vs.* stimulation frequency data from SS31 treated fibres before and after fatigue at 5 Torr (pre-fatigue, filled triangles; 30 min after fatigue, open triangles; $n = 7$). *C*, pre-fatigue force *vs.* stimulation frequency data from control fibres (filled circles; $n = 7$) and SS31 treated fibres (filled triangles; $n = 7$). *D*, force *vs.* stimulation frequency data obtained 30 min after fatigue from control fibres (filled circles; $n = 7$) and SS31 treated fibres (filled triangles; $n = 7$). $^*P < 0.05$.

(e.g. at 40 Hz; 44 ± 4% *vs.* 60 ± 7% of maximal tetanic force for control and SS31, respectively, $P < 0.01$; Fig. 2*D*).

The mitochondrial antioxidant SS31 prevented the right-shift in the force–Ca^{2+} relationship detected during PLFFD

The peak $[Ca^{2+}]_c$ detected during submaximal and maximal contractions (between 10 and 120 Hz) was equally diminished (10–20% decrease) in the control and SS31 groups 30 min following the fatigue time point compared to pre-fatigue values (Fig. 3*A*). This is similar to the changes in peak $[Ca^{2+}]_c$ reported in other studies examining PLFFD at supraphysiological P_{O_2} (Cheng *et al.* 2015). To determine whether the effects detected on force when SS31 was present were due to changes in myofibrillar Ca^{2+} sensitivity, peak $[Ca^{2+}]_c$ was plotted against peak force obtained from the force–frequency curves, before (unfatigued state) and 30 min after the fatigue protocol (PLFFD). In the control fibres, the force–peak $[Ca^{2+}]_c$ relationship was right-shifted during PLFFD (i.e. $Ca^{2+}{}_{50}$ was significantly increased from 618 ± 63 nM to 734 ± 90 nM, for pre-fatigue and 30 min post-fatigue, respectively; $P < 0.05$; Fig. 3*B*). In contrast, fibres treated with SS31 did not show changes in the $Ca^{2+}{}_{50}$ (577 ± 36 nM *vs.* 560 ± 24 nM; pre-fatigue and 30 min post-fatigue, respectively; $P = 0.273$; Fig. 3*C*). These data suggest that myofibrillar Ca^{2+} sensitivity was not different between pre-fatigue compared to 30 min after fatigue when SS31 was present. This can also be noted in Fig. 3*D* and *E*, which shows representative $[Ca^{2+}]_c$ and force traces during submaximal stimulations in a control (Fig. 3*D*) and an SS31 treated fibre (Fig. 3*E*) before and 30 min after a fatigue protocol. The decrease in the $[Ca^{2+}]_c$ 30 min after fatigue was similar for both fibres whereas the decline in the force developed was greater in the control fibre compared to SS31 treated fibre.

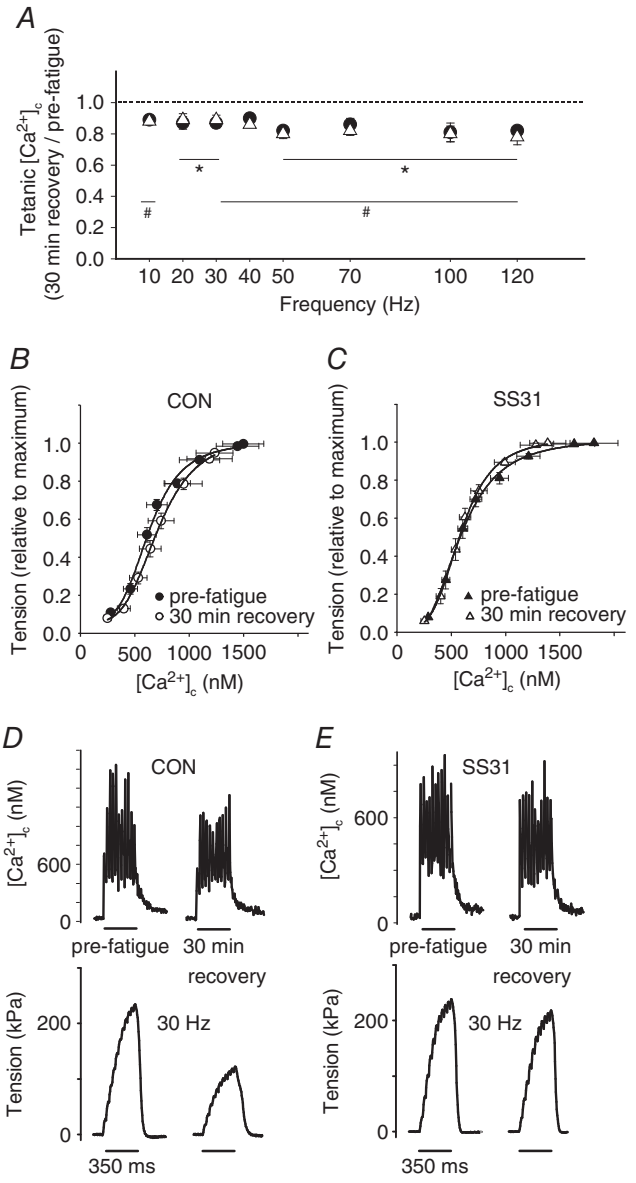

Figure 3. The prolonged decrease in myofibrillar Ca^{2+} sensitivity after fatigue at low P_{O_2} was avoided by SS31
A, tetanic $[Ca^{2+}]_c$ 30 min after fatigue relative to pre-fatigue *vs.* stimulation frequency, data from control (filled circles; $n = 7$; $^*P < 0.05$ *vs.* pre-fatigue values) and SS31 treated fibres (open triangles; $n = 7$; $^\#P < 0.05$ *vs.* pre-fatigue values). *B*, tension *vs.* $[Ca^{2+}]_c$ data from control fibres (pre-fatigue, filled circles; and 30 min after fatigue, open circles; at 5 Torr; $n = 7$). *C*, tension *vs.* $[Ca^{2+}]_c$ data from SS31 treated fibres (pre-fatigue, filled triangles; and 30 min after fatigue, open triangles; at 5 Torr; $n = 7$). *D*, representative $[Ca^{2+}]_c$ and force generation records from a single control fibre during a submaximal stimulation (30 Hz) before fatigue and 30 min after fatigue at an extracellular P_{O_2} of 5 Torr. *E*, $[Ca^{2+}]_c$ and force generation records from a single fibre treated with SS31 during a submaximal stimulation (30 Hz) before fatigue and 30 min after fatigue at an extracellular P_{O_2} of 5 Torr.

Discussion

The results of this study show that a mitochondrial antioxidant can mitigate the fatigue induced PLFFD in single skeletal muscle fibres at a very low extracellular P_{O_2}. In isolated fibres at low extracellular P_{O_2}, PLFFD resulted from a decrease in the peak tetanic Ca^{2+} concentration and in the myofibrillar Ca^{2+} sensitivity. When fibres were incubated with the mitochondrial-specific antioxidant SS31, the prolonged decrease in myofibrillar Ca^{2+} sensitivity after fatigue was blunted. Thereby, SS31 partially prevents the fatigue-induced prolonged fall in submaximal force development at low extracellular P_{O_2}.

Extracellular P_{O_2} condition in the single fibre model

We exposed single muscle fibres to a very low extracellular P_{O_2} to replicate conditions in which O_2 levels in skeletal muscle can be nearly restrictive for mitochondrial respiration during contractions. During exercise, skeletal muscle from COPD or CHF patients, or from climbers exposed to high altitude, may be subjected to hypoxic-like states and to increased intracellular oxidative stress levels (Clanton, 2007). In isolated fibres NADPH oxidase was identified as the major source of ROS during contractions, but these experiments were performed at supra-physiological extracellular P_{O_2} conditions (i.e. ambient P_{O_2}) (Michaelson *et al.* 2010; Sakellariou *et al.* 2013). However, it has been proposed that in physiological, near hypoxic conditions in skeletal muscle fibres during exercise, ROS formation by NADPH oxidase is inhibited due to its relatively high K_m for O_2 (Clanton, 2007; Brandes *et al.* 2014; Nisimoto *et al.* 2014). Since mitochondria are important sources of ROS when intact cells are exposed to low P_{O_2} (Duranteau *et al.* 1998; Guzy *et al.* 2005; Guzy & Schumacker, 2006; Clanton, 2007; Waypa *et al.* 2010; Sabharwal *et al.* 2013), the goal of the present study was to determine if treatment with a mitochondrial-targeted antioxidant can alter Ca^{2+} handling and force development in single fibres after fatiguing contractions at a low P_{O_2} condition that closely mimics *in vivo* conditions that occur during contractions.

The critical intracellular P_{O_2} reported for resting isolated fibres is ∼1.25 Torr (Richmond *et al.* 1997). As a result of contractile activity, the metabolic rate and mitochondrial oxygen consumption are elevated from resting levels and the critical P_{O_2} during contractions will be higher than at rest (Golub & Pittman, 2012; Clanton *et al.* 2013). The critical extracellular P_{O_2} during maximal work in rat plantaris muscle fibres was estimated to be between 4 and 6 Torr (Wüst *et al.* 2009). In our laboratory we have consistently observed that fatigue resistance for fibres from mice (with a diameter of ∼35 μm) contracting at an extracellular P_{O_2} of ∼5 Torr is significantly reduced compared to an extracellular P_{O_2} of 40 Torr, which is close to the *in vivo* interstitial P_{O_2} (Golub & Pittman, 2012) (authors' unpublished observations; see example in Fig. 1*A*). This suggests that during a fatiguing contractile period at an extracellular P_{O_2} of 5 Torr, oxidative phosphorylation is quite likely partially limited by oxygen diffusion from the extracellular environment to the mitochondria in isolated fibres. However, based on the model developed by our group (Stary & Hogan, 1999) the diameter of mouse fibres should be small enough to not restrict the extracellular oxygen diffusion to hinder mitochondrial respiration. Therefore, in this study the fatiguing contractile period was performed in physiological, near hypoxic, conditions, which should increase ROS generation from mitochondria (Guzy *et al.* 2005; Clanton, 2007; Clanton *et al.* 2013).

Treatment with a mitochondrial-targeted antioxidant can improve PLFFD in isolated fibres at a low extracellular P_{O_2}

The unaltered fatigue resistance observed with SS31 treatment was expected since treatments with antioxidants in general are not necessarily correlated with changes in fatigue resistance in isolated fibres (Zuo *et al.* 2011). Also, the treatment with antioxidants have been reported to have a greater effect on blunting PLFFD compared to increasing fatigue resistance (Kavdia, 2011). Enhanced levels of ROS generation resulting from muscle contractile activity has been implicated as one of the mechanisms causing a decrease in the peak tetanic $[Ca^{2+}]_c$ during PLFFD, possibly due to a decrease in SR Ca^{2+} release, and a decrease in the sensitivity of the contractile sites to Ca^{2+} (Bruton *et al.* 2008; Cheng *et al.* 2015; Watanabe *et al.* 2015). Increased levels of $O_2^{-\bullet}$ seems to impair the SR Ca^{2+} release in intact fibres (Bruton *et al.* 2008). In contrast, a prolonged exposure of unfatigued intact fibres to high amounts of H_2O_2 causes a decrease in the myofibrillar Ca^{2+} sensitivity, although the exposure to low concentrations of H_2O_2 can increase myofibrillar Ca^{2+} sensitivity (Andrade *et al.* 1998, 2001). Therefore, it has been proposed that different ROS (e.g. H_2O_2 or $O_2^{-\bullet}$) and the specific cellular sites where these oxidants are generated (e.g. mitochondrial matrix, cytosol) during contractile activity may have different effects on the myofibrillar Ca^{2+} sensitivity and the SR Ca^{2+} release in skeletal muscle fibres (Cheng *et al.* 2016). In this regard the antioxidant peptide SS31 can be used as an experimental probe to study the effects of mitochondrial ROS generation in cells (Min *et al.* 2011). SS31 accumulates specifically in the mitochondrial inner membrane and has the ability to scavenge H_2O_2 and $OH^{\bullet}$, thereby inhibiting intracellular oxidative stress such as lipid peroxidation (Zhao *et al.* 2004; Szeto, 2006). SS31 may also control mitochondrial ROS generation by stabilizing cardiolipin, a phospholipid specific to the mitochondrial inner membrane (Morin *et al.* 2003; Clanton *et al.* 2013; Szeto, 2014). Indeed, studies with sarcolemma-permeabilized skeletal muscle fibres have shown that SS31 significantly diminishes H_2O_2 emission from mitochondria (Anderson *et al.* 2009; Siegel *et al.* 2013). We observed that treatment of isolated single myofibres with SS31 ameliorated the submaximal force development during PLFFD at very low extracellular P_{O_2} conditions, which should mimic an oxygen-restricted state (i.e. near hypoxia). During PLFFD, SS31 had no effects on the peak tetanic $[Ca^{2+}]_c$ that had been diminished to a similar extent in control and SS31 treated fibres. The myofibrillar Ca^{2+} sensitivity was decreased 30 min after fatigue in the control untreated fibres, while SS31

treated myofibres did not demonstrate this reduction in Ca^{2+} sensitivity at the same time point. Similar to our results, *in vitro* SS31 treatment prevented a decrease in the myofibrillar Ca^{2+} sensitivity, and maintained force development within the control levels when ROS generation in mouse skeletal muscle was elevated by treatment with sphingomyelinase (Ferreira *et al.* 2012). In addition, SS31 prevented a decrease in myocardial force development in an *ex vivo* stunned heart model (Zhao *et al.* 2004). Thus, treatment with SS31 avoided a depression in the myofibrillar Ca^{2+} sensitivity during PLFFD at low extracellular P_{O_2}, which may possibly be due to its effect in lowering mitochondrial ROS emission in skeletal muscle (Anderson *et al.* 2009; Powers *et al.* 2011).

The extracellular P_{O_2} may impact the effects of SS31 on PLFFD

In contrast to our results, Cheng *et al.* reported that peak tetanic $[Ca^{2+}]_c$ was not smaller during fatigue recovery compared to pre-fatigue, when isolated fibres were treated with SS31 at a high, hyperoxic, extracellular P_{O_2}. Therefore they concluded that PLFFD resulted from a decline in myofibrillar Ca^{2+} sensitivity (Cheng *et al.* 2015). It is possible that these different effects of SS31 on submaximal peak tetanic $[Ca^{2+}]_c$ levels and myofibrillar Ca^{2+} sensitivity during PLFFD are due to the use of a low extracellular P_{O_2} (~5 Torr) in the present study instead of a supraphysiological P_{O_2} (≥150 Torr) in the study of Cheng *et al.* Accordingly, it has been recently suggested that an increased ROS generation resulting from the use of supraphysiological P_{O_2} in isolated muscle preparations *in vitro* may affect the development of PLFFD (Watanabe *et al.* 2015). Although controversial, in intact muscle cells, mitochondrial ROS formation can be increased in hypoxic as well as in hyperoxic conditions (Guzy *et al.* 2005; Guzy & Schumacker, 2006; Clanton, 2007; Murphy, 2009; Clanton *et al.* 2013). In isolated mitochondria ROS generation is directly proportional to the oxygen concentration (Clanton, 2007; Hoffman *et al.* 2007). Such a paradox may be explained by complex regulations and cell signalling mechanisms present in intact cells that are absent in isolated mitochondrial preparations (e.g. post-translational protein modifications, accumulation of signalling molecules and allosteric regulations) (Hoffman *et al.* 2007; Clanton *et al.* 2013). However, it is likely that the rates of ROS formation within the distinct mitochondrial compartments are different at low and high P_{O_2}. For example, in pulmonary artery smooth muscle cells, hypoxia prompts a decrease in ROS generation in the mitochondrial matrix and an increase in ROS production in the mitochondrial intermembrane space, which then diffuses to the cytosol (Waypa *et al.* 2010; Sabharwal *et al.* 2013). Notably, Cheng *et al.* (2015) have proposed that increases in the levels of superoxide radical in the mitochondria can directly affect Ca^{2+} release in adjacent SR, thereby linking ROS production in mitochondria to SR function in skeletal muscle (Eisner *et al.* 2013). Whereas, when the mitochondrial ROS is emitted to the cytosol, myofibrillar Ca^{2+} sensitivity should be decreased (Cheng *et al.* 2015). Thus, the P_{O_2} surrounding the muscle fibre may be an important factor affecting the cellular location where reactive species levels may be increased during contractions, which will ultimately define the effects of treatment with SS31.

In conclusion, our data confirm that a decrease in myofibrillar Ca^{2+} sensitivity after fatigue is one of the mechanisms causing fatigue-induced PLFFD at a low, but physiologically relevant, P_{O_2} and that a mitochondrial-targeted antioxidant treatment was able to prevent this decrease. The use of mitochondrial-targeted antioxidants may constitute a potential strategy to improve muscle function after fatiguing contractions, particularly in conditions associated with a restrictive O_2 delivery to skeletal muscle mitochondria which enhance mitochondrial ROS generation (e.g. COPD and high altitude). Since a certain level of oxidant activity is important for normal muscle function (e.g. glucose uptake, Sandström *et al.* 2006; force development, Andrade *et al.* 1998; and adaptation to exercise training, Kavdia, 2011), the use of antioxidants supplementation needs to be well deliberated to avoid blunting of ROS signalling. The use of mitochondrial specific antioxidants is a step forward for controlling oxidant activity in specific cellular compartments without drastically affecting overall cellular redox state, different cellular functions and muscle adaptation to exercise (Shill *et al.* 2016).

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Additional information

Competing interests

None declared.

Author contributions

M.C.H, L.N. and P.G.G. participated in the conception and design of the study, interpretation of the data and drafting of the manuscript. Experimental data collection was performed at the Hogan Lab at University of California San Diego by P.G.G., A.A.S. and L.N. P.G.G., A.A.S., L.N., and M.C.H critically revised the manuscript. A.A.S. was a recipient of the American Physiological Society's Undergraduate Summer Research Fellowship Program. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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